CIRCULATING BIOMARKERS IN THE STUDY AND EARLY DETECTION OF OVARIAN CANCER

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CIRCULATING BIOMARKERS FOR THE STUDY AND EARLY DIAGNOSIS OF OVARIAN CANCER

Brian Michael Nolen, PhD

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Ovarian cancer, the most lethal of all gynecological malignancies, represents a significant public health burden to women worldwide. The current challenges associated with ovarian cancer stem from a lack of effective screening strategies, an inability to detect the disease at a treatable stage, and the disappointing impact of treatment regimens over the entire disease course. A multi-faceted evaluation of circulating biomarkers of ovarian cancer was conducted in order to identify specific biomarkers and combinations which might serve as effective tools in the screening, triage, and therapeutic targeting of ovarian cancer patients.

Ovarian epithelial carcinoma (OEC) represents a heterogeneous disease characterized by several histological subtypes displaying divergent etiology, pathology, and treatment responsiveness. Serum biomarkers were identified which displayed subtype-specific alterations in a comparison of OEC patients and benign controls. These results suggest that circulating biomarkers may assist in the selection of patients for targeted therapies.

The efficient triage of women diagnosed with a pelvic mass based on risk of malignancy is known to result in a significant improvement in outcome for ovarian cancer patients and also a significant reduction in morbidity and anxiety for women with benign masses. Several multimarker panels, including the optimal combination of CA 125 and HE4, were capable of

discriminating benign from malignant pelvic masses. Based on current and previous findings, this biomarker panel may represent a novel diagnostic tool in this clinical setting.

Urine may offer several distinct advantages over serum as an analytical biofluid based on its low complexity, high stability, and lack of invasivity. An analysis of urine biomarkers revealed that several previously identified ovarian cancer biomarkers offer higher diagnostic performance in urine versus serum. Urine multimarker panels were effective in discriminating ovarian cancer cases from controls while a combination of urine and serum biomarkers resulted in the highest performance.

The current study provides compelling evidence for the use of circulating biomarkers in several capacities within the setting of ovarian cancer. The collective impact of biomarker research on the clinical management of ovarian cancer has the potential to significantly improve overall public health.

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PREFACE

The author would like to acknowledge the personnel of the University of Pittsburgh Cancer Institute Luminex Core Facility for technical and logistical assistance related to the experiments described herein.

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1.0 INTRODUCTION

1.1 OVARIAN CANCER: EPIDEMIOLOGY AND ETIOLOGY

Ovarian cancer represents the eighth most common cancer among women and the second most frequently diagnosed gynecological malignancy in the United States and Europe [1]. The overall global mortality attributed to ovarian cancer exceeds that of any other gynecological cancer with over 50% of the more than 200,000 women newly diagnosed each year expected to perish from the disease [2-5]. A critical factor in the elevated mortality associated with ovarian cancer is the lack of disease-specific symptoms. A high-profile consortium of public health organizations including the American Cancer Society, the Gynecological Cancer Foundation, and the Society of Gynecologic Oncologists recently issued a joint recommendation, termed the Ovarian Cancer Symptom Index (OCSI), which listed bloating, pelvic or abdominal pain, difficulty eating/fullness, and urinary symptoms as those more likely to occur in ovarian cancer patients than healthy women [6]. Compounding the problem of ubiquitous clinical presentation is the observation that the majority of early-stage cancers are asymptomatic resulting in over three-quarters of all diagnoses being made at a time when the disease has already established regional or distant metastases [2]. Despite aggressive cytoreductive surgery and platinum-based chemotherapy, the 5-year survival rate for patients with clinically advanced ovarian cancer is only 15-20%, although the cure rate for stage I disease is usually greater than 90% [2-4]. Thus,

improved screening methodologies aimed at detecting ovarian cancer at its earliest stages have the potential to result in substantial improvements in overall survival for this disease.

The lifetime risk of developing ovarian cancer stands at 1.39%, however this risk increases dramatically in women over the age of 45 (median age at diagnosis of 63) and in women with familial/hereditary conditions [5, 7]. In addition to age and genetic background, other risk factors associated with ovarian cancer include chronic inflammatory conditions/NSAID use, diet, ethnicity, hormone replacement therapy, hysterectomy, infertility drug use, obesity, OCP use, pregnancy, smoking, and exposure to talc or asbestos (reviewed in [8]). Hereditary ovarian cancer generally occurs within one of two distinct genetic backgrounds. The first, hereditary breast and ovarian cancer (HBOC) syndrome, is attributable to germline mutations in the *BRCA1* or *BRCA2* tumor suppressor genes [9-10], while the second is associated with hereditary non-polyposis colorectal cancer (HNPCC), or Lynch Syndrome, which is attributable to a germline mutation in one of several genes located within the DNA mismatch repair pathway [11-12]. Recent evidence supports the notion that the genetic background underlying ovarian tumorigenesis extends well beyond these familial conditions and that the development of fully malignant tumors involves the progressive acquisition of mutations in multiple genes, including *BRAF, KRAS, PTEN, Her2/neu, c-myc, p16*, and *p53* (reviewed in [13-15]). Although these molecular alterations have been identified in a significant fraction of ovarian cancers, none of these mutations are diagnostic of malignancy or predictive of tumor behavior over time. Furthermore, the frequency of several of the above mutations appears to be highly dependent on the histological subtype of the tumor [13].

The precise etiology of ovarian cancer remains poorly characterized. Factors including the rarity of the disease, its high rate of mortality, and the lack of useful experimental model

systems have contributed to the challenging landscape facing ovarian cancer researchers. A such, considerable controversy remains regarding the specific tissue origins and tumorigenic pathways involved. Although several types of ovarian tumors of non-epithelial origin occur at low frequency [16], it is widely held that the vast majority of ovarian cancers, termed epithelial ovarian cancer (EOC), arise from the coelomic epithelium of the ovary. Increasing evidence suggests that many of these tumors progress through a series of premalignant phases before becoming invasive [17-19] however, a premalignant lesion for ovarian cancer has yet to be identified. Invasive EOC can be further subdivided into the histological subtypes of serous, mucinous, endometrioid, clear cell and several other less common types. Among these, serous is by far the most prevalent representing 75-80% of all EOCs [2]. Morphological similarities between each of these subtypes and tissues of the lower genital tract have led to the proposal of an alternative hypothesis suggesting that ovarian tumors could arise directly from these tissues of Mullerian embryological origin [20]. In either case it remains plausible that the various subtypes of epithelial ovarian cancer may represent divergent etiologies given the distinct patterns of differentiation and clinical characteristics they exhibit [21-22]. Several models of ovarian carcinogenesis have been proposed which describe a multifactorial process involving environmental, genetic, and endocrine components. Popular among these models is the theory of incessant ovulation, which suggests that the repeated rupture/wounding of the ovarian surface followed by the rapid proliferation of surface epithelial cells that occurs during ovulation may facilitate malignant transformation of these cells [23]. Excessive gonadotropin and androgen stimulation of the ovary has also been postulated as a contributing factor [24]. A third theory proposes that EOC might arise as a result of exposure to toxic contaminants and carcinogens such as talc [25]. While each of these theories is supported by significant clinical evidence, none of

them are currently sufficient to describe a comprehensive mechanistic basis of ovarian cancer. Improved insights into the factors contributing to ovarian tumorigenesis, achieved through the utilization of novel methodologies, are therefore required to reconcile these models and further define disease etiology.

1.2 OVARIAN CANCER SCREENING: CURRENT TRENDS AND OBSTACLES

1.2.1 Screening Strategies

The high mortality associated with epithelial ovarian cancer can be partially attributed to the lack of effective early detection methods. Screening strategies capable of achieving the goal of early detection have the potential to dramatically enhance overall survival [4]. A substantial amount of research is now focused on the development of improved methods of evaluating women at high risk of developing ovarian cancer. The information garnered from such research will provide a better understanding of the early events associated with the neoplastic process in the ovary, which remains disappointingly uncharacterized. Although experimental evidence suggests the existence of a series of ovarian premalignant lesions demonstrating a cumulative array of molecular alterations, the definitive clinical identification of such lesions remains elusive. Currently, women designated as high-risk for ovarian cancer must rely on genetic counseling and testing, which typically includes the measurement of serum CA 125 and transvaginal sonography (TVS) [26]. The tumor marker CA 125 has demonstrated utility in monitoring the treatment response and progression of the disease, but not as a diagnostic or prognostic marker. Overall, the CA 125 assay exhibits a sensitivity of only 50-60% for stage I disease, and has been shown to be

significantly less sensitive in premenopausal women in comparison to postmenopausal women [27-29]. In light of these limitations, current recommendations do not favor the use of CA 125 for general screening. Screening based on TVS, doppler and morphological indices has provided some encouraging results, however each of these methods currently lack the specificity required of a screening test for the general population [30]. A multimodal screening approach that combines the use of tumor markers measured at specific intervals with ultrasound may yield higher sensitivity and specificity. An approach of this type has been evaluated in ovarian cancer and may represent a cost-effective strategy for early detection [31-32]. However, the current version of this strategy relies solely on CA 125 as the biomarker component and is therefore unlikely to provide sufficient sensitivity for early stage disease. Thus, there is a critical need to develop additional informative biomarkers in order to achieve the requisite diagnostic performance necessary for clinical advancement.

The requirements for a screening strategy for early stage ovarian cancer to be effective in the general population are considerable, and the feasibility of such an endeavor is the focus to two large, ongoing prospective randomized control trials (RCT): the Prostate, Lung, Colorectal, and Ovarian screening trial (PLCO, NCT00002540) sponsored by the National Cancer Institute, and the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS, NCT00058032) [33-34]. In a recent detailed meta-analysis, it was estimated that to achieve 50% sensitivity in detecting tumors before they advance to stage III, an annual screen would need to detect tumors 1.3 cm in diameter while an improvement to 80% sensitivity would require the detection of tumors less than 0.4 cm in diameter. In addition, a 50% reduction in serous ovarian cancer mortality though annual screening would require a test capable of detecting tumors 0.5 cm in diameter [35]. Considering the low prevalence of ovarian cancer in general population, any

proposed screening strategy must demonstrate a minimum specificity of 99.6% and a sensitivity of >75% for early stage disease to achieve a positive predictive value of 10% and avoid an unacceptable level of false-positive results [31-32]. Previous CA 125-based studies indicate that to meet these requirements, a first-line biomarker-based screening test would need to achieve a specificity of 98% [31-32]. As we await the results of the ongoing RCTs, a practical approach to ovarian cancer screening is the incorporation of serum biomarker testing into the evaluation of specific high-risk groups and clinical settings. The enrichment of ovarian cancer cases within these settings may permit the achievement of promising results in the short-term. With that goal in mind, the most pressing need to be addressed is the identification of novel biomarkers, or combinations of biomarkers that can detect small pre-symptomatic ovarian tumors and differentiate malignant from benign tumors with high levels of sensitivity and specificity.

1.2.2 Biomarkers of Ovarian Cancer

A number of cell-surface antigens and serum proteins are produced by ovarian tumors and can be assayed using monoclonal antibodies. Some of these assays have been applied clinically as markers of disease status and are useful in the detection of subclinical disease and in the diagnosis of recurrent ovarian cancer [36-37]. As mentioned above, of all the serum biomarkers of ovarian cancer, CA 125 has been the most extensively studied, however a growing number of additional biomarkers elevated in patients with ovarian cancer have been identified (Table 1.1) including: CA 15-3, CA 54/61, CA 19-9, TAG-72, OVX1, M-CSF, carcinoembriogenic antigen (CEA), cancer-associated serum antigen (CASA), lipid-associated sialic acid (LASA), urinary gonadotropin fragment (UGF), HER2/neu (ErbB2), EGFR, sICAM-1, VEGF, and lysophosphatidic acid [28, 38-46]. In addition, several members of the kallikrein family of proteins

have been identified as potential serum markers of ovarian cancer [47-53]. The use of gene expression array analysis has identified a number of novel markers, including HE4 [54], prostasin [55] and osteopontin [56]. HE4, or human epididymus protein 4, is a secreted glycoprotein product of the *WFDC2* gene which has shown great promise a diagnostic biomarker for ovarian cancer and has also recently been approved by the United States Food and Drug Administration for disease monitoring [57]. With the exception of HE4, the identification of additional biomarkers associated with ovarian cancer has not translated into widespread clinical implementation. Although several of these biomarkers are currently utilized clinically in other disease settings, most notably CA 15-3 and CA 19-9 for disease monitoring in breast and pancreatic cancer, respectively, none have shown significant diagnostic capabilities.

Table 1.1 Previously described biomarkers associations in ovarian cancer

Biomarker	Description	Method	Reference
CA 15-3	tumor antigen	serum ELISA	Woolas et al. [45]
CA 19-9	tumor antigen	serum ELISA	Woolas et al. [45]
TAG 72	tumor antigen	serum ELISA	Woolas et al.[45]
CA 54/61	tumor antigen	IHC	Suzuki et al.[41]
OVX1	tumor antigen	serum RIA	Woolas et al. [28]
M-CSF	growth factor	serum RIA	Woolas et al.[28]
CEA	tumor antigen	Luminex® (serum)	Yurkovetsky et al.[46]
CASA	tumor antigen	serum ELISA	Sehouli et al.[44]
LASA	tumor antigen	serum ELISA	Crump et al. [43]
UGF	tumor antigen	serum ELISA	Crump et al. [43]
HER2/neu	growth factor receptor	serum ELISA	Crump et al. [43]
EGFR	growth factor receptor	MS/serum ELISA	Baron et al.[38]
sI-CAM1	adhesion molecule	serum ELISA	Callet et al.[39]
VEGF	angiogenesis factor	serum ELISA	Oehler et al.[40]
Lysophosphaditic Acid	mitogenic factor	plasma ELISA	Xu et al.[42]
Kallikreins 4-8,11,15	proteases	various methods	Diamandis and colleagues[47-53]
HE4	tumor antigen	microarray	Schummer et al.[54]
Prostasin	protease	microarray	Mok et al.[55]
Osteopontin	bone factor	microarray	Kim et al.[56]

The limited diagnostic performance demonstrated by each of the established and emerging biomarkers of ovarian cancer has led many investigators to focus on the use of multimarker panels in hopes of achieving superior sensitivity and specificity. In preclinical testing, multimarker combinations containing CA 125 have generally demonstrated increases in sensitivity of 5-10% over CA 125 alone while maintaining a similar level of specificity. The addition of known tumor markers such as CA 15-3, TAG 72 (CA 72-4), mesothelin, and OVX1 to CA 125 has yielded promising results[29, 58-61]. Among the highest performing models is a panel consisting of CA 125, leptin, prolactin, IGF-II, MIF, and osteopontin which demonstrated a sensitivity of 95.3% at a specificity of 99.4% [62]. This report illustrates a growing trend involving the incorporation of proteins unlikely to be derived from the tumor itself into discriminatory panels. Several other examples of this include the addition of proteins such as M-CSF[29], sIL-2R[63], sFas[64], ApoA1[65], and transthyretin[65] to CA 125 to achieve improved performance. The various biomarkers utilized above represent factors originating from not only the growing tumor itself, but also from the stromal microenvironment surrounding the tumor and elements of the host response to the malignancy. Circulating levels of biomarkers derived from these distinct sources are less likely to correlate and are thus more likely to offer complementary information leading to improved diagnostic utility. Therefore, the evaluation of biomarkers conducted in the current investigation proceeded from a broad and diverse array of candidate proteins.

1.3 EXPERIMENTAL DESIGN

1.3.1 Method Development

The development of bead-based immunoassay platforms has had a significant impact on the field of serum biomarker discovery and development. Such platforms represent a synergistic combination of the reproducibility and diverse utility of solid phase ELISAs with the improved kinetics and flexibility of a liquid-phase assay. Bead-based systems also exhibit a high capacity for multiplexing which greatly reduces sample and reagent volume, conveys high throughput and automation capabilities, and permits the generation of large amounts of biomarker data in a single experiment. The technique was first conceptualized by Streefkerk in 1976 [66] leading to a patent filed in collaboration with Coulter in 1979. Multiplexed assays, based on bead size and a flow cytometric analysis platform were introduced and implemented by McHugh [67-68] and Stewart [69] from 1989 through 1994. Commercialization of the platform by Luminex Corporation (Austin, TX) in 1997 has ushered in the widespread usage of bead-based immunoassays for multiplexed biomarker analysis. The general principles regarding the Luminex® platform are diagrammed in Figure 1.1. Current protocols involve the use of a set of 5um microspheres internally labeled with a combination of two laser-reactive dyes. Each of the dyes can be loaded into the bead at 10 levels of intensity, thus allowing for 100 spectrally-distinct microsphere lots. Following the covalent coupling of each microsphere lot to a capture antibody specific to a particular antigen of interest, beads of different spectral lots can be mixed together and utilized in a multiplex format. The assay procedure then proceeds in a manner similar to that of traditional sandwich ELISA. The bead mixture is incubated with patient sera and bound analyte is detected using a biotin-labeled antigen-specific polyclonal antibody. The bead-coupled antigen/antibody

complexes are then fluorescently labeled using streptavidin-phycoerythrin (SA-PE). The bead set is analyzed using one of several Luminex® analyzers, which incorporates flow cytometry-based fluidics with dual-laser optics. While one laser excites the internal bead dye combination, the other laser simultaneously excites the PE label. The instrument then records and reports the identity of each bead, according to dye composition, along with the intensity of bound analyte, represented by PE fluorescence.

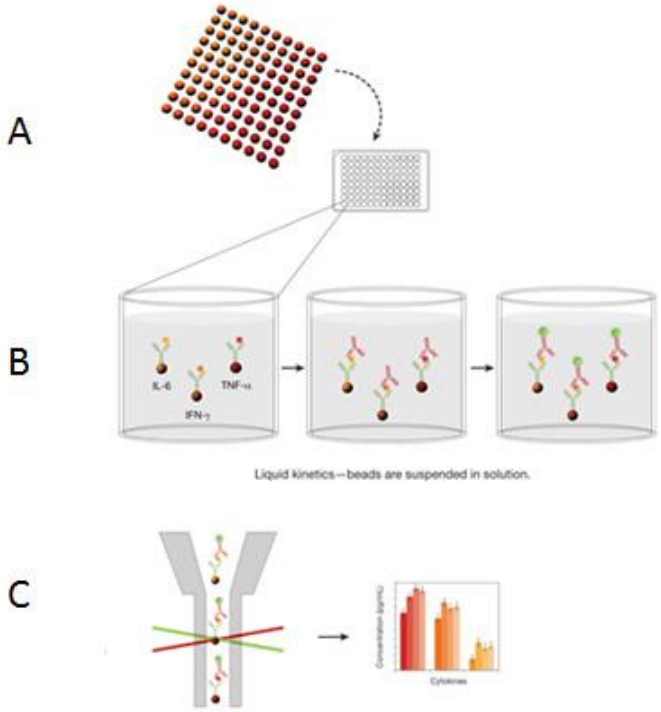

Figure 1.1 Principles of the Luminex® platform Copyright

A. 100 spectrally distinct bead lots are available for covalent coupling to distinct capture antibodies. **B.** Bead-capture antibody complexes are incubated with sample. Captured analyte is detected by biotin labeled polyclonal antibody and fluorescently tagged using SA-PE. **C.** Dual-laser excitation of sample permits the simultaneous determination of bead identity and quantitation of captured analyte. Copyright (c) 2011 Life Technologies Corporation. Used under permission.

The current investigation required the assembly of an extensive multiplex array consisting of 65 separate xMAP assays for proteins relevant to epithelial carcinogenesis (Ch. 3, Table 3). Commercially available xMAP assays do not include cancer antigens and many other important cancer biomarkers. To expand the number of biomarker assays available on the Luminex® platform, additional multiplexed panels were developed in the laboratory. Laboratory-developed assays include: CA 19-9, CA 125, CEA, CA 15-3, ErbB2, EGFR, kallikrein 10, Cyfra 21-1, AFP, IGFBP I, full-length mesothelin, HE4, small mesothelin-related protein (SMRP), tissue transglutaminase (TgII), SSC, TTR. These biomarkers have been multiplexed into six different panels, based on the absence of cross-reactivity and the required serum dilution factor. These assays were developed and validated according to industry quality control standards regarding sensitivity, inter- and intra-assay reproducibility, % recovery from serum, and correlation with conventional single analyte ELISA (when available) (Table 1.2). Monoclonal and polyclonal antibodies utilized in assay development were obtained from commercial vendors or collaborators and were evaluated individually for efficacy in the Luminex® platform. Inter- and intra-assay variability, expressed as a coefficient of variation, was calculated based on the average of 10 patient samples measured on at least three separate occasions. The performance of each assay was compared between single and multiplex formats to ensure the absence of cross-reactivity.

Table 1.2 Quality control characteristics of laboratory developed Luminex® immunoassays

Assay	Std Range	Sensitivity	% Recovery (Serum)	Intra-Assay CV (%)	Inter-Assay CV (%)	ELISA Correlation (%)
CA 19-9	.14-100 U/ml	.02 U/ml	96	5	8.2	62
CA 125	.69-500 U/ml	.16 U/ml	87	4	9.55	98
CEA	.34-250 ng/ml	.32 ng/ml	92	5	2.9	98
CA 15-3	.27-200 U/ml	.02 U/ml	114	4-5	7.12	97
ErbB2	13.7-10000 pg/ml	23 pg/ml	50	3-5	9.28	99
EGFR	137-100000 pg/ml	18.8 pg/ml	50	2-6	9.31	98
Kallikrein 10	69-50000 pg/ml	52 pg/ml	52	3-6	11.22	NT
Cyfra 21-1	137-100000 pg/ml	9 pg/ml	64	6	8.12	NT
AFP	55-40000 pg/ml	3.2 pg/ml	140	2-7	9.22	NT
IGFBP-1	13.7-10000 pg/ml	102 pg/ml	90	.7-5	6.68	NT
Mesothelin	137-1000000 pg/ml	228 pg/ml	73	3-7	8.81	NT
HE4	68-50000 pg/ml	68 pg/ml	88	5-8	12.95	60
TTR	1.37-1000 ng/ml	.8 ng/ml	85	4-8	9.59	NT

Biomarker expression levels were expressed as median fluorescent intensities (MFI) generated by analyzing 50-100 microbeads for each analyte in a single sample. The concentration of each analyte was quantitated from the MFI using standard curves generated by five-parameter curve fitting [70] to a series of known concentration standards. The Mann-Whitney non-parametric U test was used to evaluate the significance of differences in serum biomarker levels between subject groups. This test was chosen on the basis of robustness with respect to outliers, a common occurrence in the measurement of multiple serum biomarkers. The multivariate analysis used in the development of multimarker panels was performed in close collaboration with Alexsey Lomakin, a statistician at the Massachussetts Institute of Technology. Dr. Lomakin has developed a bioinformatics algorithm that is specifically designed for the construction of descriptive multianalyte panels from serum biomarker data generated by

Luminex®. This method, a Metropolis algorithm with Monte Carlo simulation (MMC) [1, 71-72] constructs a Scoring Function (SF) for a specific biomarker panel from a linear combination of logarithms of biomarker concentrations. The Monte Carlo optimization was then used to determine the coefficients in this linear combination that provides the highest sensitivity (the minimal number of misdiagnosed cases) at the desired specificity (fixed number of misdiagnosed control cases) in the case/control set. The algorithm is designed to identify the best performing panels consisting of 2-5 biomarkers. For each panel size, the panels with the highest sensitivity at the desired specificity are re-estimated for sensitivity by cross-validation. For cross-validation, 20% of subjects are randomly excluded from the data set and the rest used as a training set to build the optimal SF. The resultant model is applied to the excluded subjects, and this process is repeated 400 times in order to obtain a smooth averaged ROC curve.

1.3.2 Research Objectives

The investigation detailed herein proceeded from the hypothesis that *biomarkers present in the circulation of patients diagnosed with ovarian cancer and benign ovarian conditions can provide clinically relevant information pertaining to the development of malignancy and also a basis for the discrimination between the two conditions*. The evaluation of this hypothesis was conducted according to the following objectives: i) biomarkers levels present in the serum of patients diagnosed with several distinct histological subtypes of epithelial ovarian cancer and benign ovarian conditions were examined in order to identify alterations associated with specific disease pathology; ii) biomarker levels present in the serum of a broad group of patients diagnosed with benign or malignant adnexal masses were examined in order to identify multimarker panels capable of discriminating the two conditions with high levels of sensitivity and specificity; iii)

urine samples obtained from ovarian cancer patients, patients with benign ovarian conditions, and healthy controls were examined for biomarker levels to determine whether the use of urine as an analytical biofluid might offer advantages over serum with regards to diagnostic panel development. The workflow associated with objectives i and ii is presented in Figure 1.2.

Ovarian epithelial carcinoma can be subdivided into several distinct histological subtypes including clear cell, endometrioid, mucinous, and serous [2]. These carcinoma subtypes may represent distinctive pathways of tumorigenesis and disease development [13, 21-22]. This distinction could potentially be reflected in alterations of specific circulating biomarkers. A broad array of circulating biomarkers was analyzed in sera obtained from a diverse set of patients diagnosed with ovarian carcinoma to identify trends and relationships associated with distinct carcinoma histotypes and divergent tumorigenic pathways. Fifty-eight biomarkers including cancer antigens, oncogenes, cytokines, chemokines, soluble receptors, growth and angiogenic factors, proteases, hormones, and apoptosis and adhesion related molecules were evaluated using bead-based immunoassays. Nearly one-third of the biomarkers tested differed significantly between the cases and controls and a fair number of these alterations were subtype-specific. The results demonstrate that the divergent histology-based tumorigenic pathways proposed for ovarian epithelial carcinomas are associated with distinct profiles of circulating biomarkers. Continued investigation into the relationships between these factors should reveal new insights into the complex mechanisms underlying ovarian epithelial tumorigenesis.

The diagnosis of an adnexal mass is a prevalent issue among women in the United States while current methods of identifying those at high risk of malignancy remain insufficient [73-74]. Ineffective triage of women with malignant masses is associated with delayed or inappropriate treatment and a negative effect on disease outcome [6]. Sixty-five ovarian cancer-related

biomarkers were examined in sera obtained from women diagnosed with an adnexal mass. The subject group consisted of women diagnosed with benign masses and early and late stage ovarian cancer. Over half of the biomarkers tested were found to differ significantly between benign and malignant cases. As individual markers, HE4 and CA 125 provided the greatest level of discrimination between benign and malignant cases and the combination of these two biomarkers provided a higher level of discriminatory power than either marker considered alone. Multivariate statistical analysis identified several multi-marker panels that could discriminate early stage, late stage, and combined ovarian cancers from benign cases with similar or slightly improved SN/SP levels to the CA 125/HE4 combination, however these larger panels could not outperform the 2-biomarker panel in an independent validation set. A 3-biomarker panel with particular utility in premenopausal women was also identified. These findings serve to advance the development of blood-based screening methods for the discrimination of benign and malignant ovarian masses by confirming and expanding upon the superior utility of the CA 125/HE4 combination.

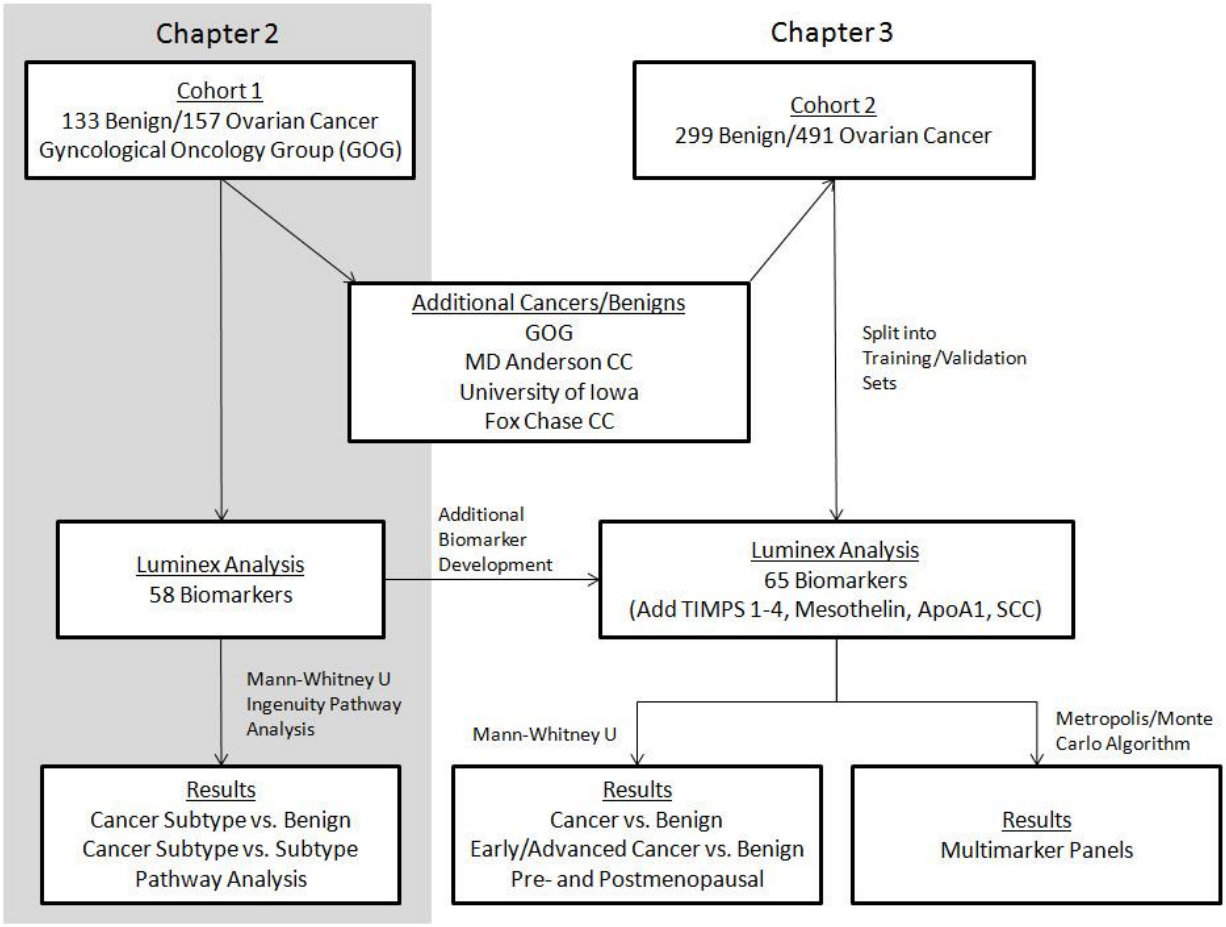

Figure 1.2 Workflow for evaluation serum biomarkers and multimarker panels in ovarian cancer patients and women diagnosed with benign ovarian conditions.

The measurement of biomarkers present in the bodily fluids of cancer patients represents an important avenue for the development of minimally invasive tests to predict tumorigenesis, disease recurrence, or treatment response. A great deal of work along these lines has already been devoted to blood, given its systemic exposure and extensive availability through tissue banks. However, blood is a dynamic biofluid with a proteome under continuous metabolic and homeostatic regulation. Alternatively, urine represents a thermodynamically stable biofluid that is inherently quiescent in that all molecular and proteolytic activity is largely complete upon sampling [75]. The urine proteome, representing the direct product of renal filtration, provides a

testing matrix of far lower complexity relative to that of serum [76]. Thus, the use of urine as an alternative or companion to serum in biomarker analyses has recently been proposed [77]. An analysis of biomarkers present in the urine of patients diagnosed with ovarian cancer was performed utilizing multiplexed bead-based immunoassays. Ovarian cancer patients were compared to healthy controls and women diagnosed with benign ovarian conditions. Nearly all of the tested biomarkers were detectable in urine and many exhibited a greater diagnostic capacity in urine versus serum. A multivariate analysis identified several urine multimarker panels capable of discriminating the cancer from the control groups with high sensitivity and specificity. The use of a 4-biomarker panel comprised of 3 urine biomarkers and one serum biomarker resulted in the discrimination of ovarian cancer patients from healthy controls with a sensitivity of 99% at 95% specificity. These results support the use of urine biomarkers as alternatives and/or companions to serum biomarkers for the early detection of ovarian cancer.

2.0 A SERUM BASED ANALYSIS OF OVARIAN EPITHELIAL TUMORIGENESIS

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A Serum Based Analysis of Ovarian Epithelial Tumorigenesis

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Abstract

Objectives: Ovarian epithelial carcinoma can be subdivided into separate histological subtypes including clear cell, endometrioid, mucinous, and serous. These carcinoma subtypes may represent distinctive pathways of tumorigenesis and disease development. This distinction could potentially be reflected in the levels of tumor produced factors that enter into the circulation and serve as biomarkers of malignant growth. Here, we analyze levels of circulating biomarkers from a diverse set of patients diagnosed with ovarian carcinoma to identify biomarker trends and relationships associated with distinct carcinoma histotypes and divergent tumorigenic pathways.

Methods. We utilize multiplexed bead-based immunoassays to measure serum levels of a diverse array of fifty-eight biomarkers from the sera of patients diagnosed with various histological subtypes of ovarian carcinoma and benign lesions. The biomarkers studied include cancer antigens, oncogenes, cytokines, chemokines, receptors, growth and angiogenic factors, proteases, hormones, and apoptosis and adhesion related molecules. Levels of each biomarker are compared statistically across carcinoma subtypes as well as with benign cases.

Results. A total of 21 serum biomarkers differ significantly between patients diagnosed with ovarian carcinomas and benign cases. Nine of these biomarkers are specific for carcinomas identified as clear cell, endometrioid, or mucinous in histology, while two biomarkers are specific for the serous histology. In a direct comparison of the histology groups, ten biomarkers are found to be subtype specific. Identified biomarkers include traditional and emerging tumor markers, cytokines and receptors, hormones, and adhesion- and metastasis-related proteins.

Conclusions. We demonstrate here that the divergent histology-based tumorigenic pathways proposed for ovarian epithelial carcinomas are associated with distinct profiles of

circulating biomarkers. Continued investigation into the relationships between these factors should reveal new insights into the complex mechanisms underlying ovarian epithelial tumorigenesis.

Keywords: ovarian carcinoma; tumor histology; serum biomarkers; ovarian tumorigenesis

2.1 INTRODUCTION

For women in the United States, ovarian cancer ranks eighth among cancers, excluding skin cancer, in terms of incidence, but moves up to fifth in a ranking of age-adjusted mortality [19]. Ovarian carcinomas, tumors of the surface epithelium, are by far the most common form of ovarian cancer [13]. The notion that ovarian carcinomas arise from the surface epithelium or postovulatory inclusion cysts following chronic exposure to hormones is met with widespread agreement [78], however a growing number of clinicians and researchers are beginning to appreciate a far greater heterogeneity concerning the development of ovarian epithelial carcinoma (OEC). Ovarian carcinomas can be classified into the histological subtypes of serous, clear cell, endometrioid, and mucinous which correspond to the different types of epithelia present in the female reproductive tract [79-80]. Serous tumors, which carry the poorest prognosis, are the most common form of ovarian carcinoma and make up roughly half of all diagnoses [81]. Serous tumors are histologically similar to cancers of the fallopian tube, and range from cystic papillary tumors to solid masses [81]. Endometrioid tumors, accounting for 15-20% of ovarian carcinomas, are characterized by endometrial-like glandular structures [82]. Mucinous tumors often contain cysts and glands lined by mucin-rich cells and constitute 10% of ovarian carcinomas [83]. Clear cell tumors represent 4-12% of ovarian carcinomas and are comprised of clear and hobnailed cells with an immature glomerular pattern [84].

Within the broad spectrum of disease states represented by OEC, there is accumulating clinical, translational, and genetic evidence for the existence of two distinct classes of carcinogenesis [13]. These classes have been termed type I, tumors comprising low-grade serous, mucinous, endometrioid, malignant Brenner, and clear cell carcinoma, and type II, tumors including high-grade serous carcinoma, malignant mixed mesodermal tumors (carcinosarcomas),

and undifferentiated carcinoma [13, 85]. Type I tumors typically present as early stage neoplasms that pursue an indolent course which may last more than 20 years [86-88]. Recent findings have traced the development of type I tumors through a stepwise series of well-described precursor lesions [85]. Benign cystadenomas and adenofibromas are believed to give rise to so-called borderline tumors which in turn develop into the type I tumors described above. In contrast to type I tumors, type II tumors are not associated with any recognizable precursors and apparently develop *de novo* from the surface epithelium or inclusion cysts of the ovary [89]. Type II carcinomas present as late stage, high grade neoplasms that are clinically aggressive, evolve rapidly and metastasize early, and are associated with a poor prognosis [13, 88]. Type II tumors are relatively chemosensitive in comparison to type I tumors [13].

Mutation screening and gene expression profiling have identified a number of molecular alterations and differences in gene expression that distinguish type I ovarian tumors from type II. These distinctions suggest a difference in prognosis and treatment response between the two groups [90-91]. Most prominent among observed genetic alterations are mutations in the *BRAF* and *KRAS* oncogenes, which occur in 28-35% of type I tumors but are largely nonexistent in type II tumors [92]. Mutations in the tumor suppressor gene *PTEN* and the *CTNNB1* gene, which encodes β-catenin, are also more prevalent in type I tumors, particularly endometrioid carcinomas [93-95]. Mutations in *TP53* are common in type II carcinomas but relatively rare in type I tumors [96-100]. Gene expression profiling and immunohistochemical analyses have identified numerous factors that are overexpressed in type II tumors when compared to type I including AKT2, human leukocyte antigen-G (HLA-G), apolipoprotein E, p53, MIB1, and bcl-2 [101-104].

Here we present an analysis of a diverse array of biomarkers found in the serum of women diagnosed with ovarian cancer. Biomarker levels are compared among patients grouped

according to carcinoma subtype as well as with those presenting with benign disease to identify markers that may contribute to or result from a particular carcinogenic pathway. In this manner, we seek to contribute to the evolving body of evidence related to ovarian epithelial tumorigenesis.

2.2 MATERIALS AND METHODS

2.2.1 Human Serum Samples

Serum samples from 157 patients diagnosed with ovarian cancer as well as 130 women with benign ovarian lesions were provided by the Gynecological Oncology Group (GOG) (Cleveland, OH) without individual identification of patients. Procedures for serum collection, processing, and storage have been previously described [105]. Written informed consent was obtained for each subject. The diagnostic breakdown of the study population is presented in Table 2.1 and represents a diverse spectrum of disease subtypes. Benign cases include a broad spectrum of non-malignant lesions representing a variety of histological origins. Patients diagnosed with endometriosis were not included in this study. Patients diagnosed with clear cell, endometrioid, and mucinous carcinomas are grouped together under the heading of "CEM Carcinoma."

Table 2.1 Histological characteristics of subjects included in tumorigenesis study

Diagnosis	N	Age Range
Benign	133	24-87
CEM Carcinoma	100	27-87
Clear Cell Carcinoma	24	
Endometrioid Carcinoma	46	
Mucinous Carcinoma	30	
Stage I & II	83	
Stage III & IV	17	
Grade 1	17	
Grade 2	11	
Grade 3	16	
Unknown Grade	56	
Serous Carcinoma	57	48-87
Stage I & II	27	
Stage III & IV	30	
Grade 1	2	
Grade 2	21	
Grade 3	29	
Unknown Grade	5	

2.2.2 Multiplexed Bead-Based Immunoassay

The xMAP™ bead-based technology (Luminex Corp., Austin, TX) permits simultaneous analysis of numerous analytes in a single sample. Fifty-eight bead-based xMAP™ immunoassays for a variety of known or potential biomarkers for ovarian and other epithelial cancers were utilized in the present study (Table 2.2). Assays were performed according to the manufacturers' protocol or as described previously [105]. Samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). For each analyte, 100 beads were analyzed and the median fluorescence intensity was determined. Analysis of

experimental data was performed using five-parameter logistic curve fitting to standard analyte values.

Assays for CA 19-9, CA 125, CA 15-3, CA 72-4, CEA, ErbB2, Kallikrein 10, EGFR, Cyfra 21-1, SMRP, tTG, HE4, osteopontin, transthyretin, and IGFBP-1 were developed in the UPCI Luminex® Core Facility [54]. The inter-assay variability of each assay was 5% to 11% and the intra-assay variability was 2% to 9%. Assays for eotaxin, Mip-1β, IP-10, IL-2R, IL-1Rα, IL-6R, DR5, TNF-R1, and TNF-R2 were obtained from Invitrogen (Camarillo, CA). Assays for MMP-2, MMP-3, and MMP-9 were obtained from R&D Systems (Minneapolis, MN). All other listed assays were obtained from Millipore (St. Charles, MO).

Table 2.2 Biomarker array utilized in tumorigenesis study

Category	**Individual Biomarkers**
Cancer Antigens/Oncogenes	α-fetoprotein, CA 19-9, CA 125, CA 15-3, CA 72-4, CEA, ErbB2
Cytokines/Chemokines/Receptors	Eotaxin, fractalkine, GM-CSF, IFNγ, IL-10, IL-12p70, IL-13, IL-1β, IL-1Rα, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-6R, IL-7, IL-8, IP-10, MIF, MIP-1β, sCD40L, TNFα, TNF-R1, TNF-R2
Growth/Angiogenic Factors	EFGR, IGFBP-1, TGFα
Proteases	Kallikrein 10, MMP-2, MMP-3, MMP-9
Hormones	ACTH, FSH, GH, LH, prolactin, TSH
Adipokines	Adiponectin
Apoptosis-related molecules	Cyfra 21-1, DR5, sFas, sFasL
Adhesion molecules	sICAM-1, sVCAM-1, tTG, tPAI-1
Other	HE4, osteopontin, SMRP, transthyretin, MPO

2.2.3 Statistical Analysis

The Mann-Whitney nonparametric *t* test was used to evaluate the significance of differences in serum biomarker levels expressed as observed concentrations between patients diagnosed with benign ovarian lesions and various ovarian carcinoma subtypes. The level of significance was $p<0.05$.

2.3 RESULTS

2.3.1 Analysis of Serum Biomarker Levels Across Ovarian Epithelial Carcinoma Subtypes

Sera from patients presenting with clear cell, endometrioid, and mucinous carcinomas, hereafter termed (CEM), were considered jointly as this group was presumed to represent type I ovarian carcinomas. Patients diagnosed with serous carcinoma presented with tumors that were almost uniformly high grade. Thus, this group was presumed to represent type II carcinomas and was considered separately. Serum biomarker levels from each of these groups were compared to each other as well as to those from patients diagnosed with benign ovarian lesions. These results are presented in Table 2.3.

Table 2.3 Serum biomarker levels across ovarian epithelial carcinoma subtypes

	Biomarker Levels (mean pg/ml ± 95% CI)			Mann-Whitney Significance		
	Benign	CEM Carcinoma	Serous Carcinoma	Benign vs. CEM	Benign vs. Serous	CEM vs. Serous
CA 125[1]	14.15±4.61	49.65±15.04	123.9±51.17	***	**	***
CA 72-4[1]	2.04±.285	13.5±7.9	4.39±2.06	***		**
CD40L	19457±4732	23522±4994	16414±5929			*
Cyfra 21-1	891±222	2323±718	2633±886	***	***	
EGFR	8548±344	7233±397	7552±636	***	**	
FSH[2]	37487±5344	24744±5182	36471±7751			**
HE4	5476±4357	74816±42866	43857±18512	***	***	
IGFBP-1	10178±1891	15509±3660	9734±3536			*
IL-10	15.67±2.63	32.48±10.78	20.79±4.31	***	**	
IL-2R	355.7±57.2	541.6±105.2	651.6±169.8	***	***	
IL-6	19.67±3.4	31.55±5.87	27.07±9.01	***		
IL-7	8.5±.864	11.6±1.85	10.2±1.69	***	*	
IL-8	15±3.91	24.8±15.17	16.9±5.24	**	*	
IP-10	49.86±5.35	42.4±5.71	72.53±15.54	*	***	***
LH[2]	19118±2435	15767±2982	22441±4496	**		**
MMP-2	150963±8262	131903±9126	137652±14133	**		
MMP-9	212302±34252	361810±62411	250084±64652	***		**
MPO	91818±21710	123134±30713	80550±30403	*		**
SMRP[3]	44227±19250	43804±15896	117660±49778		***	***
sVCAM-1	876645±68143	772258±66047	796917±70051	**		
TgII[4]	9.44±1.38	14.34±1.61	14.95±3.09	***	***	
TNF-R2	1515±137	1798±186	1836±228	*	**	
tPAI-1	35876±3432	47987±4514	36723±4949	***		**

[1]U/ml, [2]IU/ml, [3]pM, [4]mU/ml CEM: clear cell, endometrioid, mucinous carcinoma * - $p<0.05$, ** - $p<0.01$, *** -$p<0.001$

When the benign group was compared to the CEM carcinoma group, a number of significant serum biomarker level differences were observed. Among the cancer antigens and oncogenes assayed, CA 125, CA 72-4, Cyfra 21-1, and HE4 were all elevated in the CEM carcinoma group while levels of EGFR were reduced in the same group. The CEM carcinomas demonstrated higher levels of the cytokines IL-10, IL-2R, IL-6, IL-7, IL-8, and MPO as well as the cytokine receptor TNF-R2 in comparison to benign cases. IP-10 was decreased among CEM

carcinomas. Levels of MMP-2 were decreased among the CEM carcinomas while levels of MMP-9 were increased. The CEM carcinomas also exhibited increased levels of tTG and tPAI-1 and decreased levels of LH and sVCAM-1 when compared to the benign group.

Serum samples from the serous carcinoma group were compared with the benign group and several significant differences were identified. CA 125, Cyfra 21-1, HE4, and SMRP were elevated in serous carcinomas while levels of EGFR were reduced. Among cytokines and their receptors, IL-10, IL-2R, IL-7, IL-8, IP-10, and TNF-R2 were all found to be increased in serum samples from serous carcinoma patients in comparison to the benign group. Serum levels of LH and tTG were also increased in the serous carcinoma group.

The CEM carcinoma group was compared to the serous carcinoma group to identify serum biomarker level differences. Levels of CA 125 and SMRP were higher in the serous carcinoma group while CA 72-4 was higher in the CEM group. The CEM carcinomas demonstrated increased levels of CD40L and MPO and decreased levels of IP-10 when compared to the serous carcinomas. In the serous carcinomas, serum levels of MMP-9, FSH, and tPAI-1 were elevated while levels of IGFBP-1 were reduced in comparison to the CEM carcinoma group.

2.4 DISCUSSION

Tumorigenesis is a complicated and multi-faceted process that involves unchecked proliferation, immune evasion, angiogensis, stroma formation, tumor cell invasion and migration, and implantation and growth within distant tissues. To accomplish each of these feats requires a balanced and precise genetic background and tumor microenvironment, the components of which

remain largely unresolved by cancer researchers. Here we attempt to identify circulating factors associated with ovarian epithelial tumorigenesis through the comparison of a broad array of serum biomarkers in patients with distinct ovarian carcinoma histotypes.

The results of our analysis of serum biomarkers across OEC subtypes are outlined in Figure 2.1. We identified nine biomarkers that were elevated in the serum of patients diagnosed with ovarian carcinoma regardless of the disease subtype. These included the commonly used ovarian cancer biomarkers CA 125 and Cyfra 21-1 as well as the inflammatory cytokines IL-7, IL-8, and IL-10 and the receptors IL-2R and TNF-R2. These same cytokines, known to promote growth and inhibit apoptosis [106], have been previously found to be produced *in vitro* by ovarian cell lines and primary cells [107-109] and have also been observed to be elevated in the sera of ovarian cancer patients in comparison to healthy and benign controls [110]. Also increased in our ovarian cancer group were HE4 and tissue transglutaminase (tTG). HE4 is an 11kDa precursor to the epididymal secretory protein E4 and is an emerging biomarker for the detection of ovarian and endometrial cancer [111-113]. HE4 is overexpressed in ovarian carcinomas and demonstrates minimal gene expression and production in all tested normal tissues [57, 114]. tTG is highly expressed in ovarian tumors and has a proposed role in tumor invasion and migration by facilitating cell adhesion to fibronectin[115]. tTG overexpression was most recently reported to be an adverse prognostic factor in ovarian carcinoma [116]. Our analysis found that serum levels of soluble EGFR were lower in ovarian cancer patients in comparison to benign cases. Although cell surface EGFR is overexpressed in 35% to 70% of EOCs [117], it would appear from our investigation, and that of another group [38] that levels of the soluble form of EGFR present in serum are inversely correlated with ovarian cancer risk.

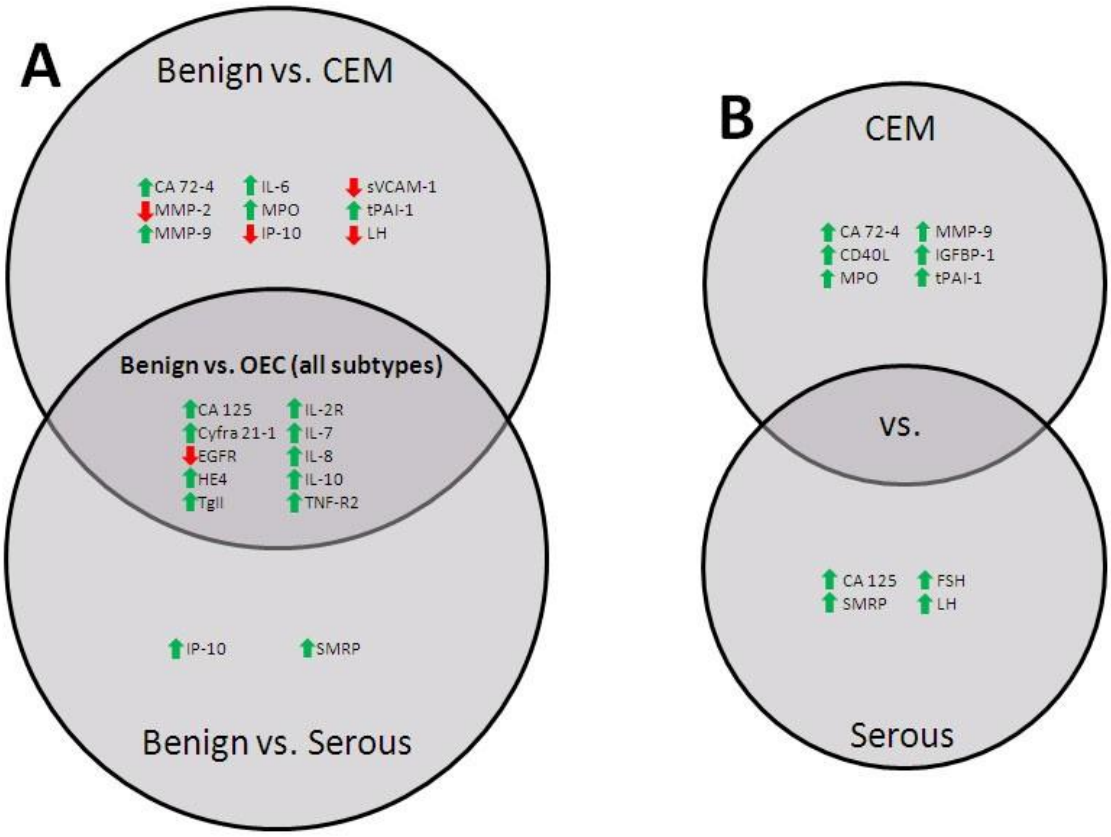

Figure 2.1 Serum biomarkers significant across ovarian epithelial carcinoma subtypes

Findings presented in Table 3 are summarized. Listed biomarkers were found to differ significantly between comparison groups. Arrow preceding each biomarker name indicates increased or decreased serum concentrations in the cancer group. **A.** Comparison between benign cases and ovarian cancer subtypes. **B**. Comparison between clear cell, endometrial, and mucinous (CEM) carcinomas and serous carcinomas.

Our primary aim in this investigation was to identify biomarkers with distinct serum levels among presumed type I and type II OECs. In comparison to benign pelvic disease, OECs of the clear cell, endometrioid, and mucinous (CEM) subtypes demonstrated significant differences in nine serum biomarkers while serous carcinomas differed among only two. Among conventional tumor markers, our observations are in agreement with a previous study that found CA 72-4 to be highly specific for mucinous ovarian carcinoma while CA 125 was specific for the

serous subtype [118]. Two additional emerging ovarian cancer biomarkers, IGFBP-1 and SMRP, were also found to be subtype specific, an observation not previously reported. Both of these markers have been implicated in ovarian cancer but remain uncharacterized [111, 119-120]. Significant among the cytokines tested were IP-10 for serous carcinomas and IL-6 and sCD40L for CEM carcinomas. These differences in serum cytokine levels were not as robust as those observed for all OECs considered together, suggesting a relative uniformity in tumor behavior. Interestingly, the CEM carcinomas demonstrated higher levels of myeloperoxidase (MPO). MPO is the chief protein product of neutrophils and is believed to play a role in the production of ROS and the oxidative activation of environmental carcinogens [121-122]. The results for the invasion, migration, and metastasis related molecules were somewhat mixed. The CEM carcinomas demonstrated relatively high serum levels of tPAI-1 and MMP-9 and relatively low levels of MMP-2 and sVCAM. Serous carcinomas did not differ significantly from the other groups for any of these markers. This is intriguing in light of the clinical observation that serous carcinomas are the most aggressive subtype of OEC and metastasize far more readily. Further investigation related to these observations would be justified. The serous carcinomas demonstrated higher serum levels of the gonadotropins LH and FSH. These hormones are important regulators of ovarian cell function and have been long implicated in the development of ovarian cancer, however the results from previous investigation concerning serum levels of the gonadotropins have been inconsistent [123]. The finding that LH and FSH play a greater role in the development of a particular histological subtype of ovarian carcinoma would be of great clinical significance.

Circulating biomarkers found in the serum of ovarian cancer patients may represent factors involved in either the cause of or the systemic response to the malignancy. These factors

may originate from a number of sources including the tumor itself, the surrounding stroma, or systemic tissues involved in the host response. It is crucial that ongoing work in the field of serum biomarkers is aimed at pinpointing the origins and functional roles of identified biomarkers. We sought to approach these questions by placing our findings within the broader context of genetic regulation of ovarian epithelial tumorigenesis. To that end, we utilized the Ingenuity Pathway Analysis (IPA) software package (Ingenuity Systems Inc., Redwood City, CA) to identify published relationships between the biomarkers we found to be informative and a consensus list of genetic markers currently under investigation in the field. A list of genes identified to be commonly mutated or overexpressed in CEM carcinomas includes: *BRAF* [85, 92], *KRAS* [85, 92], *CTNNBI* [94-95], *PTEN* [94], *MAP3K* [124], and *PI3K* [124]. This list was entered into the software package along with the list of biomarkers we identified when comparing CEM carcinomas with benign cases. The IPA software identified relationships between molecules in the two groups as shown in Figure 2.2A. A similar analysis was performed for biomarkers we identified in our comparison of serous carcinomas with benign samples utilizing a list of genes including: *AKT2* [101, 125], *APOE2* [102], *BCL2* [104], *HLA-G* [103], *MK167* [104], *TP53* [96-100], and *WT1* [84]. The results of this analysis are shown in Figure 2.2B. Several of the genes examined are established players within molecular pathways widely considered to play a role in ovarian cancer. Among these are the RAS/RAF/MAP pathway and the PI-3 kinase/PTEN pathway, both of which have been implicated in type 1 ovarian carcinomas [13, 124], and the p53 pathway active in type II carcinomas[88]. The IPA analysis demonstrates that several of the serum biomarkers identified in this study have been reported to interact with members of these pathways and further study aimed at characterizing these relationships would be well warranted.

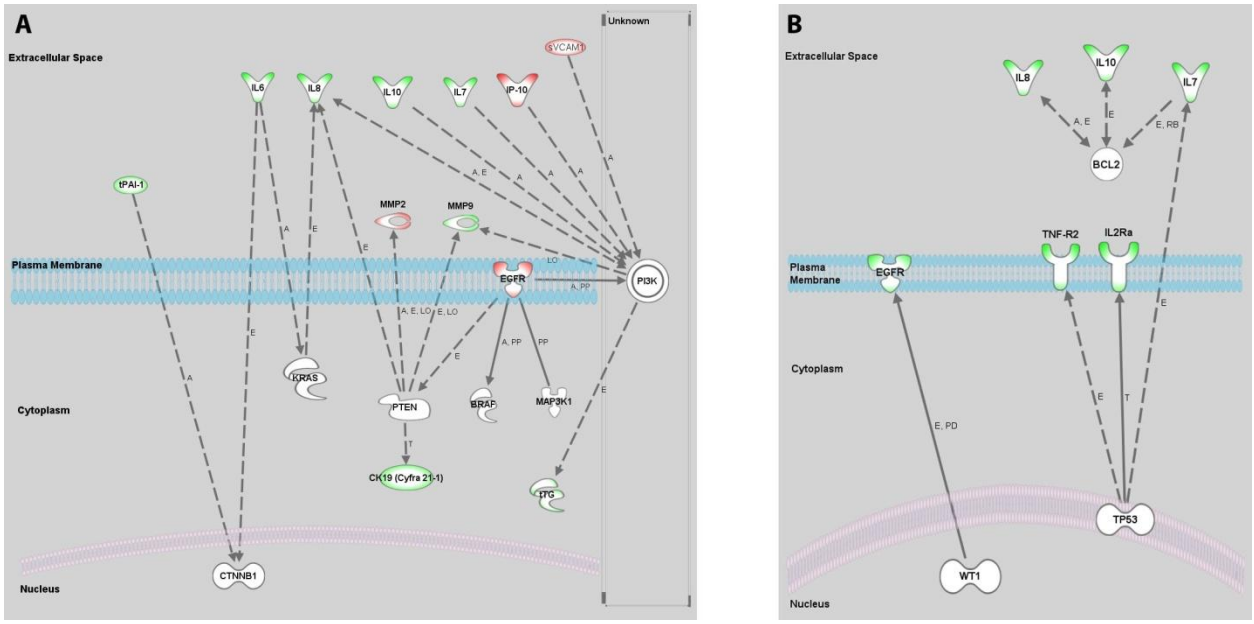

Figure 2.2 Ingenuity Pathway Analysis of identified serum biomarkers and reported molecular alterations

The Ingenuity Pathway Analysis software package (Ingenuity Systems Inc., Redwood City, CA) was used to identify relationships between identified serum biomarkers and genetic markers associated with ovarian carcinoma subtypes. **A**. Interactions identified between CEM carcinoma associated serum biomarkers and the following genes: BRAF, KRAS, CTNNBI, PTEN, MAP3K, and PI3K. **B.** Interactions identified between serous carcinoma associated serum biomarkers and the following genes: AKT2, APOE2, BCL2, HLA-G, MK167, TP53, and WT1. Biomarker outlines: green - increased in the serum of cancer patients, red – decreased in the serum of cancer patients. Interaction labels: A – activation, E – expression, PD – protein-DNA interaction, T – transcription, PP – protein-protein interaction, LO – localization, RB – regulation of binding, solid line – direct relationship, dashed line – indirect relationship.

This investigation clearly illustrates the unique and informative role of serum profiling in advancing our understanding of ovarian tumorigenesis. Our findings suggest that several traditional and emerging tumor markers, factors involved in the host cytokine and hormonal response, and adhesion- and metastasis-related proteins may be differentially utilized among OEC histological subtypes. An improved characterization of the mechanisms and molecular interactions that characterize the emerging pathways of ovarian epithelial tumorigenesis will allow for the development of improved tools and methods to better identify and capture every clinical opportunity.

3.0 SERUM BIOMARKER PANELS FOR THE DISCRIMINATION OF BENIGN FROM MALIGNANT CASES IN PATIENTS WITH AN ADNEXAL MASS

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Serum biomarker panels for the discrimination of benign from malignant cases in patients with an adnexal mass

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Abstract

Objectives: The diagnosis of an adnexal mass is a prevalent issue among women in the United States while current methods of identifying those at high risk of malignancy remain insufficient. Ineffective triage of women with malignant masses is associated with delayed or inappropriate treatment and a negative effect on disease outcome. *Methods:* We performed an evaluation of 65 ovarian cancer-related biomarkers in the circulation of women diagnosed with an adnexal mass. Our subject group consisted of women diagnosed with benign masses and early and late stage ovarian cancer. *Results:* Over half of the biomarkers tested were found to differ significantly between benign and malignant cases. As individual markers, HE4 and CA 125 provided the greatest level of discrimination between benign and malignant cases and the combination of these two biomarkers provided a higher level of discriminatory power than either marker considered alone. Multivariate statistical analysis identified several multi-marker panels that could discriminate early stage, late stage, and combined ovarian cancers from benign cases with similar or slightly improved SN/SP levels to the CA 125/HE4 combination, however these larger panels could not outperform the 2-biomarker panel in an independent validation set. We also identified a 3-biomarker panel with particular utility in premenopausal women. *Conclusions:* Our findings serve to advance the development of blood-based screening methods for the discrimination of benign and malignant ovarian masses by confirming and expanding upon the superior utility of the CA 125/HE4 combination.

3.1 INTRODUCTION

According to current estimates, 1.4% of women born today, or 1 in 72, will be diagnosed with ovarian cancer at some point in their lifetime. This year in the United States, there will be over 21,000 new cases of ovarian cancer along with over 15,000 deaths. [126] These cases arise from a much larger group of women presenting with adnexal abnormalities. The overall prevalence of adnexal abnormalities is estimated at 7% [73-74] and it is expected that 5-10% of American women will receive prophylactic surgery for suspected ovarian cancer at some point in their lives [74]. A pelvic exam is the primary clinical method by which adnexal masses are diagnosed and it is estimated that for each case of ovarian cancer identified, 10,000 pelvic exams will be performed [73]. A patient's age and menopausal status are important factors to consider upon the identification of an adnexal abnormality as the associated risk of malignancy increasess from 13% in premenopausal women to 45% in postmenopausal women [127].

While nearly all women diagnosed with ovarian carcinoma will initially present with an adnexal mass, only a small proportion of all masses detected will be malignant and the expeditious triage of these patients is the most important component of their treatment regimen. The burden of early identification of potential ovarian cancer falls predominantly upon the obstetrician/gynecologist whose training in the management of cancer patients is usually limited. While these practitioners can effectively manage the high percentage of patients diagnosed with functional cysts and benign neoplasms through observation and surgery, respectively [128-129], the clinical outcome for a patient presenting with a malignant mass can be drastically worsened if she is not immediately referred to a gynecological oncologist [6]. A series of diverse studies have

demonstrated a decrease in the relative risk of reoperation [130], and increases in disease-free interval [131] and overall survival [132] for women operated on by gynecological oncologists compared to gynecologists and general surgeons. Despite these findings, referral rates remain disappointingly low for patients diagnosed with an adnexal mass [133]. Improvements upon current screening methodologies and the emergence of new techniques should aid general gynecologists in making appropriate referral decisions and thus, improve the effectiveness of ovarian cancer treatment.

While useful in the identification of an adnexal mass, a pelvic examination is ineffective in discriminating benign and malignant lesions. Transvaginal ultrasonography has proven useful as a secondary screening tool, however its utility as a screening tool remains questionable given its demonstrated low positive predictive value and clinically insufficient levels of sensitivity [134]. Advanced imaging techniques such as CT or MRI have proven too expensive for widespread use given their limited SN and SP. In addition to a family history, pelvic examination, and imaging, the CA 125 blood test is a standard component in the complete evaluation of an adnexal mass. Despite its widespread use as a biomarker, CA 125 has demonstrated disappointingly low SP and SN in all evaluated patient cohorts and particularly in pre-menopausal patients [135]. Although CA 125 is associated with ovarian cancer in 80% of tested women over the age of 50, this association drops to less than 25% for women below that age [136]. The development of improved diagnostic screening tests for ovarian cancer is paramount in efforts to effectively triage patients presenting with an adnexal mass. Recently, Richard Moore and collaborators, in an analysis of serum concentrations of CA 125, SMRP, HE4, CA72-4, activin, inhibin, osteopontin, epidermal growth factor receptor (EGFR), and ErB2 (Her2) from women undergoing surgery for an adnexal mass

demonstrated the clinical utility of a CA 125/HE4 combined test for the discrimination of benign and malignant ovarian masses with 76.4% SN at 95% SP [112, 137].

We performed an extensive analysis of 63 additional circulating proteins found in the serum of a large group of patients diagnosed with an adnexal mass. Our objective was the identification of a biomarker panel that will surpass the CA 125/HE4 combination for discrimination of benign from malignant disease.

3.2 MATERIALS AND METHODS

3.2.1 Human serum samples

The training and premenopausal sets consisted of serum samples obtained from four sources. Cancer patients were women with histologically diagnosed epithelial ovarian cancer, while the benign group consisted of women diagnosed with a spectrum of benign adnexal lesions. Patients diagnosed with pelvic inflammatory disease (PID) were not included. The complete diagnostic breakdown of the study population is presented in Table 3.1. The training and validation sets consisted of postmenopausal women. A distinct group of premenopausal patients was considered separately and a cutoff age of 48 was utilized to establish menopausal status. FSH serum levels in women age 48-55, obtained during biomarker testing, were used to confirm menopausal status with levels >25mIU/ml indicating postmenopausal. Procedures for serum collection, processing, and storage have been previously described [105]. Written informed consent

was received from each subject and protocols were approved by appropriate institutional review boards.

Table 3.1 Clinical characteristic of study population included in adnexal mass study

	Training		Validation		Premenopausal	
	N (%)	Age Range (Median)	N (%)	Age Range (Median)	N (%)	Age Range (Median)
Benign	141 (100)	48-88 (63)	140 (100)	48-84 (65)	18 (100)	15-47 (42)
Histology						
Mucinous	24 (17)		18 (13)		6 (33)	
Serous	51 (36)		57 (41)		7 (39)	
Other/Unknown	66 (47)		65 (46)		5 (28)	
Ovarian Cancer	264 (100)	48-87 (63)	169 (100)	48-86 (62)	58 (100)	27-47 (41)
Stage						
Stage I-II	132 (50)		63 (37)		31 (53)	
Stage III-IV	132 (50)		106 (63)		27 (47)	
Histology						
Clear Cell	26 (10)		11 (7)		4 (7)	
Endometrioid	58 (22)		26 (15)		9 (16)	
Mucinous	11 (4)		4 (2)		14 (24)	
Serous	119 (45)		113 (67)		20 (34)	
Other/Unknown	50 (19)		15 (9)		11 (19)	

3.2.2 Multiplex bead-based immunoassay

The xMAP™ bead-based technology (Luminex Corp., Austin, TX) permits simultaneous analysis of numerous analytes in a single sample. Sixty-five bead-based xMAP™ immunoassays for a variety of known or potential biomarkers for ovarian and other epithelial cancers were obtained and utilized in the present study (Table 3.2). The training set was analyzed for the complete set of 65 biomarkers. The premenopausal group was analyzed for a subset of 19 biomarkers chosen based on the results of the training set analysis and other published findings. The validation set was analyzed for the most informative markers identified

in the training set. Multiplexed assays, data acquisition, and analysis were performed according to the manufacturer's protocol or as described previously [105].

Table 3.2 Biomarker array utilized in adnexal mass study

Biological groups	Proteins	Plex No.	Source
Inflammatory Mediators	IP-10, TNFR I, II, IL-1R□, IL-2R, IL-6R	1	1
	Eotaxin-1, interleukins 1b, 2, 4, 5, 6, 7, 8, 10, 12p70, 13, GM-CSF, IFN-γ, TNF-α, MIP-1β, MIF, CD40L, fractalkine	2	2
	MPO	8	2
Growth/angiogenic factors	EGFR, Her2/neu	3	3
	IGFBP-1	4	3
	TGFα	2	2
Tumor-associated antigens	CA 125, CA 15-3, CEA, CA 19-9, CA 72-4	3	3
	AFP	4	3
	HE4	12	3
Apoptotic proteins	Cyfra 21-1	3	3
	DR5	1	1
	sFas, sFasL	5	2
Proteases/Inhibitors	Kallikrein 10	4	3
	MMP 2, 3, 9	6	4
	TIMPS 1-4	7	4
	tPAI-1	8	2
Adhesion molecules	sICAM, sVCAM	8	2
Hormones	prolactin, TSH, LH, ACTH, FSH, GH	9	2
Adipokines	Adiponectin	8	2
Other markers	Mesothelin	4	3
	SMRP	10	3
	Osteopontin, tissue transglutaminase	11	3
	apolipoprotein A1	13	2
	TTR, SCC	14	3

Source No: 1 - Invitrogen/Biosource, Camararillo, CA; 2 - Millipore/Linco, St. Louis, MO; 3 – Luminex Core Facility, University of Pittsburgh, Pittsburgh, PA; 4 – R&D Systems, Minneapolis, MN
Plex No. indicates multiplexed panel, i.e. biomarkers that were analyzed simultaneously

3.2.3 Statistical Analysis

The Mann-Whitney U test was used to evaluate the significance of differences in serum biomarker levels expressed as observed concentrations between patients diagnosed with benign adnexal masses and ovarian cancer. The minimum level of significance was $p<0.05$. The multivariate analysis of the biomarker data was performed using the Metropolis algorithm with Monte Carlo simulation[138]. All development of multivariate statistical models for distinguishing ovarian cancer cases from benign controls was restricted to the training set. All possible panels consisting of 2, 3 and 4 biomarkers were evaluated for SN at 85% SP. Optimal panels were chosen that offered high cross-validated SN for both early and late stage ovarian cancer and high specificity for benign pelvic disease. These panels and method of combination were evaluated on the validation set to estimate, free from selection bias, the models' SN and SP. Premenopausal cancer patients were considered without stage stratification due to the limited number of late stage cases.

3.3 RESULTS

3.3.1 Analysis of individual biomarker levels in benign and malignant adnexal masses

Of the 65 biomarkers tested in the training set, 34 demonstrated significant differences between benign and malignant cases (Table 3.3). Ovarian malignancy was associated with an increase in

circulating levels of 26 tested biomarkers and a decrease in levels of 8 biomarkers. The comparison involving late stage ovarian cancer resulted in 33 biomarkers demonstrating significant differences, compared with 21 biomarkers for early stage cancer and 28 biomarkers for the combined set of cancer patients. CA 125 provided the highest level of discrimination of benign from malignant cases among early stage tumors while HE4 performed best in the late stage disease group.

Table 3.3 Serum biomarker levels across adnexal mass diagnoses for subjects in training set

	Mean Biomarker Level (pg/ml | p-value[4])						
Biomarker	**Benign**	**All Cancer**		**Stage I-II**		**Stage III-IV**	
ACTH	36.4	28.1	0.05	32.0	NS	22.1	0.01
ApoAI[3]	2213	2088	0.01	2120	0.05	1858	0.01
CA 19-9[1]	0.130	0.347	0.001	0.315	0.001	0.471	0.001
CA 125[1]	3.6	34.5	0.001	23.7	0.001	72.4	0.001
CA-153[1]	0.49	0.83	0.001	0.66	0.01	1.06	0.001
CA72-4[1]	8016	11724	0.001	9874	0.001	14748	NS
Cyfra 21-1	669	1421	0.001	1355	0.001	1840	0.001
DR5	120	173	0.001	135	NS	294	0.001
EGFR	19592	16902	0.001	18212	0.001	13713	0.001
EOTAXIN	169.5	185.5	NS	161.5	NS	218.0	0.001
ErbB2	1854	1948	NS	1791	NS	2412	0.001
Fas	4152	4248	NS	4118	NS	4890	0.001
HE4	2645	9621	0.001	5612	0.001	29779	0.001
IGFBP-1	48784	40957	NS	58629	0.01	24078	0.001

Table 3.3 (Continued)							
IL-10	11.25	18.50	0.001	17.10	0.001	21.85	0.001
IL-1Ra	1193	1288	NS	1035	NS	1751	0.01
IL-2R	578	803	0.001	645	0.05	1401	0.001
IL-6	15.85	24.05	0.001	21.25	0.01	31.95	0.001
IL-7	8.01	10.50	0.001	10.20	0.001	11.65	0.001
IL-8	8.41	12.45	0.001	11.20	0.01	14.95	0.001
IP-10	78.4	79.7	NS	66.6	NS	106	0.001
Kallikrein 10	47082	50163	0.001	48504	0.05	53054	0.001
MMP-2	172917	152645	0.01	154843	0.05	150823	0.001
MMP-9	184359	249059	0.01	241996	0.05	256590	0.01
MPO	38480	59049	0.05	50142	NS	74169	0.01
sFasL	43.75	29.60	0.01	36.05	NS	23.80	0.001
sVCAM-1	821776	716309	0.05	721565	NS	700190	0.05
TG II[2]	61.80	82.25	0.001	NA		NT	
TIMP-1	104826	121653	0.001	NA		NT	
TNF-a	6.69	7.23	0.001	6.88	NS	7.96	0.001
TNF-RI	3090	4132	0.001	3410	0.01	5860	0.001
TNF-RII	2240	2806	0.001	2590	0.01	3545	0.001
tPAI 1	33723	42974	0.001	41068	0.01	49028	0.001
Transthyretin	2273	1708	0.001	1921	0.01	1362	0.001

[1]U/ml, [2]mU/ml, [3]ng/ml, [4] minimum level of significance determined by Mann-Whitney U test for comparison of cancer vs. benign; NS – not significant, NT – not tested, NA – not applicable

Sera obtained from 76 premenopausal subjects were tested for 19 biomarkers chosen based on the results from the postmenopausal subjects and other published findings. Eleven biomarkers demonstrated significant differences between cancer and benign cases (Table 3.4). CA 125 provided the highest level of discrimination of benign from malignant cases for any single biomarker evaluated in this group. With the exception of eotaxin-1, all trends in biomarker levels between benign and malignant cases were consistent for the premenopausal and postmenopausal analyses, however the level of significance for many of the tested biomarkers was lower in premenopausal subjects, possibly a result of the smaller sample size. Eotaxin-1 was found to be significantly decreased in the sera of late stage ovarian cancer patients in

comparison to benign cases for premenopausal subjects, the opposite of what was found in postmenopausal subjects.

Table 3.4 Serum biomarker levels across adnexal mass diagnoses for premenopausal subjects

	Mean Biomarker Levels (pg/ml | p-value[3])						
	Benign	**All Cancer**		**Stage I-II**		**Stage III-IV**	
CA 125[1]	3.37	43.75	0.001	37.40	0.001	63.00	0.001
CA72-4[1]	6.28	17.10	0.001	17.20	0.001	16.50	0.05
Cyfra 21-1	0.71	59.90	NS	0.70	NS	643	0.05
EGFR	16802	14841	0.001	15080	0.01	13974	0.05
EOTAXIN	143	101.5	0.05	107	NS	91.1	0.05
FSH[2]	8129	5155	NS	4143	0.05	10108	NS
HE4	2180	5768	0.01	5904	0.05	4121	0.05
IL-2R	478	699	0.01	692	0.05	773	0.05
sV-CAM	688369	601087	NS	611459	NS	552433	0.05
TNF-RI	2512	3216	0.01	3177	0.01	3337	NS
tPAI 1	38178	51939	0.01	52048	0.01	50542	NS

[1]U/ml, [2]μIU/ml; [3] minimum level of significance determined by Mann-Whitney U test for comparison of cancer vs. benign biomarker levels
NS – not significant

3.3.2 Multivariate analysis of biomarker levels utilizing the MMC algorithm

The classification performance for the best single, 2-, 3-, and 4-biomarker panels identified in our analysis are presented in Table 3.5. The combination of CA 125 and HE4 was the best performing 2-biomarker combination of all studied 2-biomarker combinations (data for other 2-biomarker combinations are not shown) classifying cancer from benign cases at a SP of 85% with a SN of 74.2% in early cancers, 91.7% in late cancers, and 83% in the combined group. This combination outperformed either CA 125 or HE4, considered individually, in all evaluated

disease classes. Our analysis identified three 3-biomarker panels that demonstrated a classification power that was equal to or modestly better than the CA 125/HE4 combination in the training set. Each of these panels contained the CA 125/HE4 combination along with a third biomarker: CEA, Cyfra 21-1, or EGFR. The best 4-biomarker panel consisted of CA 125, HE4, CEA, and Cyfra 21-1 and demonstrated improved SN over the CA 125/HE4 combination in each disease class in the training set. However, when applied to the validation set, each of the identified 3- and 4-biomarker panels performed at a level equal to but did not improve upon the CA 125/HE4 combination.

Table 3.5 Biomarker panels that discriminate benign from malignant cases

Panel	Training					Validation				PreM	
	SP	SN			ROC	SP	SN			SP	SN
		All	Early	Late	AUC		All	Early	Late		
CA 125	85	79.5	72.0	87.1	0.860	82.1	76.3	61.9	84.9	87.5	70.7
HE4	85	70.5	50.8	90.2	0.835	84.3	83.4	69.8	91.5	93.8	43.1
CA 125, HE4	85	83.0	74.2	91.7	0.868	77.9	89.4	79.4	95.3	87.5	62.1
CA 125, HE4, CEA	85	83.0	73.5	92.4	0.872	77.1	90.5	82.5	95.3	87.5	63.8
CA 125, HE4, Cyfra 21-1	85	84.1	76.5	91.7	0.875	79.3	85.8	71.4	94.3	81.3	69.0
CA 125, HE4, EGFR	85	83.3	75.0	91.7	0.889	74.3	89.3	81.0	94.3	87.5	75.9
CA 125, HE4, CEA, Cyfra 21-1	85	86.4	78.0	94.7	0.878	75.0	90.0	81.0	95.3	87.5	62.1

All values (with exception of AUC) represent percentages (%); PreM: premenopausal subjects

Each of the single and multi-marker panels identified in the analysis of the training set was subsequently applied to the set of premenopausal subjects (Table 3.5) as the small size of this group precluded any meaningful development of panels based on it alone. In this group, CA 125 alone provided the highest SN and SP at 70.7% and 87.5% respectively. The addition of HE4 to CA 125 did not improve the SN and SP of the test, however a 3-biomarker panel consisting of CA 125, HE4, and EGFR did significantly improve the classification power,

demonstrating a SN of 75.9% at 87.5% SP. None of the other identified multi-marker panels provided any appreciable improvement over CA 125 alone in the premenopausal group.

3.4 DISCUSSION

With the exception of highly invasive procedures such as biopsy and surgery, the evaluation of circulating biomarkers offers the most definitive means of distinguishing benign from malignant pelvic masses. Several recent studies have evaluated various panels of circulating biomarkers in ovarian cancer patients and benign cases [62, 112, 139-141], however our study is the largest and most diverse to date to utilize biomarker profiles to discriminate between the two conditions. Of the 34 descriptive biomarkers identified in our study (Table 3.3), 24 are in agreement with observations made in the above-referenced studies and the reader is referred to those works for a discussion of the proposed role of each biomarker in the development of ovarian cancer. To the best of our knowledge, we are the first to describe significant differences in circulating levels of CA 15-3, Her2/neu, ACTH, DR5, sFas, sFasL, IGFBP-1, eotaxin-1, TNFRI, and kallikrein 10 in patients diagnosed with benign and malignant adnexal masses. Although most commonly associated with breast cancer, the tumor markers CA 15-3 and Her2/neu have both been previously implicated in the development of ovarian cancer [142-144]. Secretion of the pituitary hormone ACTH has been observed in a number of ovarian tumors and cell lines and has been implicated in the development of Cushing's syndrome among ovarian cancer patients in rare cases [145-146]. The apoptotic mediators DR5, Fas, and FasL have each been previously investigated in ovarian cancer resulting in the observations that elevated DR5

expression correlates with decreased overall survival [147], and the macrophage infiltrate in ovarian cancer expresses high levels of Fas and FasL [148]. Although specific roles for IGFBP-1, TNFRI, and Kallikrein 10 remain to be characterized, these biomarkers have all been shown previously to be associated with the development and progression of ovarian cancer [149-151]. The CC chemokine eotaxin-1 is an emerging biomarker for ovarian cancer with recently described serum level correlates and *in vitro* tumorigenic effects [152].

The biomarker analysis described herein provides a revealing cross-section of the physiological conditions resulting from ovarian malignancy in comparison to benign disease. Further identification of the precise roles and origins of these biomarkers will greatly improve our understanding of ovarian tumorigenesis. While the nature of our analysis does not permit such a complete characterization, we do provide a fairly comprehensive foundation for subsequent targeted biomarker studies. An overview of our findings regarding individual biomarker levels is presented in Figure 3.1. As shown in the heat map (Figure 3.1A), HE4 was the most highly elevated biomarker among cancer patients, followed by Cyfra 21-1, CA 125, and CA 19-9. This observation is reflected in the inclusion of several of these markers in our most powerful discriminatory multimarker panels. In the vast majority of cases, the magnitude of biomarker level changes, both up-regulated and down-regulated, was significantly more pronounced in late stage disease and for many biomarkers, the observed differences were significant in only the late stage patients. These findings illustrate the challenges associated with the detection of early stage disease and the need to identify more informative biomarkers. Aside from the preponderance of tumor-associated antigens near the top of the heat map, no appreciable trend in biomarker category distribution is apparent in this analysis. Trends in biomarkers levels according to category are presented in Figure 3.1B. The distribution reveals a

complex network of biological factors mediating inflammation, proliferation, apoptosis, and tissue remodeling at work during ovarian tumorigenesis. Additional work aimed at further characterizing this biomarker network in terms of function and origin is underway in our laboratory and should add valuable insight to diagnostic efforts.

A

	All Cancer	Stage I-II	Stage III-IV
HE4	1095.9	316.8	2775.6
Cyfra 21-1	380.8	158.4	857.4
CA-125	314.2	100.5	744.0
CA 19-9	300.0	376.7	135.9
IL-1Ra	*201.9*	*60.0*	483.7
CA72-4	129.0	183.0	*14.8*
IL-10	93.3	82.2	116.8
DR5	83.1	*30.5*	181.0
CA-153	78.0	44.1	152.8
IL-6	69.3	42.9	125.7
IL-2R	67.9	32.4	151.1
IL-8	66.2	75.5	46.4
TNF-RI	51.5	18.5	127.6
IL-7	35.9	31.6	45.2
MMP-9	35.8	38.2	31.2
TNF-RII	31.6	17.0	61.1
IGFBP-1	*27.9*	72.6	-66.7
tPAI 1	22.8	15.6	37.9
MPO	20.0	*16.1*	28.3
ErbB2	*16.5*	*5.1*	40.9
IP-10	*13.6*	*-6.2*	64.2
Fas	*12.0*	*2.6*	32.1
TNF-a	11.5	*-2.4*	41.2
Kallikrein 10	9.1	7.4	12.6
EOTAXIN	*5.7*	*-6.8*	32.2
sVCAM-1	-7.2	*-6.5*	-8.6
Apolipoprotein AI	-7.7	-5.6	-14.0
MMP-2	-10.3	-6.8	-17.8
EGFR	-13.1	-7.4	-25.3
sFasL	-17.2	*-13.7*	-24.8
TTR	-19.6	-11.7	-43.8
ACTH	-26.9	*-17.3*	-47.3

B

Biomarker Group	Elevated Throughout	Elevated Early	Elevated Late	Decreased
Inflammatory Mediators	IL-10 IL-1Ra IL-2R IL-6 IL-7 TNFR-1 TNFR-2	IL-8	IP-10 eotaxin-1 MPO TNFa	
Growth/Angiogenic Factors		IGFBP-1	Her2/neu	EGFR IGFBP-1
Tumor-Associated Antigens	CA 125 CA 15-3 HE4	CA 19-9 CA 72-4		
Apoptotic Proteins	Cyfra 21-1 DR5		sFas	sFasL
Proteases/Inhibitors		MMP-9	Kallikrein 10 tPAI-1	MMP-2
Adhesion Molecules				sVCAM-1
Hormones				ACTH
Other Markers				ApoA1

Figure 3.1 Summary of biomarker changes observed in the sera of women diagnosed with benign and malignant adnexal masses

A. Biomarker Heat Map. Values represent the percentage change over observed biomarkers levels in the benign group for the training set: 141 women with benign masses and 264 women diagnosed with ovarian cancer. Values are based on results presented in Table 3. Red indicates an increase in cancer levels over benign while green indicates a decrease. The minimum level of significance in differences was $p<0.05$ by Mann-Whitney U test (values in italics were non-significant). **B. Biological Trends.** Trends in biomarker level changes observed in malignant versus benign masses are organized according to each biological category of biomarkers evaluated.

Our analysis reaffirms the superior utility of the CA 125/HE4 combination reported by Moore et al. for the diagnosis of ovarian cancer [112, 137] as this combination was able to discriminate cancer patients from benign cases with sensitivities ranging from 74.2% for early stages to 91.7% for late stages at 85% SP and was also included in each of the high performing 3- and 4-biomarker panels we identified. This observation is significant given the much larger array of biomarkers examined and the more diverse set of subjects with regard to disease stage and menopausal status utilized in our study. The individual and combined sensitivities and specificities of CA 125 and HE4 observed here are very similar to those observed by Moore et al. [112, 137] , as is the observation that the two biomarkers display diagnostic complementation as each improves upon the discriminatory power of the other. In addition to CA 125 and HE4, we identified 3 other circulating biomarkers in our training analysis that offered at least modest improvement in discriminatory power when added to the 2-biomarker combination. Carcinoembryonic antigen (CEA) has been used to monitor colorectal cancer for decades and is reported to be elevated in 30-65% of ovarian epithelial cancers [153] although as an individual marker several limitations have been noted [154]**.** **A** fair amount of work has been devoted to the assessment of the Cyfra21-1 test in a variety of human cancers including lung [155], esophageal [156], head and neck [157], and cervical [158]. Recently, Baron et al. reported an increase in serum levels of Cyfra21-1 in ovarian cancer patients in comparison to benign cases and an association with disease stage [159]. Our findings regarding EGFR reaffirm those of several other groups in that lower serum levels of this marker are associated with ovarian cancer in comparison to benign cases [38, 112, 139]. Although the results of our validation analysis do not support the inclusion of CEA, Cyfra 21-1, and EGFR in a diagnostic panel at this time, our results further implicate these

biomarkers in the clinical development of ovarian cancer. Future investigations utilizing additional refinements in screening methodology may uncover more precise roles for these biomarkers in the diagnostic setting.

In our analysis of premenopausal subjects, we found that CA 125 alone provided the highest SN and SP, 70.7% and 87.5%, respectively, of any individual biomarker tested. This runs counter to most current notions concerning a lack of specificity for CA 125 in premenopausal women. One plausible explanation for this is the enrichment of CA 125 positive women in our limited patient set. All of the women in this set were initially evaluated for an adnexal mass and CA 125 results would be expected to receive priority consideration in patients in this age group, for which malignancy is more uncommon. Our finding that HE4 testing provided a lower SN than CA 125 and resulted in a reduced SN and SP when added to CA 125 also disagrees with the findings of Moore et al [112]. However, such a comparison may not be valid given the considerable discrepancies in experimental design between the two studies and the relatively small number of patients studied. Our premenopausal benign group was considerably smaller, 18 versus 82 subjects, and was compared to an age matched group of cancer cases that contained a high percentage of early stage disease. In the study by Moore et al, the premenopausal benign group was compared to a combined group of cancer patients with a mean age of 65 years and only a small percentage of early stage disease. The biomarker panels identified in our analysis of postmenopausal women were subsequently evaluated in the premenopausal group. It should be noted that this approach may not be optimal given the demonstrated clinical and biochemical differences present in the two populations. We chose this approach based on the small size of the premenopausal group, which prevented independent panel development, and also to evaluate the broader utility of our multimarker panels. We

observed that a 3-biomarker panel consisting of CA 125, HE4, and EGFR provided the highest SN and SP of any single biomarker or combination in the premenopausal group. This observation is significant in light of the recent findings of Baron et al. [38]. In that study, the authors conclude that decreased serum levels of EGFR represent a significant risk factor for ovarian cancer with particular relevance to younger, premenopausal women. Thus, our findings expand upon the notion that EGFR may offer subset-specific clinical utility as a biomarker for the early detection of ovarian cancer.

Here we report the identification and evaluation of several novel biomarker panels for the discrimination of benign from malignant cases in women diagnosed with an adnexal mass. Our findings were the result of an extensive analysis of ovarian cancer related biomarkers in the serum of a diverse group of subjects, including a large number of both early and late stage patients. Our results both corroborate and advance several recent reports regarding the importance of CA 125 and HE4 in this clinical setting and their combined use as a diagnostic test. Continuing efforts to further characterize and implement these developments should lead to improved triage methodologies for women diagnosed with adnexal masses and a positive impact on overall disease outcome.

4.0 URINE BIOMARKERS OF OVARIAN CANCER

4.1 INTRODUCTION

Efforts to identify and validate biomarkers present in the bodily fluids of ovarian cancer patients are ongoing. Investigators hope to utilize these findings in the development of minimally invasive tests to predict tumorigenesis, disease recurrence, or treatment response. The bulk of this work has focused on blood, given its systemic exposure and extensive availability through tissue banks. The analysis of blood, either through the use of serum or plasma, carries with it several inherent limitations which have hindered the development of clinically useful biomarker assays. Foremost among these limitations is the relatively high level and complex nature of the protein repertoire found in blood. Components of the blood matrix, including clotting and other serological factors, carrier proteins, immunoregulatory proteins, and active enzymes, all have the capacity to interefere with biomarker measurements. The clotting process itself, employed during the preparation of serum, has been shown to involve enzymatic activity which results in the cleavage of unrelated proteins of interest [160-161]. The invasive nature of blood testing also limits accessibility to repeated measurements and presents a risk of infection to both the patient and healthcare professionals along with the added cost of minimizing this risk.

Recently, urine has been proposed as an alternative biofluid for analytical biomarker studies on the basis that the systemic nature of such testing might be preserved while several of

the limitations inherent to blood testing could be eliminated. Urine is available in larger quantities than blood through less invasive means, allowing for repeated measurements aimed at patient surveillance or establishment of assay reproducibility. The urinary proteome is proposed to contain over 100,000 peptides, with 5000 of those present at high frequency [162], while studies have shown that this proteome is stable for hours at room temperature, days at 4˚C, and years at -20˚C [163]. The urinary proteome is a direct product of renal filtration and consists of low molecular weight, soluble peptides which are highly amenable to proteomic analysis and may represent disease specific cleavage processes [164]. Renal filtration also results in a less complex matrix than that of blood, containing fewer factors known to interfere with biomarker assays [76]. The use of urine as a diagnostic biofluid does present unique challenges including a high variability in protein concentrations due to differences in fluid intake. However, this barrier has been overcome successfully through normalization based on levels of creatinine or other common urinary peptides [165-166]. What remains in the development of urine-based analytical platforms is evidence that systemic disease-specific biomarkers are released into this biological compartment in a manner which can be reliable measured.

Traditionally, investigations focused on urinary biomarkers have been limited to those related to disorders of the urogenital system, although it is estimated that only 70% of the urinary proteome originates from the kidneys or urinary tract with the remaining 30% resulting from glomerular filtration of blood plasma [167]. Urine, therefore, can be considered a systemic biofluid with expanded clinical applications. A number of significant findings have been reported through the analysis of urine obtained from ovarian cancer patients. Several early reports characterized the use of urinary gonadotropic peptide (UGP) as a general biomarker of gynecologic malignancy [168-170]. The combination of UGF and serum CA 125 proved particularly

useful in the diagnosis of ovarian serous carcinomas, providing a SN/SP of 86/89 [169]. More recently, several other biomarkers including HE4 [171], mesothelin [172], Bcl-2 [33], and angiostatin [173] were found to be differentially present in the urine of ovarian cancer patients and controls. In their respective studies, urinary HE4 was found to discriminate ovarian cancer patients from controls at a level similar to that of serum HE4, while urinary mesothelin outperformed its serum counterpart. Proteomic-based studies performed by several independent research groups have identified a number of additional urinary biomarkers and biomarker panels offering diagnostic potential for ovarian cancer [70, 76, 174-176]. Notable among these findings are a 3-biomarker panel which, in combination with CA 125, could discriminate malignant from benign pelvic masses with an AUC of .96 [175], and the combination of glycosylated eosinophil-derived neurotoxin (EDN) and C-terminal osteopontin fragments which provided a SN of 94% at a SP of 72% for early stage ovarian cancer compared with benign controls [76].

In the current study, urines obtained from a heterogeneous group of ovarian cancer patients, women diagnosed with benign ovarian disease, and a group of healthy control women were evaluated for levels of various biomarkers previously identified to be useful in several serum biomarker analyses. Nearly all of the tested biomarkers were readily detectable in urine and many demonstrated highly significant alterations between the case and control groups. Several multiplexed urine biomarker panels were identified which provided a high level of discrimination between the groups. Overall, these results demonstrated that certain urine biomarkers and multimarker panels are capable of outperforming similar serum-based tests for diagnostic purposes and the combined use of urine and serum biomarker testing may provide a superior means of patient classification.

4.2 MATERIALS AND METHODS

4.2.1 Human Urine Samples

Urines were collected from women diagnosed with epithelial ovarian cancer (n=109), benign ovarian or pelvic lesions (n=118), and healthy control women (n=72) seen at the University of Texas MD Anderson Cancer Center, Houston, TX (Table 4.1). Women diagnosed with epithelial ovarian cancer underwent full surgical staging or tumor debulking as clinically indicated. Benign subjects were women diagnosed with an ovarian cyst or pelvic mass, some of whom were scheduled to undergo surgical resection of the lesion. All surgical tissues were examined by a gynecologic pathologist and final surgical pathology reports were obtained and recorded. Healthy controls were women seen within the University Health System with no history of malignancy or other gynecological disorder and were included in the study based on age-matching. Serum CA-125 measurements, performed using the Architect CA125II assay (Abbott Diagnostics, Abbot Park, IL) were obtained from women diagnosed with epithelial ovarian cancer (n=108) and benign lesions (n=101) as clinically indicated. Serum CA-125 measurements were also obtained from Healthy controls (n=61) when available. All urines were collected prior to surgery or treatment. Samples were collected and frozen at -80°C on the day of collection. Written informed consent was received from each subject, and protocols were approved by the local institutional review board. Urines were shipped frozen to UPCI for biomarker testing. No more than two freeze/thaw cycles were permitted throughout the testing process.

Table 4.1 Patient characteristics for urine biomarker analysis

Group	Age	Histology	Stage
Healthy	Range 49-85		
N=72	Median 62		
	Average 59		
Benign	Range 47-86	Non-malignant neoplasms (n=41)	
N=118	Median 62	Benign cysts (n=36)	
	Average 61	LMP tumors (n=15)	
		Other benign lesions (n=26)	
Cancer	Range 48-87	Serous (n=72)	I (n=5)
N=108	Median 63	Endometrioid (n=7)	II (n=5)
	Average 62	Mucinous (n=3)	III (n=85)
		Mixed (n=19)	IV (n=13)
		Undifferentiated/Unknown (n=7)	

LMP – low malignant potential

4.2.2 Urine Biomarker Testing

Each urine was tested for fifteen biomarkers chosen on the basis of previous performance in several serum-based biomarker analyses of ovarian cancer[46, 177]. The tested biomarkers included: HE4, cytokeratin 19 (Cyfra 21-1), sEGFR, sErbB2, sIL-2R, sICAM-1, CEA, Eotaxin-1, sVCAM-1, CA 15-3, tPAI-1, CA 125, MMP-9, MPO, and CA 72-4. Assays for sIL-2R, sICAM-1, Eotaxin-1, sVCAM-1, and MPO were obtained from Millipore (Billerica, MA). The assay for MMP-9 was obtained from R&D Systems (Minneapolis, MO). All other assays were developed by the UPCI Luminex® Core Facility (Pittsburgh, PA) as described in Introduction section 1.3.1. All biomarker assays were performed according to manufacturer's protocol or as described in Introduction section 1.3.1. Urine creatinine measurements were determined for a subset of the study cohort using the PARAMETER Creatinine ELISA kit (R&D Systems, Minneapolis, MO), performed at the collection site.

4.2.3 Statistical Analysis

Individual biomarker levels were compared between the ovarian cancer and control groups using the Mann-Whitney non-parametric U test. The False Discovery Rate was controlled at 5% using the method of Benjamini and Hochberg[178]. After controlling, the minimum level of significance was $p<0.04$. Reciever operating characteristic (ROC) curves were generated from the biomarker results using GraphPad PRISM (La Jolla, CA) and area under the curve (AUC) values were computed for the classification of ovarian cancers from controls. The ROC AUC analysis was repeated in several previously collected and reported serum biomarker datasets [46, 177] for purposes of comparison. The multivariate analysis of the biomarker data was performed using the Metropolis Algorithm with Monte Carlo simulation (MMC) (described in Introduction section 1.3.1). Serum CA-125 results, obtained from the site of collection were included in this analysis. All possible multimarker panels consisting of 2, 3, or 4 biomarkers were evaluated for sensitivity (SN) at fixed specificities (SP) of 95% (ovarian cancer vs. healthy subjects) or 90% (ovarian cancer vs. benign subjects). These SP values were chosen in order to provide a basis for comparison with previous findings[46, 177]. ROC curves and AUC values were generated for the top performing panels using GraphPad PRISM (La Jolla, CA).

4.3 RESULTS

4.3.1 Individual Urine Biomarker Analysis

The complete results for the individual analysis of urine biomarkers in ovarian cancer patients, benign subjects, and healthy controls are presented in Table 4.2. Each of the 15 evaluated biomarkers were detectable in urine with the exception of CA 72-4. The remaining 14 urine biomarkers all differed significantly in the comparison of ovarian cancer patients and healthy controls. Of these, 11 were observed at elevated levels in the ovarian cancer group while 3 were decreased. The most significantly altered urine biomarker in this comparison was HE4, followed by Cyfra 21-1, sEGFR, sErbB2, and sIL-2R. Seven biomarkers differed significantly in the comparison of ovarian cancer patients and benign subjects, each of them observed at higher levels in the cases. HE4 was also the most significantly altered biomarker in this comparison, followed by CA-125, sIL-2R, and sVCAM-1.

Table 4.2 Urine biomarker levels in ovarian cancer patients and benign and healthy control subjects

Biomarker	Units	Median			p-value†	
		Healthy	Benign	Cancer	Cancer vs. Healthy	Cancer vs. Benign
HE4	ng/ml	40.7	208.3	1897.7	2.21×10^{-26} (I)	2.11×10^{-25} (I)
Cyfra 21-1	U/ml	1937.9	23087	28859.2	8.57×10^{-25} (I)	0.00073 (I)
sEGFR	pg/ml	121.9	340.9	375.6	3.48×10^{-20} (I)	NS
sErbB2	pg/ml	73.3	237.9	273.8	6.46×10^{-19} (I)	NS
sIL-2R	pg/ml	176.15	251.1	547.5	9.24×10^{-18} (I)	3.39×10^{-11} (I)
sICAM-1	pg/ml	1718.9	4200.3	5627.8	1.62×10^{-14} (I)	0.00030 (I)
CEA	pg/ml	4655.6	1447.3	1079.4	2.5×10^{-14} (D)	NS
Eotaxin-1	pg/ml	1.9	2.2	2.3	6.35×10^{-12} (I)	NS
sVCAM-1	pg/ml	787.7	776.9	3811.6	1.48×10^{-11} (I)	1.18×10^{-10} (I)
CA 15-3	U/ml	7.2	39.6	37.5	6.14×10^{-10} (I)	NS
tPAI-1	pg/ml	5.5	5.8	5.9	2.50×10^{-9} (I)	0.03337 (I)
CA-125	U/ml	1.4	1.3	5.7	3.61×10^{-9} (I)	5.53×10^{-15} (I)
MMP-9	pg/ml	991.9	293.1	404.2	0.00119 (D)	NS
MPO	pg/ml	5625	2262	2409	0.01548 (D)	NS

†p-value determined by Mann-Whitney nonparametric U test , FDR controlled at 5%

To evaluate the need to adjust individual biomarker levels based on factors such as fluid intake or kidney function, urine creatinine levels were determined in a subset of ovarian cancer patients (n=38) and healthy controls (n=29). The distributions of creatinine levels within the two groups were markedly similar and the mean creatinine levels did not differ significantly (Figure 4.1). When the urine biomarker results were normalized based on creatinine levels in the evaluated subset, the statistical significance of each of the analytes listed above remained unchanged although a general elevation of p-values was observed (data not shown).

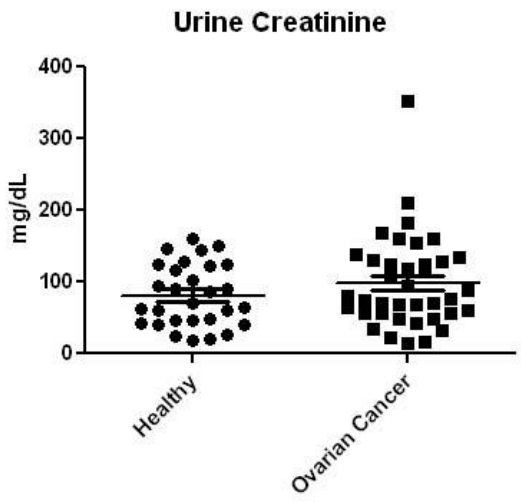

Figure 4.1 Urine creatinine levels in ovarian cancer patients and healthy controls

Urine creatinine levels were measured in a subset of ovarian cancer patients (n=38) and healthy controls (n-29) by ELISA. Mean with 95% confidence interval shown.

4.3.2 Comparison of Urine and Serum Biomarkers

Each of the 14 detectable urine biomarkers was evaluated for the capacity to discriminate cases from controls by ROC analysis. The AUC values for each of these biomarkers was compared to those obtained in two previous studies of the same biomarkers measured in serum (Table 4.3, Figure 4.2). In the comparison of ovarian cancer patients and healthy controls, 9 of the 14 biomarkers demonstrated higher AUC values in urine vs. serum with completely non-overlapping 95% confidence intervals (CI). Two biomarkers, CA 125 and MPO, provided significantly greater AUCs in serum vs. urine. The three highest performing individual biomarkers in either urine or serum were urine HE4, urine Cyfra 21-1, and urine sEGFR. When the ovarian cancer group was compared to the benign group, sVCAM-1 and HE4 provided significantly higher AUC values in urine while sEGFR performed significantly better in serum.

Urine HE4 was the most diagnostic biomarker out of any tested for this comparison, while the performance of CA 125 was nearly equivalent in both urine and serum.

Table 4.3 Classification performance of individual urine and serum biomarkers

Cancer vs Healthy (AUC (95% CI))[†]			Cancer vs Benign (AUC (95% CI))[†]		
	Urine	Serum[‡]		Urine	Serum*
ErbB2	.892 (.844-.940)	.530 (.396-.664)	sVCAM-1	.749 (.685-.812)	.579 (.520-.639)
sICAM-1	.834 (.780-.898)	.525 (.419-.632)	sIL-2R	.757 (.693-.820)	.651 (.597-.704)
sEGFR	.906 (.864-.949)	.605 (.535-.675)	HE4	.903 (.860-.946)	.799 (.756-.841)
CEA	.836 (.777-.896)	.551 (.494-.608)	sICAM-1	.640 (.567-.712)	.555 (.493-.616)
sIL-2R	.880 (.831-.928)	.692 (.627-.758)	CEA	.573 (.498-.648)	.532 (.476-.588)
Eotaxin	.804 (.739-.868)	.631 (.559-.702)	ErbB2	.550 (.475-.626)	.532 (.476-.589)
sVCAM-1	.798 (.734-.861)	.628 (.566-.690)	CA 125	.802 (.744-.859)	.799 (.754-.845)
HE4	.970 (.949-.991)	.858 (.805-.911)	Eotaxin	.518 (.442-.594)	.516 (.459-.574)
Cyfra 21-1	.954 (.924-.984)	.860 (.819-.902)	MPO	.555 (.450-.630)	.559 (.500-.619)
tPAI-1	.763 (.692-.834)	.675 (.612-.739)	Cyfra 21-1	.630 (.558-.703)	.649 (.598-.700)
CA 15-3	.774 (.702-.845)	.822 (.741-.902)	MMP-9	.558 (.483-.634)	.603 (.536-.670)
MMP-9	.643 (.560-.726)	.706 (.593-.819)	tPAI-1	.582 (.508-.657)	.641 (.585-.696)
CA 125	.760 (.692-.829)	.905 (.866-.944)	CA 15-3	.556 (.478-.633)	.648 (.594-.702)
MPO	.607 (.521-.693)	.830 (.739-.920)	sEGFR	.523 (.448-.599)	.706 (.657-.754)

[†]Determined by ROC analysis; Serum biomarker results based on analysis reported in [‡]Yurkovetsky et al. JCO (2010)[46] and *Nolen et al. Gyn Onc (2010)[177]; AUCs values in bold are significantly greater based on non-overlapping 95% CI

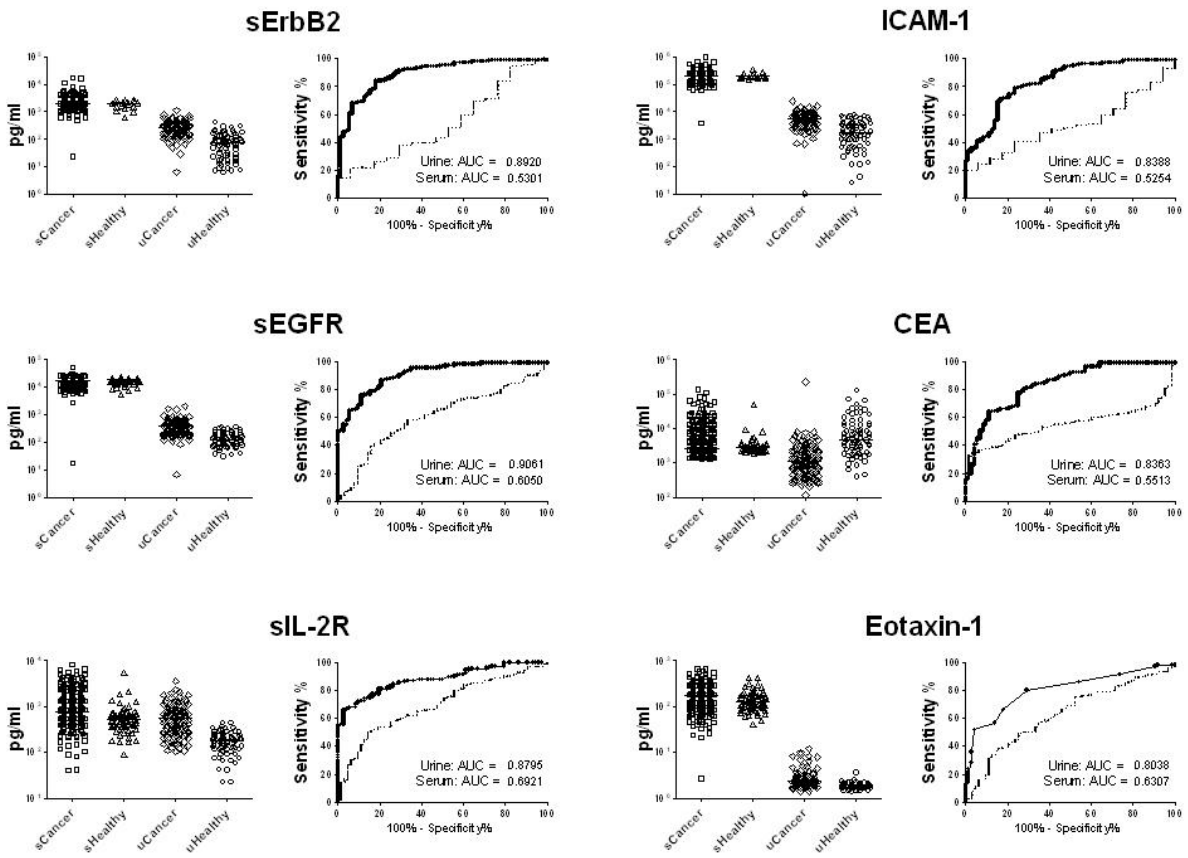

Figure 4.2 Comparative performance of urine and serum biomarkers

4.3.3 Multivariate Analysis

The MMC algorithm identified a number of urine biomarker panels which were capable of discriminating ovarian cancer cases from controls with high SN and SP (Table 4.4, Figure 4.3A). In the analysis of ovarian cancer patients and healthy controls, several 3-biomarker panels were identified which offered significant improvement over serum CA-125. Each of these panels outperformed all possible urine 2-biomarker panels while the addition of a fourth urine biomarker did not result in an improvement in classification ability. The highest performing three biomarker panel, comprised of u(urine)HE4, uCEA, and uCyfra 21-1, provided a SN of 96% at a SP of 95%. This panel also correctly classified 100% (n=10) of the stage I/II ovarian cancer cases. Replacing uCyfra21-1 with either uEotaxin-1 or uCA 15-3 resulted in only small reductions in SN, while the replacement of uCyfra 21-1 with uEGFR caused a more significant decrease. In order to investigate the efficacy of combining biomarker information from urine and serum, s(serum)CA-125 was combined with the urine biomarker panel of uHE4, uCEA, and uCyfra 21-1. This combined urine/serum panel achieved a SN of 99% at a SP of 95% and was also 100% accurate in the identification of early stage disease.

Table 4.4 Performance of multimarker panels for the discrimination of ovarian cancer patients from healthy and benign subjects

Cancer vs. Healthy	SN	SP	Cancer vs. Benign	SN	SP
uHE4,uCEA, uCyfra 21-1, sCA-125	99	95	uHE4, uCEA, sCA-125	85	90
uHE4, uCEA, uCyfra 21-1	96	95	uHE4, sCA-125	84	90
uHE4, uCEA, uEotaxin-1	94	95	uHE4, uCA-125	77	90
uHE4, uCEA, uCA 15-3	94	95	uHE4	78	90
uHE4, uCEA, uEGFR	91	95	sCA-125	71	90
sCA-125	87	95			

Panels identified and characterized using Metropolis algorithm with Monte Carlo simulation (MMC); u – urine biomarker, s - serum biomarker

For the discrimination of ovarian cancer patients from benign subjects, a two biomarker panel consisting of uHE4 and uCA-125 performed optimally, providing a SN of 77% at a SP of 95%, however this combination failed to outperform uHE4 alone. When sCA-125 was substituted for uCA-125, SN was significantly improved to 84% (Table 4.4), although the ROC AUC of the panel was relatively unchanged (Figure 4.3B). The addition of uCEA to this urine/serum combination resulted in only a minor improvement in SN.

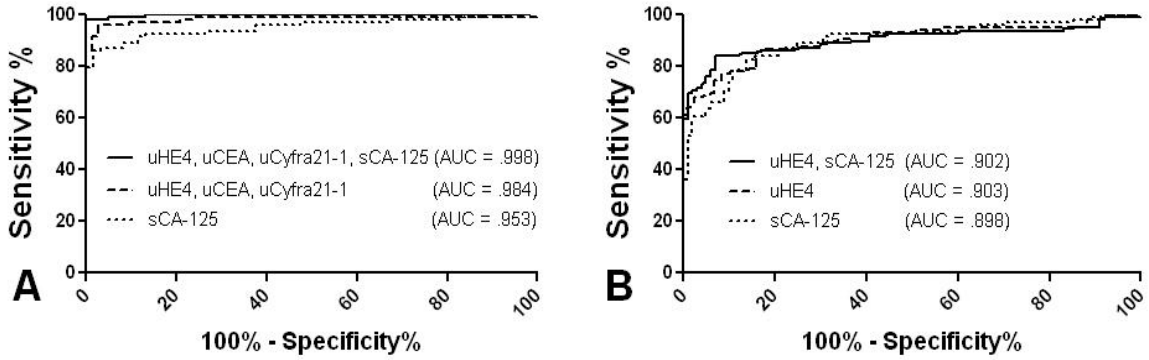

Figure 4.3 Top performing multimarker panels for the diagnosis of ovarian cancer

4.4 DISCUSSION

The topic of routine screening for ovarian cancer has received considerable attention from clinical researchers despite a high level of controversy. Such controversy largely centers on the large number of false-positive test results associated with efforts to detect a condition of very low prevalence. The result is a high degree of unnecessary treatment, invasive diagnostic testing, and patient anxiety. The minimally acceptable positive predictive value (PPV) of 10% (1 case identified for every 10 individuals tested) required for effective screening necessitates

diagnostic tools which provide a high level of SN and SP [31-32]. Currently used tools such as CA-125 testing in blood and imaging procedures such as transvaginal ultrasound (TVS) have failed to perform to this standard [30]. A number of studies, including two large randomized control trials [179-180], are currently investigating the combined use of CA-125 testing and TVS for screening purposes, however, improvements upon the individual performance of CA-125 are certainly needed for such a strategy to succeed. Although many additional blood-based biomarkers for ovarian cancer have been identified and evaluated, little progress has been achieved in the development of diagnostic tests. The current study demonstrates that several previously identified serum biomarkers of ovarian cancer provide greater levels of diagnostic utility when evaluated in urine. The results of this preliminary analysis suggest that urine biomarker panels may provide levels of SN and SP for the discrimination of ovarian cancer patients from healthy controls approaching those required for routine screening. The expanded use of urine as a testing matrix may not only result in the improved performance of previously identified biomarkers, but may also provide a basis for the identification of additional useful biomarkers, as in the study conducted by Ye et al [76].

Each of the 14 urine biomarkers observed to be altered between cases and controls in this study have been examined previously within the setting of ovarian cancer. The five biomarkers shown to be most useful with regard to diagnostic panel development: HE4[46, 137, 181], CEA[46, 182], Cyfra 21-1[159, 183], Eotaxin-1[152, 184], and CA 15-3[185], have all shown some promise as markers of early detection, prognosis, and disease monitoring. Interestingly, CA 125, the most widely studied and utilized serum biomarker of ovarian cancer, was not found to be useful in urine. Urine CA 125 did not productively contribute to diagnostic panel development and the individual performance of serum CA 125 in the discrimination of ovarian cancer cases from

healthy controls significantly exceeded that of urine CA 125. A plausible explanation for these observations stems from the considerable size of the CA 125 glycoprotein, estimated at 3-5MDa, and the estimated molecular weight cutoff associated with glomerular filtration, 30-50kDa. CA 125 was detectable in urine, suggesting that fragments of the molecule do indeed pass through the glomerulus in a form which can be recognized by the immunoassay, however the observed results indicate that cleavage processes responsible for such fragmentation are not reliable indicators of serum CA 125 levels. Several other biomarkers included in this investigation are also relatively large, with molecular weights greater than 100kDa, including CA 15-3, sEGFR, CEA, sVCAM-1 and MPO. A recent study examining the mechanisms of glomerular filtration concluded that in addition to molecular size, additional factors such as molecular conformation, charge, and deformability account for the ability of an individual molecule to be filtered [186]. The authors of that study demonstrate that molecules as large as 350-500kDa are rapidly cleared intact through the glomerulus. Such a phenomenon may indeed play a role in the detection of protein biomarkers listed above, however is also likely that the observed urine biomarker levels represent specific proteolytic cleavage processes. The latter notion is supported by the observation that several urine biomarkers including CA 125, EGFR, MMP-9, MPO, and sVCAM-1 exhibited differing trends among the cancer, benign, and healthy groups than their serum counterparts.

The accurate and efficient triage of women presenting with a pelvic mass based upon risk of malignancy is a unique clinical setting in which diagnostic biomarker panels might provide a significant benefit in the short-term. Effective and timely triage of this clinical group not only serves to reduce the number of invasive diagnostic procedures for the vast majority of those women whose masses are benign, but has also been shown to decrease morbidity and improve

overall survival through the referral of patients with malignancies to appropriately trained gynecological oncologists [6, 187-190]. Several recent reports investigating the efficacy of biomarker panels within this setting have identified the combination of CA 125 and HE4 as an effective diagnostic tool capable of discriminating benign from malignant pelvic masses with high SN and SP [112, 177]. This combination later demonstrated efficacy in a prospective study [137] and was subsequently incorporated into a scoring model termed the Risk of Ovarian Malignancy Algorithm (ROMA) [191]. In the current study, the combination of urine HE4 and serum CA 125 was optimal for the classification of ovarian cancer patients and benign controls. As was the case in the comparison of ovarian cancer patients and healthy controls, the use of urine CA 125 was not beneficial. Here, uHE4 outperformed sHE4 on an individual basis in both group comparisons and the SN and SP of the uHE4/sCA 125 panel in this study is superior to that of sHE4, sCA 125 in a previous study [177]. In a separate similarly designed study, uHE4 was reported to perform at a level on par with sHE4 [171], however the sampling sizes used in that study were considerably smaller than those here. Indeed, additional work aimed at the differential use of the HE4 biomarker is warranted.

An investigation into the potential benefits of urine as an analytical biofluid for biomarker development demonstrated that the diagnostic utility of a number of previously identified serum biomarkers of ovarian cancer was augmented upon testing in urine. The study design does have several limitation which should be addressed going forward. The benign subject group contained a small number of women diagnosed with low malignant potential (LMP) tumors and endometriosis. While these conditions reflect distinct pathologies which may serve to confound biomarker experiments, their presence within this control group is indicative of the clinical setting under investigation. Additional biomarker studies focusing particular

attention upon these groups should further refine efforts to properly triage these patients. It should be noted that nearly all of the LMP tumors were classified as "cancer" by the uHE4, sCA 125 model. Such a classification appears most prudent at this time. This investigation was also limited by the small number of early stage cases included. While the best performing model of uHE4, uCEA, and uCyfra 21-1, with or without the inclusion of sCA 125, correctly classified 100% of the stage I/II ovarian cancers, additional studies utilizing larger cohorts of early stage patients will be needed to further demonstrate the efficacy of urine biomarker panels. In conclusion, urine biomarkers used as an alternative to or in combination with serum biomarkers offer a minimally invasive and effective means of ovarian cancer detection.

5.0 SUMMARY AND CONCLUSIONS

5.1 BIOMARKERS AND TARGETED THERAPIES FOR OVARIAN CANCER

5.1.1 Introduction

Throughout the course of the last three decades, the incremental optimization of surgical techniques and chemotherapeutic regimens has achieved a meaningful impact on the overall management of ovarian cancer. The current standard-of-care use of combination carboplatin and paclitaxel as a first-line therapy now yields response rates of over 80%, with complete response rates of 40-60% [192-198]. Despite these advances, current treatment regimens remain characterized by disappointment due to a failure to extend progression-free survival in advanced patients, a persistently high rate of relapse following first-line therapy, and an overall inability to produce a cure at diagnosis [195, 199]. Thus, the identification and development of targeted therapies has moved to the forefront of ovarian cancer research. A number of promising therapeutic targets have been identified in recent years, with a large number of clinical trials initiated (Table 5.1). Novel drugs, in the form of monoclonal antibodies and small molecule inhibitors, are under development which target specific components of the multiple molecular pathways shown to play a role in the development of ovarian cancer. The high degree of biological heterogeneity which characterizes ovarian cancer, wherein the dysregulated tumorigenic pathway varies on an

individual basis, has hindered the clinical impact of targeted therapies and emphasizes the need for improved tools aimed at identifying those patients most likely to benefit from a particular treatment.

Table 5.1 Recent, ongoing, and planned clinical trials of targeted agents in ovarian cancer

Agent	Target	Class	Phase	Clinical Trials
Bevacizumab	VEGFA	Monoclonal Antibody	I-III	14
Aflibercept	VEGF	Inhibitor	II	1
Sunitinib	VEGFR	Inhibitor	II	1
Cediranib	VEGFR	Inhibitor	II-III	3
Sorafenib	VEGFR, PDGFR, c-Kit	Inhibitor	I-II	5
Pazopanib	VEGFR	Inhibitor	II-III	2
Cetuximab	EGFR	Monoclonal Antibody	II	3
Matuzumab	EGFR	Monoclonal Antibody	II	1
Erlotinib	EGFR TK	Inhibitor	I-III	5
Gefetinib	EGFR TK	Inhibitor	II	5
Trastuzumab	ErbB2	Monoclonal Antibody	II	1
Olaparib	PARP	Inhibitor	II	2
Farletuzumab	α-FR	Monoclonal Antibody	II	1

The findings presented in chapter 2 of this dissertation provide evidence that serum biomarker profiles can provide information regarding the histological subtype of epithelial ovarian cancer. Previous findings aimed at characterizing these disease subtypes have revealed a number of distinct molecular alterations and differential clinical behavior associated with each of these subtypes. A bioinformatic pathway analysis of the results presented in chapter 2 revealed a number of experimentally defined links between the current biomarker findings and previously reported molecular alterations. Taken collectively, this work suggests that serum biomarkers may serve as effective tools in the identification of specific patients and groups of patients likely to benefit from a given type of targeted therapy for ovarian cancer. A detailed and informed analysis of serum biomarkers could not only provide information regarding the histology of the

disease, but also the specifically dysregulated biological pathways underlying the development of that disease. In the following sections, several of the most promising avenues for targeted therapeutic development in which there is an unmet need for improved patient selection are discussed.

5.1.2 Targeting tumor angiogenesis

The process of angiogenesis is a critical element in the development of virtually all types of solid tumors. The formation of new blood vessels through angiogenesis is required for tumor growth beyond 1-2mm and the initiation of this process often marks a transition from tumor dormancy to full malignancy [200-201]. Although the process of angiogenesis represents a complex and highly regulated series of mechanisms, several prominent players, namely the vascular endothelial growth factor (VEGF) family and its receptors (VEGFR1-3), have been identified [202]. These factors have served as targets for therapeutic development in a number of malignant settings, with anti-angiogenic agents currently moving from Phase II to Phase III clinical trials in ovarian cancer [203].

The most intensely evaluated anti-angiogenic agent is Bevacizumab, a recombinant humanized monoclonal antibody directed against VEGFA. Bevacizumab has been evaluated in several clinical trials of ovarian cancer with response rates ranging from 16-21% and a six-month progression-free survival (PFS) of 40.3% [204-205]. Two large randomized trials (GOG 218, ICON 7) of Bevacizumab as a first line therapy in combination with carboplatin and paclitaxel are currently underway with the aim of assessing PFS in comparison to chemotherapy alone. Other types of anti-angiogenic agents currently in clinical trials in ovarian cancer include VEGF trap (Aflibercept) [206], a fusion protein consisting of the VEGF binding domains of VEGFR1/2 and the

Fc region of IgG, and several small molecule inhibitors of receptor tyrosine kinases association with VEGF signaling: cediranib, sunitinib, sorafenib, and pazopanib [207-211].

Several studies have identified subtype-specific properties of angiogenesis within the setting of ovarian cancer. These findings indicate that agents targeting VEGF or VEGF signaling may be particularly useful in the treatment of ovarian clear cell and mucinous carcinomas [212-213], however much additional work is needed in order to further define the differential use of angiogenic mechanisms. Biomarkers which convey information regarding the reliance of individual ovarian tumors or tumor subtypes on specific VEGF receptor/ligand interactions and downstream signaling events would greatly enhance the effectiveness of anti-angiogenic agents.

5.1.3 Targeting the EGFR family

The epidermal growth factor receptor (EGFR) family and its ligands have well-documented roles in the development of ovarian cancer (reviewed in [144]). As such, a number of agents targeting EGFR have been developed and evaluated in ovarian cancer. These include several monoclonal antibodies directed against the receptor itself (cetuximab, panitumumab, and matuzumab), and also several small molecule tyrosine kinase inhibitors (erlotinib and gefitinib). The efficacy of each of these agents in clinical trials has been extremely limited [214-228]. Differential levels of soluble EGFR in the sera and urines of ovarian cancer patients and controls were noted in each of the studies presented in this dissertation and the diagnostic potential of serum EGFR in ovarian cancer has been reported previously [38, 46]. While additional work is necessary in order to characterize the relationship between circulating levels of EGFR and EGFR-dependence in tumors, further examination into the predictive value of circulating EGFR with regard to EGFR-

targeted therapy appears warranted. Likewise, while the results of Chapter 2 indicate no significant difference in EGFR levels between Type I and II ovarian carcinomas, additional studies focusing on individual carcinoma subtypes may yield more informative results.

An additional member of the EGFR family, ErbB2, is an important tumor marker in breast cancer and is also overexpressed in a subset of ovarian cancers [144]. The ErbB2-directed monoclonal antibody trastuzumab (Herceptin) has been evaluated in ovarian cancer with modest efficacy [229]. In the current set of studies, serum levels of sErbB2 were significantly increased in ovarian cancer patients and this increase was more apparent in urines. Additional serum or urine-based studies evaluating the predictive properties of this biomarker would be warranted. ErbB2 may be particularly overexpressed in mucinous ovarian carcinomas, indicating a potential avenue for improved efficacy [230].

5.1.4 PARP inhibitors

The Poly-ADP-ribose polymerase (PARP) proteins have emerged as popular targets for anticancer therapy given their documented roles in several oncogenic pathways including cell-cycle control, cellular differentiation, and DNA repair [231]. Treatment with PARP inhibitors leads to the accumulation of single-strand DNA breaks in tumor cells [232], and this observation has prompted the clinical investigation of these agents within the setting of *BRCA1/2*-related ovarian cancer. Previous work has shown that tumor cells harboring mutations in the *BRCA* genes are highly sensitive to PARP inhibitors, likely due to the DNA double strand break repair deficiencies displayed by those cells [233]. The PARP inhibitor olaparib has demonstrated a dose-dependent high response rate in a phase II trial of this type [234].

Phenotypic similarities between *BRCA*-associated ovarian tumors and high-grade serous tumors, type-2 ovarian carcinomas, suggest the expanded use of PARP inhibitors in this subtype of sporadic disease [235]. Indeed, several studies have reported the loss of function of a number of DNA-repair pathway related proteins, including BRCA1/2 in high grade serous tumors [236-237]. In light of these findings, the targeting of type 2 ovarian carcinomas through the aid of specific biomarkers for treatment with PARP inhibitors may prove effective.

5.1.5 Additional targets

The alpha folate receptor (α-FR) is expressed on over 70% of primary and 82% of recurrent ovarian tumors [238]. Expression of α-FR is particularly high in non-mucinous carcinomas and correlates with tumor grade, suggesting the potential for subset targeting. A monoclonal antibody to α-FR, farletuzumab, has shown promising activity in preclinical and clinical studies [239-240].

The insulin-like growth factor (IGF) family of proteins represents an important group of regulators of cell proliferation and survival. Members of this family have emerging roles in carcinogenesis and tumor progression and are the targets of novel therapeutic development [241-245]. Several IGF-related proteins have been evaluated in serum as potential biomarkers of ovarian cancer [46, 62], in addition to the evaluation of IGFBP-1 within this current study. A monoclonal antibody directed against the IGF-1 receptor, AMG 479 has demonstrated potent inhibition of the PI3-Akt pathway in xenograft mouse models of pancreatic cancer and also enhances the anti-tumor effects of several anti-EGFR targeted agents [246]. Clinical trials involving AMG 479 in ovarian cancer are planned.

Activation of the PI3-Akt pathway in ovarian cancer appears to play an important role in ovarian carcinogenesis and my represent a mechanism of resistance to therapies targeting the EGFR signaling axis [221, 247]. *AKT2* alterations are prominent in ovarian tumors, particularly in the more aggressive type II carcinomas [101, 125], while mutations in *PI3k* have been associated with endometrioid ovarian carcinomas [248]. These observations indicate that careful targeting will be required to achieve an optimal therapeutic impact for these targets. A number of inhibitors of PI3k and Akt family members (rapamycin, temsirolimus, everolimus, deforolimus) have shown promising preclinical results and are now entering phase I clinical trials [249].

5.2 BIOMARKER PANELS AS SCREENING TOOLS FOR OVARIAN CANCER

5.2.1 Introduction

The development of multimarker panels for the diagnosis of ovarian cancer is currently advancing on two fronts. The first of these fronts includes investigators seeking improved tools for use in screening strategies for ovarian cancer in the general population. Given the limited performance of currently used imaging techniques and CA 125 testing as well as the overall rarity of ovarian cancer, routine population-based screening is not recommended by any of the major relevant professional societies [250]. It also remains unlikely that any standalone biomarker-based screening test will be capable of overcoming the 10% PPV level required for implementation. However, work has persisted based on the notion that biomarker testing may prove effective in sufficiently defined high risk groups or as part of a multimodal screening strategy involving TVS or an equivalent imaging method as a second-line test. The second front

in the clinical use of ovarian cancer biomarkers pertains to the use of multimarker panels in the triage of women presenting with a pelvic mass. Effective and timely triage of this clinical group not only serves to reduce the number of invasive diagnostic procedures for the vast majority of these women with benign masses, but has also been shown to decrease overall morbidity and improve overall survival through the referral of patients with malignancies to appropriately trained gynecological oncologists within specialized centers of excellence [6, 188, 190].

The findings described in Chapters 3 and 4 of this dissertation represent important measures of progress within each of the developmental fronts outlined above. The identification of several biomarker panels useful in the discrimination of benign and malignant pelvic masses, including the optimal performance of the CA 125/HE4 combination, is an advancement upon previous findings within this clinical setting and a foundation for ongoing work. The results presented in Chapter 4 demonstrate the potential for improved performance of biomarker-based screening tests through the use of urine. Further advancement along these lines should bring such screening tests closer to widespread implementation. Recent efforts in the development of biomarker-based screening tools are discussed in this section.

5.2.2 Biomarker panels for routine screening

Despite the lofty performance standards currently in place for ovarian cancer screening tests, a number of research groups have reported findings which offer considerable promise and warrant further attention (Table 5.2). Perhaps most notable among these reports is that of a six-biomarker panel comprised of CA 125, leptin, prolactin, osteopontin, IGF-II, and macrophage inhibitory factor (MIF) which offered 95.3% SN at 99.4% SP in the discrimination of ovarian cancer patients from healthy controls [62]. Following a high level of initial enthusiasm and the

subsequent marketing of this panel under the trade name OvaSure, a number of deficiencies in study design have been identified which illustrate the challenges facing biomarker development efforts in general. Most prominent among these deficiencies was the drastic overestimation of PPV based on inaccurate calculation of ovarian cancer prevalence [251-252]. This observation coupled with the lack of evaluation in a large prospective study led to performance revisions and the withdrawal of the commercial kit. In a recent report, our group sought to more adequately address the issue of disease prevalence by utilizing a subject cohort which included more than 2000 healthy women [46]. While it should be noted that our cohort included only postmenopausal women and the prevalence of ovarian cancer within the cohort remained elevated with respect to the general population, our identified panel of CA 125, HE4, carcinoembryonic antigen (CEA), and vascular cell adhesion molecule-1 (VCAM-1) was found to discriminate early-stage ovarian cancer from the control group with 86% SN at 98% SP.

Table 5.2 Multimarker panels which discriminate ovarian cancer cases from healthy controls.

Panel	Cases	Controls	SN	SP	Reference
CA 125, leptin, prolactin, osteopontin, IGF-II, MIF	156†	362†	95.3	99.4	Visintin et al.[62]
CA 125, HE4, CEA, VCAM-1	456†	2000†	86-93	98	Yurkovetsky et al.[46]
CA 125, HE4, Glycodelin, Plau-R, MUC1, PAI-1	200	396	80-89	87	Havrilesky et al. [253]
CA 125, CRP, SAA, IL-6, IL-8	150†	212†	94.1	91.3	Edgell et al. [254]
CA 125, HE4, SI*	74	137	84	98.5	Andersen et al. [255]
CA 125, TTR, ApoA1	200	82	89	92	Su et al. [256]
CA 125, IL-6, IL-8, VEGF, EGF	44	45	85	95	Gorelik et al. [105]
CA 125, ApoA1, TTR, H418	200†	142†	74	97	Zhang et al. [65]
CA 125, CA 72-4, M-CSF	123†	224†	70	98	Skates et al. [60]

*Symptom index; †includes independent validation set

In Chapter 4 of this dissertation, it is demonstrated that three of the four biomarkers included in the panel above (HE4, CEA, and VCAM-1) provide a greater level of discrimination in urine compared to serum. While a direct comparison of similarly designed urine and serum biomarker panels has yet to be performed, the current results indicate that a urine panel of this

type may offer superior performance. An expanded analysis of urine biomarkers, including several found to be useful in serum by other groups, appears to be warranted. For example, a panel derived from plasma was recently found to perform well in the discrimination of early stage ovarian cancer with a reported SN of 94.1% at a SP of 91.3% [254]. Here, CA 125 was combined with the inflammatory cytokines IL-6 and IL-8, and the acute-phase proteins C-reactive protein (CRP) and serum amyloid A (SAA). These same cytokines along with the growth factors vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), were also included within a high performing panel identified by our group through an investigation of circulating inflammatory molecules which complement the diagnostic ability of CA 125 [105]. Other factors prominent among those identified in multimarker panel development include ApoAI and transthyretin (TTR), two emerging biomarkers associated with malignancy [65, 256].

5.2.3 Biomarker panels in the triage of women with a pelvic mass

A collaborative group of investigators led by Robert Bast, Steven Skates and Richard Moore has provided the most promising results to date regarding the use of biomarker panels for the discrimination of benign from malignant pelvic masses. The work of this group and several other notable reports are summarized in Table 5.3. Early efforts in this diagnostic setting were characterized by the use of CA 125 in combination with several other glycoprotein tumor antigens including CA 72-4, CA 15-3, OVX-1, and LASA [142, 185, 257]. More recent reports reflect the emergence of HE4 as a biomarker of ovarian cancer and its effective use in this clinical setting. In a series of publications, Moore et al. first established in a retrospective study that the combination of CA 125 and HE4 could discriminate benign from malignant masses with a SN of

76.5% at a SP of 95% [112]. This panel was then used to prospectively categorize patients as high or low risk for malignancy resulting in 93.8% correct classification of epithelial ovarian cancer patients [137]. Lastly, measurements of CA 125 and HE4 were incorporated into a scoring model termed the Risk of Ovarian Malignancy Algorithm (ROMA) which outperformed the Risk of Malignancy Index (RMI) yielding a SN of 94.3% at a SP of 75% [191]. Another group further demonstrated the utility of this combination of biomarkers in the discrimination of ovarian cancer from ovarian endometriotic cysts [258].

Table 5.3 Multimarker panels which discriminate benign from malignant pelvic masses

Panel	Cases	Controls	SN	SP	Reference
CA 125, MDK, AGR2	46	61	95.2	97.7	Rice et al.[259]
CA 125, OVX1, LASA, CA 15-3, CA 72-4	192	237	90.6	93.2	Woolas et al. [142]
CA 125, G-CSF, IL-6, EGF, VEGF	44	37	86.5	93	Gorelik et al. [105]
CA 125, CA 72-4, CA 15-3, M-CSF	90†	228†	71	98	Zhang et al. [185]
CA 125, IL-7	187	45	69	100	Lambeck et al. [110]
ROMA*	145	312	94.3	75	Moore et al. [191]
CA 125, HE4	491†	299†	83	85	Nolen et al. [177]
CA 125, HE4	129	352	92.3	75	Moore et al. [137]
CA 125, CA 15-3, CA 72-4, LASA	182†	237†	87.5	79	Zhang et al. [257]

*Risk of ovarian malignancy algorithm; †includes independent validation set

The results reported in Chapter 3 of this dissertation describe the independent identification of the CA 125/HE4 combination as the best possible biomarker panel for the discrimination of benign and malignant pelvic masses. The current study utilized a somewhat larger patient cohort than that of Moore et al. [112], and also evaluated a much larger pool of candidate biomarkers. A subsequent study utilizing urine as the testing matrix (Chapter 4) identified the same panel and suggested an optimal combination of urine HE4 and serum CA 125. Thus, accumulating evidence indicates a high degree of clinical utility for a biomarker-based diagnostic tool based on this combination, with implementation possible in the near future.

5.3 FUTURE PERSPECTIVES

The use of biomarkers in targeted therapies for ovarian cancer will require the concerted development of novel therapeutics and predictive biomarkers. As our knowledge regarding the specific etiologies of the various subtypes of ovarian cancer continues to expand, so must the identification and development of biomarkers associated with each subtype. The tailoring of treatment regimens based on disease subtyping, through the aid of biomarker testing, is a likely first step toward personalized treatment of ovarian cancer. The implementation of targeted agents earlier in the course of treatment should also facilitate the identification of predictive biomarkers. In addition to such predictive markers, biomarkers of tolerability will be equally useful in efforts to identify combinations of targeted agents which are safe and effective.

Several significant hurdles remain before any biomarker-based diagnostic model can be implemented clinically on a widespread basis. Foremost is the need to evaluate the most promising panels in prospective randomized clinical trials. Additional preclinical validation will be required to more fully characterize the efficacy of selected panels before this significant next step is warranted. A key component of this validation process is the evaluation of such panels in samples obtained from prediagnostic ovarian cancer patients. Progress towards this type of validation is greatly hindered by the rarity of this sample type, however several significant findings have been reported by a group under the direction of Nicole Urban. In a pair of reports, this group first describes elevated levels of CA 125, HE4, and mesothelin in the sera of symptomatic ovarian cancer patients [260] and then in the sera of patients 0-3 years prior to diagnosis, although an optimal lead time of 1 year is noted [261]. Recently, a collaborative study was performed to assess pre-diagnostic performance of candidate biomarkers in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Study [262]. The study demonstrated that CA 125

offers robust performance in the interval 0-6 months prior to diagnosis, however the multivariate analysis did not yield a biomarker panel with an appreciable improvement in sensitivity over that of CA 125 alone for this interval. The performance of CA 125 significantly diminished to a SN of 33% at 95% SP during the 6-12 months prior to diagnosis, and further decreased to 12% at 12-18 months prior to diagnosis. Unfortunately, none of the studied biomarkers either individually or in combination could offer a better performance for these pre-diagnostic intervals.

The promising performance of urine biomarkers in the current study suggests the possibility that other alternative biofluids may offer similar advantages. The saliva proteome is known to consist of 1166 distinct proteins including a spectrum of full length proteins, peptides, hormones, and enzymes [263]. Although saliva contains a relatively low overall protein concentration [264], modern assay methodologies displaying improved levels of sensitivity now permit the reliable detection of low abundance proteins. As expected, much of the work regarding the use of salivary biomarkers for cancer diagnosis has focused on oral cancer [265-266], however several groups have extended this type of work to cancers of remote origins with promising results. Gao et al. performed an analysis of salivary biomarker profiles of melanoma and non-small cell lung cancer using a mouse model system which not only identified several descriptive profiles but also characterized the origins of these factors as a combination of local and remote secretion [267]. Elsewhere, Streckfus et al. identified the soluble fragment of c-erbB-2 in saliva samples taken from patients diagnosed with breast cancer but not in samples obtained from healthy or benign control subjects [268]. Additional work by this group, employing proteomic methodology, suggests that many additional breast cancer-related proteins are present in saliva [269]. Recently, a separate group reported on the ability of panel of salivary biomarkers consisting of both proteins and nucleic acids to discriminate breast cancer cases from controls with a SN of

83% at a SP of 97% [270]. The evaluation of salivary biomarkers has yet to be applied to ovarian cancer in a significant capacity, however several studies have examined the relationship between serum and salivary CA 125 with mixed results. In two separate studies focusing on breast [271] and ovarian cancer [272], salivary CA 125 was found to be significantly elevated in both groups of cancer patients in comparison to their respective control groups, however serum and salivary levels of CA 125 were only correlated in the former study. In a third study which examined a limited number of ovarian cancer patients, salivary CA 125 was found to provide a lower SN than serum CA 125, however the false-positive rate in saliva was also significantly reduced leading the authors to conclude that salivary CA 125 may offer improved diagnostic potential [273]. Collectively, these findings reflect considerable promise for the expanded analysis of salivary biomarkers in ovarian cancer.

The body of work contained herein outlines the vast and diverse potential for the use of non-invasive biomarkers of ovarian cancer. The continued development of ovarian cancer biomarkers should not only permit the improved detection of the disease at a stage when curative treatment is far more likely, but also the improved triage and targeting of individual patients so that the impact of treatment can be maximized. Synergistic coupling of biomarker development and advances in treatment options should greatly reduce the impact of this devastating disease.

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