

**TUMOR-STROMAL INTERACTIONS IN TYPE I AND TYPE II ENDOMETRIAL
CANCER: THE ROLE OF CXCL12/CXCR4 AND HGF/C-MET/BFGF IN A LARGE
COHORT OF ENDOMETRIAL CANCER PATIENTS**

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ABSTRACT

Endometrial cancer (EC) is the most common gynecologic malignancy in the United States. In 2010, 43,470 cases were newly diagnosed with 7,950 deaths reported. Certain subtypes of EC are responsible for a disproportionate number of deaths each year. Specifically, high-grade Type I, papillary serous (PS), and clear cell (CC) tumors account for 60% of all deaths, despite accounting for only 20% of new cases. The molecular mechanisms related to poor survival in these subtypes are unknown. The tumor microenvironment refers to the complex milieu of supporting cells, i.e. stromal cells, which co-exist with the primary tumor. Broad classes of stromal cells including inflammatory cells, endothelial cells, and fibroblasts, support the growth and dissemination of the primary tumor through their interactions with cancer cells. The primary goal of this research was to determine the prognostic roles of two stromal-related pathways [CXCL12 and CXCR4; hepatocyte growth factor (HGF), c-Met, and basic fibroblast growth factor (bFGF)] in a sample of EC cases (N=216) treated at Magee-Womens Hospital. Paraffin-embedded tissue blocks were retrieved from the Pathology Department at Magee-Womens Hospital and protein expression was measured using immunohistochemistry (IHC). Chi-square

tests, Kaplan-Meier plots, log-rank tests, and Cox proportional hazards models were used to examine the relationship between tumor and stromal protein expression, clinicopathologic factors, overall survival (OS), and recurrence-free survival (RFS). In the first microenvironmental pathway, positive CXCL12 expression was significantly associated with better OS (hazard ratio, HR: 0.17 95% CI 0.05, 0.59) and RFS (HR: 0.10 95% CI 0.02, 0.57) in high-grade Type I cases. In the second pathway of interest, better OS was detected in HGF positive, stromal bFGF positive patients compared to HGF positive, stromal bFGF negative patients (HR: 0.14, 95% CI 0.03, 0.60). Additionally, worse RFS was observed in HGF positive, tumor bFGF positive patients compared to patients with negative expression of both markers (HR: 9.88, 95% CI 2.63, 37.16). This study provides evidence that tumor microenvironmental proteins can serve as independent prognostic biomarkers in EC. The public health implications include a better understanding of the biology of EC and potential targets for molecularly-targeted treatments in EC.

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PREFACE

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1.0 INTRODUCTION

Despite being a highly curable cancer, endometrial cancer (EC) accounts for a large proportion of cancer-related deaths each year. In 2010 the American Cancer Society estimated that 43,470 cases of EC were newly diagnosed and approximately 7,950 deaths occurred. Risk factors related to estrogen exposure are implicated in the etiology of the majority of ECs (Type I), with obesity showing the largest relative risk. Other important estrogen-related risk factors include early menarche, late menopause, hormone replacement therapy, nulliparity, polycystic ovary syndrome, and diabetes. The mechanism describing the association between estrogen exposure and EC risk is the unopposed estrogen hypothesis. This hypothesis states that estrogen, when unopposed by progesterone, has mitogenic effects on the endometrium which can result in a higher incidence of oncogenic mutations. In general, survival associated with estrogen-driven EC is favorable.

A subgroup of ECs appears to be estrogen-independent (Type II) and little is known about the etiology of these tumors. Identification of risk factors associated with development of Type II EC is limited by the low incidence of these tumors, which precludes effective prevention of these tumors. Patients with Type II tumors are more likely to experience poor outcomes such as recurrence, metastasis, and death compared to their Type I counterparts. Additionally, the molecular pathways associated with poor outcomes in these patients are unknown. Understanding the molecular epidemiology of poor-prognosis tumors could potentially lead to better treatment options and reduce morbidity and mortality associated with these cancers, while adding to the general body of knowledge regarding these tumors.

The tumor microenvironment refers to the extracellular matrix and the supporting cast of cells that are genetically normal but phenotypically altered due to their interaction with neoplastic cells. In normal tissue homeostasis, stromal cells provide supportive functions to epithelial cells and facilitate communication between cells. Following the carcinogenesis of a primary tumor, the tightly regulated interactions between transformed epithelial cells and stromal cells become dysregulated. Cancer cells are able to co-opt the multiple functions of stromal cells to serve their metabolic needs and migratory ambitions. Evidence from other common cancers suggests that the tumor microenvironment contributes to the invasive and aggressive phenotypes seen in cancers with poor survival.

Two microenvironmental pathways have been examined in other common cancers. The first, CXCL12 and CXCR4, is involved in directional migration of cells and therefore may contribute to metastasis, a common cause of cancer-related death. The second pathway, hepatocyte growth factor (HGF), c-Met, and basic fibroblast growth factor (bFGF) is associated with angiogenesis, the formation of new blood vessels from preexisting vessels. The initiation of angiogenesis is a requirement for the growth of tumors as well as distant metastasis.

The goals of this research are to further describe Type I and Type II ECs through completion of the following three primary aims: 1.) explore risk and prognostic factors associated with Type I and Type II EC patients, 2.) examine the association between CXCL12/CXCR4 expression, clinicopathologic factors, and survival in EC, and 3.) examine the association between HGF/c-Met/bFGF expression, clinicopathologic factors, and survival in EC.

2.0 LITERATURE REVIEW

2.1 ENDOMETRIAL CANCER (EC) EPIDEMIOLOGY

2.1.1 EC incidence and mortality

EC is the most common gynecological malignancy in the United States; an estimated 43,470 cases were newly diagnosed in 2010 [1]. Joinpoint analyses using data from the Surveillance, Epidemiology End Results (SEER) program indicate that EC incidence rates in the U.S. declined significantly between 1975 and 1988 and increased between 1988 and 1998. More specifically, between 1988 and 1998, the annual percentage change (APC), a statistic which describes the rate of change for a particular cancer statistic, increased yearly at a rate of 0.6% [2]. Thereafter, the APC decreased at a rate of 1% per year between 1998 and 2004.

Analyses of EC incidence rates by race indicate important trends over time (Figure 1). In the early 1970's, Caucasian women had a two-fold higher incidence of EC compared to African-American and women of other race. Following the peak in 1975, age-standardized incidence rates in Caucasian women began to decline dramatically. By 1989, the difference in incidence between Caucasian and African-American women was less than 3 cases per 100,000 persons (White: 14.1 cases per 100,000 women; African-American: 11.4 cases per 100,000 women). Joinpoint analyses stratified by race indicate that African-American, Asian or Pacific Islander,

and American Indian/Alaska Native women had positive APC's of 1.0%, 0.7%, and 2.2% respectively between 1995 and 2004, whereas Caucasian women had a statistically significant APC decline of 0.5% during the same time period [2].

Regardless of race, as women transition into menopause, the incidence of EC increases dramatically (Figure 2). Age-specific incidence rates show that EC incidence begins to dramatically increase at age 50 which coincides with the menopausal transition for most women. The highest incidence of EC among Caucasian and African-American women occurs between ages 75 and 79, with approximately 54 cases per 100,000 women being diagnosed in each race. American Indian/Alaska Native women experience bimodal peaks in incidence rates at ages 65-69 and 75-79. Finally, Asian or Pacific Islander women experience their highest age-specific EC incidence at age 55-59, with 27.5 cases per 100,000 women occurring. Subsequently, incidence rates gradually decline over the next twenty years of life for Asian or Pacific Islander women.

Although 75% of ECs are diagnosed early in the disease process, mortality from EC has increased dramatically over the past 20 years [3]. Likely contributors to this increase include an increasing life span in the U.S. and coexisting medical co-morbidities [3]. Historically, EC mortality rates have been higher in African-American women compared to Caucasian women, despite a two-fold higher incidence of EC in white women. The disparity in incidence has narrowed between the two races in the last decade, but the disparity in mortality has not. Espey et al. [2] reported the EC mortality rate to be 7.1 per 100,000 African-American women while the mortality rate for Caucasians was 3.9 per 100,000 women between 2000 and 2004. Several plausible explanations exist for this disparity in mortality: 1) differences in stage, grade, and histology at time of diagnosis 2) lower socioeconomic status of African-American women 3) greater clinical co-morbidities and 4) differences in treatment [4].

EC mortality rates among other ethnic minorities appear to be increasing as well.

Tammemagi [5] reported the age-adjusted mortality rates from EC to be 2.2, 2.6, and 3.2 per 100,000 women for Asian Pacific Islanders, American Indian/Alaska Native, and Hispanic/Latino women, respectively, during the 1992-2002 time period, indicating an increase in cancer associated mortality.

2.1.2 Factors related to EC survival

Survival following EC diagnosis and treatment is generally favorable, however survival decreases significantly with increasing stage at diagnosis. Using data from SEER, the relative survival for EC in the U.S. is plotted in Figure 3. Between 1996 and 2006, women diagnosed with localized tumors had a relative survival of 94% after 9 years of follow-up. Patients with regional spread had a relative survival of 60% after 9 years of follow-up, while patients with distant metastases present at diagnosis had poor survival rates throughout the 8-year duration follow-up period. In the SEER cohort, unstaged patients had a relative survival slightly lower than patients with regional endometrial tumors.

In addition to stage, age and tumor histology have a significant impact on survival in EC [6]. Compared to patients less than 45 years of age at the time of diagnosis, Zaino et al. [7] reported that patients 55 years of age or older had a two-fold higher risk of EC death while patients 65 years of age had a 3.40 times higher risk, and patients greater than 75 years of age had a 4.70 times higher risk. Poor prognosis associated with increasing age may be due to a variety of factors including: 1.) older patients may develop more aggressive histology subtypes of EC which carry a worse prognosis, 2.) older patients may have a weaker immunologic capacity against cancer, and 3.) less aggressive therapeutic options are used in elderly patients [8, 9].

Finally, numerous studies show that tumor histology is an important independent predictor in EC survival. Clear cell (CC) and papillary serous (PS) histology subtypes comprise between 10% and 20% of all EC cases, however they account for approximately half of all EC-related deaths, signifying an important disparity in EC survival [10].

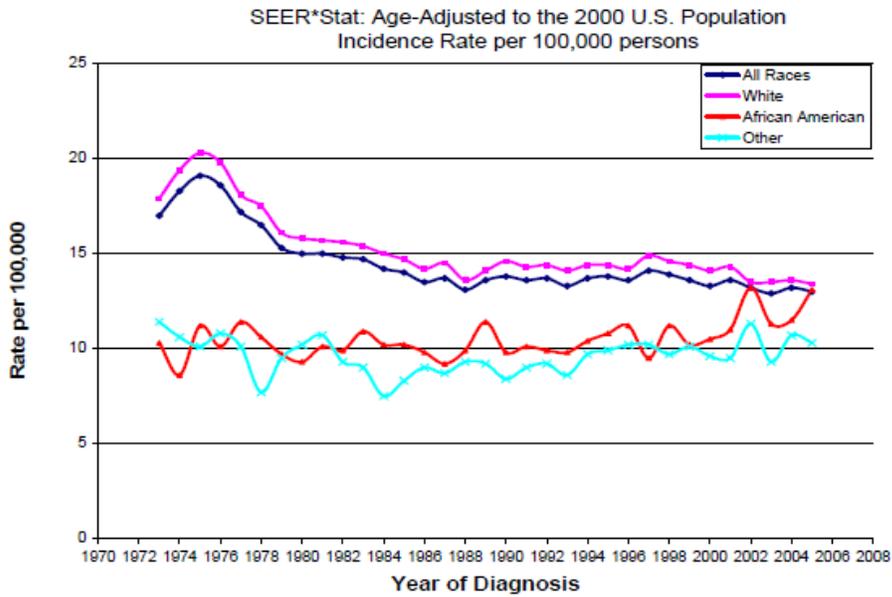


Figure 1 EC incidence among U.S. women by race, SEER*Stat, 1973-2005

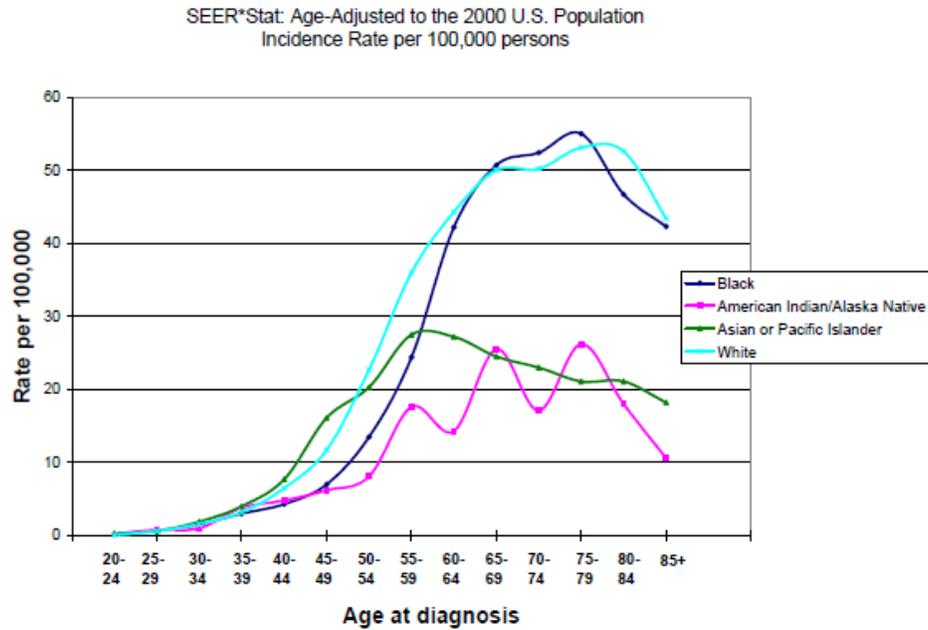


Figure 2 Age-specific endometrial cancer incidence rates among U.S. women by race, SEER*Stat, 2000-2004

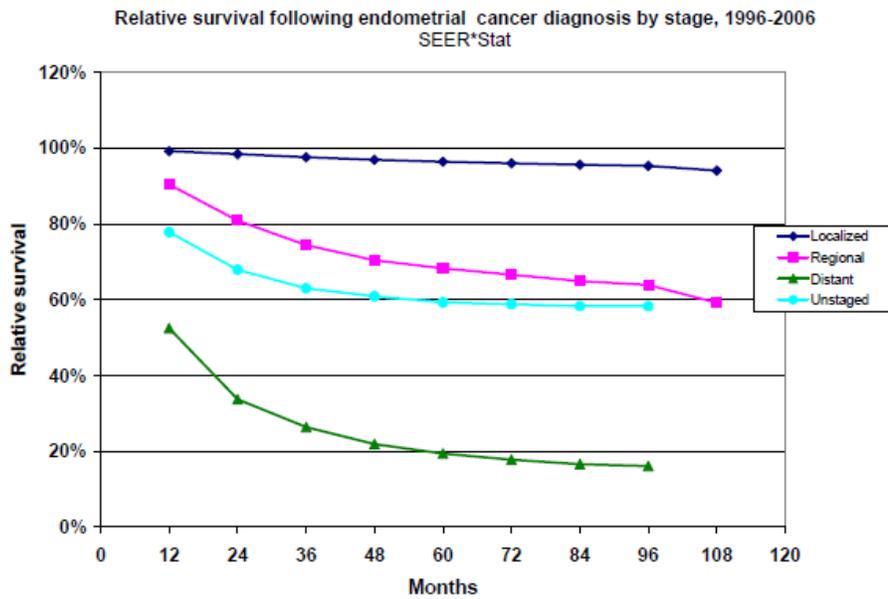


Figure 3 Relative survival following endometrial cancer diagnosis by stage, 1996-2006, SEER*Stat

2.2 EC RISK FACTORS

The etiology of EC is multifactorial and varies considerably based on the histology of the tumor. Two classes of endometrial tumors are recognized: Type I, also referred to as endometrioid, is associated with the estrogen pathway, often arises from atypical hyperplasia, and presents as a low-grade, well-differentiated tumor [11]. Typically, estrogen and progesterone receptors are expressed in these tumors [12]. Type II endometrial tumors, also known as the nonendometrioid type, arise from an atrophic endometrium and appear to be unresponsive to estrogen stimuli. The two histology subtypes of Type II EC are PS and CC [13]. Patients with these tumors are usually diagnosed in advanced stages with relatively low 5-year survival rates compared to patients with Type I endometrial tumors [13].

Many of the commonly cited risk factors for Type I EC contribute to a chronic low-level increase in circulating estrogen exposure that is not counterbalanced by progesterone [14]. Namely, obesity, unopposed estrogen therapy, and reproductive characteristics, such as parity, age at menarche and menopause, and oral contraceptive use are strongly associated with EC risk. Recent studies have implicated other lifestyle risk factors, particularly diet and the metabolic syndrome, in the carcinogenic process related to Type I EC. Conversely, the risk factor profile for those who develop Type II endometrial tumors is unknown. Relatively fewer studies have explored the epidemiologic profile of Type II patients, however these patients tend to be African-American, multi-parous, and of normal weight [15].

In addition to the environmental component of EC risk, a genetic basis for EC has been described. Hereditary non-polyposis colorectal cancer syndrome (HNPCC), also known as Lynch syndrome, is caused by mutations in one of five DNA mismatch repair genes, *MLH1*, *MSH2*, *MSH6*, *PMS1* and *PMS2*; mutations in *MLH1* and *MSH2* account for the majority of cases,

whereas *MSH6* mutation is implicated in 15% of cases [16]. Germline mutations in these genes result in microsatellite instability (MSI) which is a progressive accumulation of alterations at microsatellite loci [17]. EC is the most common phenotype of this cancer syndrome following colorectal cancer [17]. Some studies even report EC to be the most common malignancy for women with HNPCC, not colon cancer [18]. Females with HNPCC have a ten-fold increased lifetime risk of developing EC compared to women not belonging to HNPCC families.

Hereditary EC is more likely to occur at a younger age and is characterized by high stage and poor differentiation [17].

The increasing prevalence in many of the known risk factors for EC, such as obesity, highlights the potential for an increased future burden of EC in the U.S. The modifiable nature of many of these risk factors underscores the need for public health interventions.

2.2.1 Endogenous estrogens and EC

Endometrial tissue is one of the many target tissues responsive to sex steroid hormones [14]. Sex steroid hormones share a basic chemical structure, and include androgens, estrogens, and progesterone [19]. Important regulatory properties maintained by these hormones include apoptosis, cellular proliferation, and differentiation; processes that are frequently dysregulated in malignant transformation. The unopposed estrogen hypothesis describes the relationship between steroid hormones and EC risk. Estrogen, when unopposed by progesterone, has proliferative effects on the endometrium. Consequently, women with high levels of bioavailable estrogens and low plasma progesterone have an increased risk of EC. This hypothesis resulted from two important observations: 1.) endometrial proliferation rates are greater during the follicular phase of the menstrual cycle when progesterone levels are low and estrogen levels are at normal

concentrations and 2.) the risk of EC is higher for women using exogenous estrogen therapy without a progestin component [14]. Factors associated with high circulating levels of estrogen are described below.

2.2.1.1 Obesity

The role of obesity in endometrial carcinogenesis has been documented extensively. In fact, EC is the cancer most commonly associated with obesity [19]. Many epidemiologic studies show a substantially greater risk with increasing weight. In obese pre- and post-menopausal women the increase in risk of EC ranges from two to five-fold [14]. In a meta-analysis of international cohorts, Renehan et al. [20] reported the overall relative risk of EC to be 1.59 times higher for each 5 kg/m² increase in body mass index (BMI). Although this association has been demonstrated convincingly, the exact mechanisms through which increased adiposity confers a higher EC risk are not fully understood. Implicated in the disease process are two interrelated hormonal pathways: the estrogen/progesterone pathway and the insulin and insulin-like growth factor 1 (IGF-1) axis pathway.

Following menopause, the major source of estrogen is from the conversion of adrenal androgens (androstenedione) to estrogen (estrone) by aromatization in adipocytes [21]. In postmenopausal women, excess weight can potentially affect the bioavailability of estrogen through two mechanisms. First, adiposity raises plasma levels of aromatase and 17 β -hydroxysteroid dehydrogenase, enzymes responsible for conversion of androstenedione and testosterone into estrone and estradiol, respectively. In fact, BMI is positively associated with levels of estradiol and estrone in healthy control women [22]. The second mechanism through which excess weight influences the availability of estrogen is by increasing circulating levels of both insulin and IGF1 [19]. Excess weight is associated with insulin resistance due to increased

concentrations of fatty acids which are released from adipose tissues [14]. Elevated levels of fatty acids lead to metabolic changes that limit the ability of hepatic and muscular tissue to absorb and utilize glucose in energy metabolism. Consequently, a reduction in insulin receptors occurs, leading to a state of insulin resistance. Insulin resistance causes plasma levels of IGF-1 to increase, which lower hepatic synthesis of sex-hormone binding globulin (SHBG). SHBG is responsible for binding estradiol in women, with lower levels of this protein resulting in greater amounts of bioavailable estrogens [19].

2.2.1.2 Metabolic conditions

Metabolic conditions, particularly diabetes, have been shown to be positively related to an increased EC risk. Although excess weight has played a mediating role in this association, recent analyses show that EC risk is associated with diabetes independent of weight. Weiderpass et al. [23] explored this association in a population-based case-control study in Sweden. Adjusted for age, hormone replacement therapy use, parity, age at menarche, and recent BMI, EC cases were 1.70 times more likely to have diabetes compared to randomly selected population controls. Furthermore, this study examined if hypertension, another component of the metabolic disorder, modified the risk of EC associated with diabetes. When adjusted for recent BMI, history of hypertension was not significantly associated with EC risk (Odds Ratio (OR): 1.10, 95% CI 0.90, 1.30). Hypertension did modify the risk of EC in obese patients; cases were 1.40 times more likely to be obese and hypertensive compared to controls (95% CI 1.00, 2.20) [23]. Among normal weight women no increase in risk of EC for hypertensive women was observed (OR: 1.00, 95% CI 0.70, 1.20).

Two potential mechanisms exist for the increased EC risk in relation to diabetes. As previously mentioned, increased levels of circulating insulin can result in decreased synthesis of

SHBG which is associated with increased circulating estrogen. Conversely, insulin may act as a direct growth factor on the endometrium, irrespective of changes in sex steroid hormone levels [23].

2.2.1.3 Parity

Related to unopposed estrogen exposure, nulliparity has consistently been shown to be a risk factor for EC. Most studies report a 10-40% reduction in EC risk for parous vs. nulliparous women [24-28]. Pregnancy is a time when progesterone levels are high which may account for the reduced EC risk through opposition of estrogen [29]. In a population-based case-control study, Brinton et al. [30] reported that Polish women who ever had a full-term pregnancy were half as likely to be cases compared to women who never had a full-term birth (OR: 0.51, 95% CI 0.40, 0.70). Additionally, a statistically significant trend in the number of full-term births was observed; women with 3 or more full-term births had a 70% lower risk of EC compared to nulliparous women. Furthermore, this study examined the potential relationship between timing of pregnancy and EC. Age at last birth and intervals since last birth variables were constructed to examine the effect on EC risk. Although the findings did not support a role for such a relationship, other studies have reported important relationships between timing of pregnancy and EC risk [29, 31]. Parrazzini et al. [29] and Lambe et al. [31] reported that protection of pregnancy against EC risk decreased significantly with longer time since last birth.

Several studies have examined whether infertility is associated with risk of EC. The World Health Organization defines infertility as not being able to conceive within one year of trying; however, many disorders could be at the root of this condition which makes infertility a difficult risk factor to study [32]. Furthermore, infertility is usually treated with fertility drugs that stimulate multiple ovulations per cycle. As these drugs increase serum levels of hormones, these

agents may underpin the reported associations between infertility and EC risk [33]. Two large studies examined the risk of EC in cohorts of infertile patients [34, 35]. In a cohort of Australian women, no significant difference in EC risk was observed for women who utilized in vitro fertilization (IVF) compared to the general population (SIR 1.09, 95% CI, 0.45-2.61) [34]. In women who did not receive ovarian stimulation, the observed incidence of EC was significantly higher compared to the expected incidence (SIR: 2.47, 95% CI 1.18, 5.18). This study implies that untreated IVF patients have an increased risk of EC. Importantly, this study only controlled for age; factors that may confound the relationship between infertility and EC risk were not taken into account.

Modan et al. [35] studied the risk of EC in a cohort of women diagnosed as infertile between 1964 and 1974. In unadjusted analyses, the incidence of EC was significantly greater than expected (SIR: 4.80, 95% CI 3.00, 7.40). The role of ovulation induction treatments was also assessed in this study. Women treated with fertility drugs had a SIR of EC two times greater than women not treated with fertility drugs (6.80 vs. 3.10), however this was not statistically significant. Because of the conflicting evidence, carefully planned studies are needed to understand this relationship in the presence of potential confounders.

2.2.1.4 Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a relatively common disorder in pre-menopausal women that is characterized by anovulation and progesterone deficiency. In 1990 the National Institutes of Health (NIH) established diagnostic criteria for the disorder: chronic anovulation and presence of hyperandrogenism (diagnosed either clinically or biochemically) with the exclusion of other causes of hyperandrogenism such as adult-onset congenital adrenal hyperplasia (CAH), hyperprolactinemia, and androgen-secreting neoplasms [36]. More recently, the 2003 Rotterdam

consensus workshop revised the previous guidelines to include polycystic ovaries as a diagnostic criterion [37]. Because PCOS is a heterogeneous syndrome, the Rotterdam consensus concluded that 2 out of 3 of these criteria are sufficient for diagnosis. An association between PCOS and EC was first suggested in 1949 by Speert from the observation that women with EC had a high incidence of cystic ovaries [38]. Since then, several studies have been conducted to further explore this relationship, however design issues have precluded a definitive answer regarding this relationship [39]. Many studies have examined whether EC risk was elevated in women with anovulatory infertility, which is one of the presenting symptoms of the syndrome and may occur from causes unrelated to PCOS. Others have observed an association between risk of endometrial hyperplasia and PCOS [39].

In a cross-sectional study, the prevalence of polycystic ovaries, a marker of PCOS, was similarly distributed in patients with EC and patients with benign gynecological conditions. When stratifying by age, PCOS was more common in EC cases less than 50 years of age compared to similarly aged women with benign gynecological conditions (62% vs. 27%, $p=0.03$) [39]. Although this cross-sectional study cannot establish the timing of events, i.e. if cystic ovaries preceded EC, this study does lend favorable support to the association. Prospectively planned studies, where PCOS is defined based on diagnostic criteria, are needed to determine the relationship between PCOS and EC risk. Based on biological plausibility it would appear reasonable to suggest that women with PCOS have a higher probability of EC as anovulation is associated with decreased circulating levels of progesterone, which would lead to higher levels of unopposed circulating estrogen [40].

2.2.2 Exogenous estrogens and EC

2.2.2.1 Estrogen replacement therapy

Risk of EC is strongly associated with unopposed estrogen replacement therapy (ERT). ERT use rose dramatically in the United States from the mid 1960's until 1975 for the relief of menopausal symptoms. During this time period, EC rates increased at an alarming rate, which prompted investigations into an association between ERT and EC risk [41]. In a meta-analysis of 29 observational studies, the combined relative risk (RR) for EC was 2.30 times higher for ERT users compared to non-users. Additionally, EC risk increased as the duration of ERT usage increased [42].

In a meta-analysis including 7 observational studies exploring the association between estrogen plus progestin therapy and risk of EC found no association (RR: 0.80, 95% CI 0.60, 1.20), further supporting a role for unopposed estrogen and EC risk [42]. Similarly, the Women's Health Initiative trial reported no statistically significant increase in the incidence of EC after 5 years of follow-up in women who used estrogen plus progestin therapy (HR: 0.83, (95% CI 0.29, 2.32) [43].

2.2.2.2 Selective estrogen receptor modulators

Selective estrogen receptor modulators (SERMs) have been used in the treatment of hormonally related cancers. SERMs resemble the chemical structure of estrogens and compete for binding at estrogen receptors, antagonizing the effects of endogenous estrogen [44]. Two major SERMs, tamoxifen and raloxifene, have been commonly used in the treatment of breast cancer. Although tamoxifen antagonizes estrogen receptors in the breast, it is associated with EC risk via stimulation of endometrial estrogen receptors. The estrogenic effects of tamoxifen in the uterus

are associated with a two to seven-fold increased risk of EC [45]. In a large cohort of women who developed endometrial tumors after being diagnosed with breast cancer, Hoogendoorn et al. [46] reported that EC was significantly more likely to occur in the women treated with tamoxifen. Additionally, tamoxifen-treated patients had unfavorable prognostic characteristics and worse survival compared to EC patients not treated with tamoxifen.

Conversely, raloxifene has anti-estrogenic effects in both the breast and uterus. DeMichele et al. [44] reported a 50% reduction in the odds of EC associated with raloxifene use compared to never users. Moreover, raloxifene is associated with a 33% reduction in EC risk adjusted for age, race, BMI, and family history of breast cancer.

2.2.2.3 Oral contraceptive use

The risk of EC associated with oral contraceptive use varies based on the formulation of the drug. Sequential oral contraceptive (SOC) formulations, although commonly used until the 1970's, were discontinued when an increased risk of EC was observed [47]. Sequential formulations deliver high doses of estrogen for up to 16 days of the monthly cycle, consequently increasing circulating levels of unopposed estrogen [14]. Use of the SOC Oracon, was seven-fold more frequent in women with EC than women from the same population without EC [48].

Similar to estrogen plus progestin treatments, combination oral contraceptive (COC) formulations contain estrogen and progesterone, and are given for approximately 22 days of the monthly cycle [14]. Sixteen case-control studies and three cohort studies have examined the relationship between COC therapy and risk of EC [47]. The results of these studies show that risk of EC is essentially halved for women who use COC formulations [47]. Moreover, duration of use was associated with a decreased risk of EC. In the Cancer and Steroid Hormone Study, women who used COC formulations for less than one year had the same risk of developing EC

as those women who had never used COCs. Compared with never-users, women who used COCs for more than 5 years were 0.40 times as likely to develop EC [49].

2.2.3 Age and EC

Increasing age is a risk factor for many cancers. The two distinct types of EC, Type I and Type II, have different age distributions, which may point out important differences in the etiologies of the two types of EC. The median age for Type I EC onset is 61 years of age, with 90% of cases occurring in women older than 50 years of age [3]. Mean age at diagnosis of Type I EC is 61 years whereas women with Type II EC are typically diagnosed five to ten 10 years later than their Type I counterparts [50]. Age-specific graphs for EC incidence, regardless of type, illustrate the dramatic increase in diagnoses as women age that appears to coincide with the menopausal transition (Figure 2).

EC in women less than 50 years of age is uncommon. Studies vary regarding the incidence of EC in this demographic but most report between 5% and 30% of cases occur in women younger than 50 years of age [51]. In general, women younger than 50 years of age are premenopausal. Several epidemiologic studies have examined risk factors associated with EC development in patients younger than 50 years of age, as well as prognostic factors and survival rates in these cases [52-55]. In general, young EC cases are more likely to be obese and have advanced stage disease at the time of diagnosis compared to older EC cases, while hypertension and diabetes are reportedly more common in older EC cases [52, 53]. Furthermore, survival was generally better for young cases in these two case-series; at the end of follow-up almost all EC cases designated as “young,” were alive at the end of follow-up. Lu et al. [54] examined the frequency of germline mutations in *MLH1*, *MSH2*, and *MSH6* in a cohort of EC cases younger

than 50 years at the time of diagnosis. MSI and immunohistochemistry (IHC) analyses revealed 9% of young EC cases to carry a germline mutation in one of three DNA repair genes. Notably, all patients with germline HNPCC syndrome mutation had a first-degree relative with an HNPCC-related cancer [54]. In this setting, family history as well as age at diagnosis can play an important role for clinicians in recommending DNA testing for HNPCC.

In a Danish case-control study, Parslov and colleagues [55] compared EC cases to age-matched non-cancer controls. In the multivariable model, family history of EC (OR: 2.10, 95% CI 1.10, 3.80), having two or more children (OR: 0.30, 95% CI 0.20, 0.60), increasing age at first birth (OR: 0.10, 95% CI 0.04, 0.10), induced abortion (OR: 0.50, 95% CI: 0.30, 0.90), oral contraceptive use between 1-5 years (OR: 0.20, 95% CI 0.10, 0.30), and hormone replacement therapy use between 1-5 years (OR: 3.10 95% CI 1.40, 7.00) were significantly associated with EC risk in women younger than 50 years of age.

Schmeler et al. [51] examined the incidence of EC in young, normal weight women. While obesity is a major risk factor for EC, a substantial proportion of young women with EC is normal weight. In their cohort of young premenopausal women who developed EC before the age of 50, 25% had a BMI less than 25kg/m², which was defined as normal weight in this study [51]. HNPCC status was assessed using the revised Amsterdam criteria and patient medical records. Among the normal weight cases, 4% were identified as having HNPCC compared to 14% in the overweight group. Nulliparity was most prevalent in the normal weight and obese groups compared to the overweight group. Infertility was higher among normal weight patients compared to overweight and obese cases (17% vs. 7% and 14%, respectively). Diabetes was least frequent in the normal weight cases (4% vs. 13% and 35% for overweight and obese cases, respectively). Irregular menses was more common among obese cases compared to normal

weight cases (61% vs. 30%). Histology subtype, differentiation, and stage of the tumors were similar between normal weight, overweight, and obese EC cases.

2.2.4 Diet and EC

Although the evidence for an association between dietary factors and EC is somewhat contradictory, studies suggest that consumption of soybean-containing foods (phytoestrogens), fruits, vegetables, diets low in fat and high in fiber are protective against EC [56]. In a case-control study using a diet history questionnaire to determine food intake, Goodman et al. [57] reported that EC cases were less likely to consume legumes, dietary fiber, and fruit fiber compared to healthy controls. Compared to the lowest quartile of vegetable and fruit intake, those in the highest quartile had ORs of 0.47 and 0.54, respectively, after adjustment for pregnancy history, oral contraceptive use, ERT use, and history of diabetes. High consumption of soy products was associated with a decreased risk of EC (OR: 0.46, 95% CI 0.26, 0.83) for the highest quartile compared with the lowest quartile of soy intake. Conversely, energy intake from fat sources was associated with an increased risk in EC, adjusted for total energy intake.

The reduced risk of EC as a result of dietary soy and fiber is biologically plausible. Soy diets provide high doses of phytoestrogens which share a similar chemical structure to estrogen. Therefore, phytoestrogens may antagonize estrogen by binding at the estrogen receptor [57]. Furthermore, high dietary intake of fiber is positively associated with plasma levels of SHBG, which may reduce the amount of circulating estrogen [57].

2.2.5 Smoking and EC

Although a risk factor for many human cancers, cigarette smoking has an inverse relationship with EC risk. A meta-analysis including 10 prospective and 24 case-control studies found that women who reported ever-smoking had a significantly reduced risk of EC in prospective studies (RR: 0.81, 95% CI, 0.74,0.88) and case-control studies (OR: 0.72, 95% CI, 0.66, 0.79) [58]. An even greater reduction in risk was evident for current smokers; the overall summary estimate from prospective studies was 0.74 (95% CI 0.64, 0.84) while the summary estimate from case-control studies was 0.63 (95% CI 0.55, 0.72). When stratifying women based on menopausal status, a reduction in EC risk remained for postmenopausal women (RR: 0.71, 95% CI 0.65, 0.78), but not in pre-menopausal women (RR: 1.06, 95% CI, 0.88, 1.28).

Mechanistically, several explanations may account for an association between smoking and a reduced risk of EC. Michnovicz et al. [59] reported that cigarette smoking induces an increase in estradiol 2-hydroxylation. This pathway yields 2-hydroxyestrogens, which possess minimal peripheral estrogenic activity and are cleared rapidly from the circulation, thereby reducing the bioavailability of estrogen. Others have suggested that smoking may negatively affect ovarian function. Pre-menopausal women who smoke have fewer ovarian follicles which may result in earlier menopause and consequently reduce lifetime estrogen exposure [60]. This hypothesis was not supported by the meta-analysis findings by Zhou et al. [58], as no decreased risk of EC was reported among pre-menopausal women who smoke. As the mechanism for an association between smoking and EC risk is unknown, further study into this relationship is warranted.

2.3 CLINICAL FEATURES OF ENDOMETRIAL TUMORS

2.3.1 Histopathology

The major EC histology subtype is Type I (endometrioid), which accounts for 75-80% of all ECs [61]. Adenocarcinoma is the most commonly diagnosed histology subtype among Type I EC patients, however adenocarcinoma with squamous differentiation occurs in approximately 25% of cases [62]. The next most common EC histology subtypes, CC and PS, account for less than 20% of all endometrial tumors, and are collectively referred to as Type II EC. Finally, mucinous, squamous, and undifferentiated tumors comprise the remaining EC histology subtypes [61].

2.3.2 Precursor Lesions

2.3.2.1 Type I EC: Endometrial intraepithelial neoplasia (EIN)

Endometrial intraepithelial neoplasia (EIN) is the precursor lesion of Type I EC [3]. The histological appearance of this lesion is characterized by a glandular area that exceeds the stromal area; left untreated, these precursors progress to invasive carcinoma [63]. EIN arises from a hyperplastic endometrium. Endometrial hyperplasia (EH), a noninvasive proliferation of the uterine lining, occurs in the setting of unopposed estrogen stimulation [64]. Unopposed estrogens produce a disordered proliferative endometrium and over time an increasingly irregular distribution of endometrial glands results [65]. Classification of EH is defined by architectural complexity (simple or complex) and cytological atypia (present or absent) [65]. Hyperplasia with atypia is the least common type of hyperplasia and is strongly associated with EIN and further

progression to Type I EC. In fact, women diagnosed with atypical hyperplasia are ten times more likely to develop EC compared to women diagnosed with non-atypical hyperplasia [63].

2.3.2.2 Type II EC: Endometrial glandular dysplasia (EmGD)

Endometrial intraepithelial carcinoma (EIC) was previously thought to be the precursor lesion in PS EC, however recent studies have suggested that a newly identified lesion, endometrial glandular dysplasia (EmGD), is the precursor to both EIC and PS. Morphologically and clinically, EIC resembles an early form of PS rather than a precursor [66]. EIC is characterized by a complete replacement of surface epithelium by glands that resemble the PS cells with high grade nuclei [66]. EmGD on the other hand appears to represent the earliest morphologically identifiable precursor lesion in PS development for several reasons: first, EmGD transitions frequently into EIC, and EIC transitions into PS, however there is no direct transition from EmGD to PS. Second, in PS tumors, areas of EmGD are usually noncontiguous with the main tumor mass, however EmGD lesions are proximal to EIC. Finally, p53 overexpression, the most common molecular alteration in PS tumors, ranges on a continuum in various endometrial tissues: p53 overexpression scores are lowest in the benign endometrium, moderately high in EmGD lesions, and more frequent in EIC [66].

2.3.3 Staging of EC

Guidelines for the staging of EC have been defined by the International Federation of Gynecology and Obstetrics (FIGO). Stage I refers to cancer confined to the corpus uteri; stage II involves the corpus and the cervix but has not extended outside the uterus; stage III extends outside of the uterus but is confined to the true pelvis with or without lymph node involvement;

stage IV involves the bladder or bowel mucosa or has metastasized to distant sites [12]. In order to properly stage EC a hysterectomy and lymphadenectomy should be performed [61].

Stage of presentation varies based on the histology subtype of the tumor. In Type I tumors, roughly 72% of ECs are stage I, 12% are stage II, 13% are stage III, and the remaining 3% are diagnosed at stage IV [3]. Type II tumors are usually diagnosed at an advanced stage compared to Type I tumors. Approximately 54% of Type II tumors are stage I, 8% are stage II, while the remaining 38% are either stage III or IV [15]. Five-year survival rates for stage I-IV ECs are as follows: stage I: 81-90%; stage II: 72-80%; stage III: 39-63%; and stage IV: 17-20% [3].

2.4 EC TREATMENT

Several thorough reviews have been written regarding the treatment of EC [67-70]. Typically, women between 55 and 65 years of age presenting with bleeding unrelated to the menstrual cycle and/or pelvic pain have an endometrial biopsy or dilation and curettage [61]. After identifying cancerous cells, most patients will undergo a hysterectomy with bilateral salpingo-oophorectomy as the initial treatment. Contraindications to surgery (old age, morbidly obese, poor health, etc) occur in a minority of cases, which precludes surgical intervention. At the time of surgery, lymph node sampling (i.e. lymphadenectomy) is usually performed to inform adjuvant treatment options [67]. In patients with Type I (endometrioid) tumors, surgery alone is sufficient for stage IA, grade 1 or 2 disease and stage IB, grade 1 disease due to the low risk of recurrence and death [67].

Intermediate risk Type I EC patients are a heterogeneous group and typically have a combination of one or more of the following pathologic risk factors: any degree of myometrial

invasion and moderately to poorly differentiated grade, lymphovascular invasion, and age older than 70. The efficacy of adjuvant radiotherapy in this subgroup was addressed in the Gynecological Oncology Group (GOG) trial 99 which concluded that adjuvant radiotherapy in this subgroup decreases the risk of recurrence (HR: 0.42, 95% CI 0.25, 0.73), but not all-cause mortality [71].

The role of adjuvant therapy in high-risk EC cases (stage III and IV, any histology) was examined in the GOG trial 122, which scrutinized the role of whole-abdominal irradiation vs. doxorubicin and cisplatin chemotherapy. Chemotherapy significantly improved progression-free survival (PFS) and overall survival (OS) compared with whole-abdominal irradiation in this high-risk subgroup. Although toxicities were more commonly reported in patients in the chemotherapy arm they appeared to be well-tolerated [72].

By definition, CC and PS ECs of any stage are considered to be at high risk for recurrence and death. The initial treatment for these tumors is similar to intermediate and high-risk Type I tumors; surgical resection of the uterus, removal of the ovaries, and lymph node dissection followed by chemotherapy and/or radiotherapy is generally employed. Only one prospective trial has examined the role of radiotherapy in early stage CC and PS cases; due to a large proportion of recurrences in the irradiated areas, the authors of this trial concluded that chemotherapy should be used in this subgroup of EC cases [73]. Several clinical phase III trials of chemotherapy have included patients with CC and PS EC subtypes [74-78]. Typically, these trials examine varying combinations and doses of doxorubicin, cisplatin, and paclitaxel. Fleming et al. [74] examined the efficacy of cisplatin and doxorubicin with or without paclitaxel in 263 advanced or recurrent EC cases. Cases with CC and PS tumors made up 20% of the patient population. Treatment with all three therapies significantly improved response rates, PFS, and

OS compared to the two therapy regimen. In 2009, a phase III clinical trial examined a protocol including surgery, volume-directed radiotherapy of the pelvic and para-aortic lymph nodes, and cisplatin/doxorubicin chemotherapy with or without paclitaxel [78]. In this trial, CC and PS histologies made up 18% of all cases. The addition of paclitaxel to cisplatin, doxorubicin, and radiotherapy did not improve recurrence-free survival (RFS), however significant toxicities were observed in this treatment arm.

To date, no phase III clinical trials utilizing molecularly targeted therapies have been performed in EC. Dedes et al. [79] reviewed the phase II clinical trials that target specific alterations known to occur in EC. The PI3K/Akt/PTEN/mTOR signaling pathway (to be discussed in section 2.5) is the most comprehensively tested pathway; approximately 16 clinical trials have been completed or are ongoing. Other molecular treatment targets include epidermal growth factor receptors (EGFRs) and vascular endothelial growth factor (VEGF). In general, these trials have not shown significant improvements in complete response, partial response, or stabilization of disease, however this is likely due to the fact that patients in these trials previously underwent chemotherapy regimens and no stratification based on molecular subtype was performed.

2.5 MOLECULAR ALTERATIONS IN EC

2.5.1 Type I EC

A dualistic model of endometrial carcinogenesis based on clinical and prognostic factors was first proposed by Bokhman in 1983 which led to the acceptance of two main types of EC, Type I

and Type II [80]. Epidemiological evidence suggests that the multi-step carcinogenic process of Type I endometrial tumors begins with complex EH and progresses to EIN which is the precursor lesion of this type [64]. The most commonly reported molecular alterations in Type I ECs occur in PTEN, K-ras, β -catenin, BRAF, DNA repair genes which result in MSI, and certain cell cycle genes.

2.5.1.1 Tumor suppressor genes

The phosphatase and tensin homolog (PTEN) is an important tumor suppressor gene commonly mutated in Type I ECs [17]. The PTEN gene product has both lipid and protein phosphatase activities which confer different functions [81]. The lipid phosphatase activity of PTEN causes cell cycle arrest at the G1/S checkpoint while the protein phosphatase activity of PTEN modulates signal transduction pathways by acting on the second messenger, phospholipid phosphatidylinositol-(3,4,5)-triphosphate (PIP3). Inactivation of PTEN results in an increase in PIP3 which leads to phosphorylation and upregulation of AKT. The overall result is an increase in cell proliferation and survival [17].

Between 40% and 83% of Type I endometrial tumors have altered PTEN expression [82]. Additionally, 55% of precancerous lesions have altered PTEN expression, signifying that loss of PTEN function is an early event in EC formation [17]. Mutation and loss of heterozygosity without mutation result in loss of PTEN [50]. IHC studies have examined the association between PTEN expression and prognostic variables; based on semi-quantitative scoring, loss of PTEN expression was significantly associated with poor differentiation, positive lymph node involvement, and shorter OS compared to tumors with positive PTEN expression [83].

2.5.1.2 Oncogenes

The K-ras gene is a cellular GTPase belonging to the ras gene family [84]. K-ras functions as a molecular switch during cell signaling and plays an important role in tumor growth and differentiation [11]. When K-ras is present in its constitutively active state, i.e. GTP-bound state, continual propagation of intracellular signaling occurs, which allows the cell to proliferate. Mutations in the K-ras gene have been reported in 10-30% of Type I tumors, however this alteration is rarely seen in Type II tumors [11]. Importantly, K-ras mutations occur in approximately 16% of EHs indicating an early event in the carcinogenic process [50]. A single amino acid change is responsible for an activating mutation in this gene [84].

β -catenin is an adherens junction protein, involved in the maintenance of epithelial layers in coordination with E-cadherin [84]. By mediating adhesions between cells, communicating with neighboring cells, and anchoring the cytoskeleton of the cell, β -catenin plays a major role in normal cell growth and tissue architecture [84]. Mutations in β -catenin lead to overexpression of the protein in the nucleus due to the inability of the ubiquitin proteasome to degrade β -catenin [11]. Consequently, constitutive activation of target genes such as cyclin D1 occurs which favors cell transformation [12]. Additionally, β -catenin is an activator of the downstream Wnt signaling pathway; disturbances in Wnt signaling promote human cancers [81]. Nuclear expression of β -catenin ranges from 31-47% in Type I endometrial tumors. Similar to K-ras and PTEN mutations, β -catenin mutations are present in approximately 10% of endometrial hyperplasias, signifying β -catenin loss as an early event in tumorigenesis [85].

BRAF belongs to the raf family of cytoplasmic serine/threonine protein kinases [84]. The BRAF protein plays a role in the mitogen-activated protein kinase (MAPK) pathway which affects cell division, differentiation, and secretion [84]. Feng et al. [86] conducted an

experimental study to assess the frequency of BRAF mutations in normal endometrial tissues, atypical hyperplastic tissues, and EC. Direct sequencing of DNA samples demonstrated that BRAF mutations were present in 23%, 11%, 11%, and 0% of Type I, Type II, atypical hyperplastic lesions, and normal endometria respectively. The low percentage of BRAF mutations in the precursor lesion suggests that the BRAF gene mutation may be important for cancer progression rather than the early stages of carcinogenesis in Type I EC.

HER2/*neu* is a proto-oncogene that encodes for a transmembrane receptor tyrosine kinase which is involved in various cell signaling pathways, notably MAPK and phosphatidylinositol-3 kinase (PI3K) [84]. These pathways play major roles in cellular functions such as proliferation, survival, and aging [84]. Although less prevalent in Type I endometrial tumors, overexpression of HER2/*neu* has been reported in 10 to 30% of high-grade Type I tumors suggesting this alteration is an important event in tumor progression rather than tumor initiation [50].

2.5.1.3 Microsatellite instability

MSI refers to a progressive accumulation of alterations at microsatellite loci. Specifically, frame shift mutations in short segments of repetitive DNA are found throughout noncoding DNA [81]. These alterations arise due to DNA repair errors made during replication. Between 20 and 45% of Type I endometrial tumors have MSI and these tumors are also more likely to have PTEN, K-ras, and β -catenin mutations [12]. In EC, the most commonly inactivated mismatch repair genes are *MSH2* and *MLH1*. *MLH1* inactivation occurs due to hypermethylation of CpG islands in the promoter region of the gene. Moreover, this is the most common cause of MSI in Type I EC, however the mechanism of MSH-2 inactivation is still unknown [50].

Data on the clinicopathologic impact of MSI in EC were reported by An et al [87]. In a subset of Type I ECs, an MSI-high phenotype was denoted by the presence of MSI at 2 or more

loci while specimens were classified as “microsatellite stable” if no MSI or one MSI locus was present. Among MSI-high patients, 58% had positive lymphovascular invasion, 40% had deep myometrial invasion, and 50% had poorly differentiated tumors, signifying an aggressive phenotype.

2.5.1.4 Cell cycle genes

Cyclins and cyclin-dependent kinases (CDKs) are the regulatory subunits that govern progression through the mammalian cell cycle. Cyclins D1, E, A, and B1 form complexes with their respective CDK partners, and phosphorylate target substrates which leads to transcription and subsequently cell growth [88]. Overexpression of cyclins/CDKs is reported in many cancer phenotypes and upregulated cyclin/CDK protein levels are responsible for uncontrolled cell proliferation.

In EC, aberrant expression of cell-cycle regulators has been reported. Compared to normal endometrial glands, endometrial tumors had significantly higher rates of immunostaining of cyclin D1, cyclin E, cyclin A, cyclin B1, CDK2, CDK4, CDC2, p53, p21, and p27. Analysis by stage indicates that cyclin D1, CDK4, and p53 expression were significantly higher in advanced stages compared to early stage tumors [88].

In terms of survival, cyclin A overexpression was found to be an important indicator of poor prognosis in a multivariable model. In patients with cyclin A-positive tumors, OS following diagnosis was 20 months less than patients with cyclin-A negative tumors [88]. Cyclin A acts during the late G1 phase through the M phase; amplification of the cyclin A gene has been shown to induce anchorage independence in cultured cells, which is an important characteristic of transformation. The authors concluded that the adverse prognosis associated with cyclin-A positivity may be due to the growth potential this regulator maintains [88].

2.5.2 Type II EC

Type II EC comprises the remaining 10-20% of sporadic endometrial tumors. The two most common histologies of this type are CC and PS. Although these two histology types are commonly grouped together, IHC and genetic studies have shown that the alterations commonly observed in PS tumors are relatively infrequent in CC tumors [12].

Overall, the epidemiology and biology of Type II tumors are not well characterized, however the carcinogenic process appears unrelated to estrogenic stimuli. These tumors typically develop in women who are multiparous and normal weight, which contrasts with the tumorigenesis of Type I EC [15]. Moreover, Type II tumors generally develop from an atrophic endometrium in older women [14]. The clinical course of these tumors is generally more aggressive than Type I as five-year survival rates are significantly lower [13, 15]. Table 1 summarizes the major clinicopathologic features present in Type I and Type II ECs and Table 2 reports the frequency of genetic alterations that commonly occur in these two tumor types.

2.5.2.1 Tumor Suppressor Genes

The most commonly mutated tumor suppressor gene in Type II EC is p53. Approximately 71-85% of Type II tumors carry mutations in this gene [50]. p53 is a nuclear protein which responds to cellular stress caused by DNA damage, hypoxia, and oncogene activation; following damage, nuclear p53 accumulates due to interactions between MDM2 and p14ARF [89]. High levels of p53 signal transcription of p21 which induces cell cycle arrest by inhibiting cyclin D1 phosphorylation of the Retinoblastoma (Rb) gene [11]. Numerous cancers are associated with aberrant p53 signaling. Although p53 mutations occur in both types of EC however, this mutation is an early event in Type II tumorigenesis. p53 mutations occur in Type I tumors, but is

often described as a late event [90]. In contrast to other tumor suppressors, mutations in this gene result in an overexpression of the gene product. Mutant p53 protein is non-functional, however it resists degradation and exerts a dominant negative effect on wild-type p53 [17]. Positive p53 staining in IHC analyses is often associated with advanced stage, lymph node metastases, and the Type II subtype [83].

Inactivation of p16 is another molecular alteration reported to occur in Type II endometrial tumors [81]. p16 is a tumor suppressor gene that binds to CDK4 and inhibits the cyclin-CDK subunit from progressing past the G1 checkpoint of the cell cycle [91]. Therefore, reduced expression of the p16 protein is associated with uncontrolled cell growth [81]. Engelsen et al. [91] reported loss of p16 expression occurs in approximately 25% of endometrial tumors; moreover, low expression of p16 was significantly associated with CC and PS histology and advanced stage. The mechanism of inactivation is poorly understood however the three mechanisms that alter the p16 gene in other common cancers are homozygous deletion, promoter hypermethylation, and rarely, point mutations [92]. Recently, Ignatov and colleagues [92] reported that loss of p16 expression by gene deletion and promoter hypermethylation was significantly associated with invasive tumor behavior and development of metastases. Approximately 93% of primary tumors with metastases had alterations in the p16 gene compared to 58% of primary tumors with no metastases.

2.5.2.2 Oncogenes

The *HER2/neu* protein is a transmembrane tyrosine kinase receptor, similar in function to EGF-R [17]. Importantly, this protein does not have its own ligand binding domain, however it binds tightly to other ligand-bound EGF-R family members [84]. Due to these interactions, *HER2/neu* is an important regulator in cell growth and differentiation. *HER2/neu* overexpression is

commonly reported in EC, however the frequency of overexpression varies considerably by study. Recently, HER2/*neu* gene amplification measured by fluorescence in situ hybridization (FISH) was conducted in a large cohort of Type I and Type II EC [13]. HER2/*neu* gene amplification was significantly higher in Type II EC when compared with Type I EC (17% vs. 1%, $p < 0.001$). HER2 gene amplification was observed in 16% of the CC specimens and 17% of PS specimens. In Type II ECs HER2/*neu* and p53 are the most frequently studied molecular alterations. Other genes that have been recently explored in the carcinogenesis of Type II tumors are zinc-finger E-box-binding homeobox 1 (ZEB1) and folate receptor alpha [93, 94].

Table 1 Clinical and pathological features in Type I and Type II EC

Characteristic	Type I	Type II
Incidence	80%-90%	10%-20%
Age	Pre/peri-menopausal	>65 years
Primary exposure	Unopposed Estrogen	Unknown
Background endometrium	Hyperplastic	Atrophic
Histology	Endometrioid	Clear cell, papillary serous
Grade	Low or high grade	High grade

Table 2 Common genetic alterations in Type I and Type II EC

Genetic Alteration	Gene Type	Type I EC (%)	Type II EC (%)	Reference
PTEN inactivation	Tumor Suppressor	55	11	[95]
p53 mutation	Tumor Suppressor	16-40	80-90	[17, 95]
p16 inactivation	Tumor Suppressor	23	43	[91]
K-ras mutation	Oncogene	13-26	0-10	[95]
β -catenin mutation	Oncogene	25-38	rare	[95]
HER2/ <i>neu</i> mutation	Oncogene	10-30	45-80	[81]
BRAF mutation	Oncogene	20	rare	[86]
MSI	DNA repair genes	17	5	[95]

2.6 TUMOR-STROMAL INTERACTIONS

In normal tissues epithelial cells form fixed, ordered structures and perform tissue-specific functions [96], while the extracellular matrix (ECM) and stromal cells provide supportive functions to epithelial cells. The microenvironment of a particular tissue comprises a vast network of mobile cells that supply the epithelium with paracrine growth factors which control cellular responses [97]. Interactions between stromal cells (fibroblasts, endothelial cells, and inflammatory cells) and epithelial cells can occur either by direct cell contact or cytokine signaling.

EC originates in the epithelial cells either due to inherited mutations or an accumulation of somatic mutations in oncogenes and tumor suppressor genes. As endometrial epithelial cells acquire gene mutations, the ability of the local microenvironment to regulate cell growth becomes disrupted and results in an activated stroma, characterized by increased quantities of collagens, proteoglycans, and glycosaminoglycans [98]. Consequently, the activated stroma recruits inflammatory cells and fibroblasts which support the survival and proliferation of carcinoma cells due to abnormal paracrine signaling [97]. The relationship between tumor cells and stromal cells allows for the continued growth and invasion of the primary tumor mass.

Two stromal-related pathways have been studied in EC cell lines, in vivo mouse models, and human EC tissues: CXCL12/CXCR4 and hepatocyte growth factor (HGF)/c-Met/basic fibroblast growth factor (bFGF). Findings from experimental studies have yet to be replicated in large samples of patients with EC. Furthermore, reported relationships between expression of these proteins and prognostic factors are inconsistent. Understanding the association between expression of these potentially important genes and clinical parameters may inform therapeutic protocols to improve survival outcomes.

2.6.1 The CXCL12/CXCR4 pathway

2.6.1.1 Physiological role

CXCL12 (also called stromal cell-derived factor-1alpha), a chemokine of the CXC family and its receptor, CXCR4 (C-X-C motif receptor 4) are involved in proliferation, adhesion, chemotaxis and tumor metastasis in several malignancies [99]. Chemokines are a family of chemotactic cytokines that direct the movement of cells; cells which express the appropriate chemokine receptors migrate towards high concentrations of chemokines along a chemokine gradient [100]. Furthermore, chemokines are known to play an important role in immune responses, and recent evidence suggests that CXCR4 is the predominately expressed chemokine receptor in human cancers [101].

The ability of CXCL12 to induce cancer cell migration has been reported in other cancers. Research in breast, ovarian, and thyroid cancer shows that CXCL12 directly stimulates cancer cell migration and angiogenesis by interacting with its cognate receptor, CXCR4 [102-104]. Similar to expression patterns in other human solid cancers, CXCL12 and CXCR4 are expressed in an inverse manner in ECs. Compared to EC tissue, expression of CXCL12 is significantly higher in the normal tissues of the endometria while expression of the CXCR4 receptor is significantly higher in EC compared to normal endometrial tissue [105].

2.6.1.2 *In vitro* and *ex vivo* studies of CXCL12/CXCR4 and EC

Four studies have analyzed the CXCL12/CXCR4 axis in EC, but the conclusions regarding the prognostic role of these proteins are contradictory. Mizokami et al. [106] studied the relationship between tumor grade and expression of CXCL12 and CXCR4 in 41 Type I EC cases. Tissue resected from each case was analyzed using IHC and staining was classified on a 3-titered scale:

negative or weakly positive, moderately positive, and strongly positive. Both CXCL12 and CXCR4 expression were inversely related to tumor grade in the carcinoma compartment of the tissue [106]. In low-grade Type I tumors, CXCL12 expression was significantly higher compared to high-grade Type I tumors ($p<0.05$). Similarly, CXCR4 expression was significantly higher in low-grade Type I vs. high-grade Type I tumors ($p<0.05$) [106]. Stromal expression of CXCL12 and CXCR4 were assessed, however due to weaker expression in the stromal compartment no significant difference in stromal CXCL12 or CXCR4 expression among different grade cancers was observed.

Similarly, Kodama et al. [107] reported CXCR4 expression to be significantly lower in patients with characteristics of advanced EC. Fifty-five patients with Type I EC were evaluated. CXCR4 expression was analyzed by IHC and dichotomized as positive (more than 50% of cells stained) or negative (less than 50% of cells stained). CXCR4 was significantly lower in patients with advanced stage tumors ($p=0.004$), deep muscular invasion ($p=0.05$), lymph node metastasis ($p=0.03$), ovarian metastasis ($p=0.003$), and positive peritoneal cytology ($p=0.001$); all of which indicate an aggressive cancer phenotype. Additionally, survival following surgery was examined with a Cox multivariable model; CXCR4 expression was not an independent predictor of EC survival adjusted for other known prognostic factors [107]. Results from these two studies conflict with the notion that the SDF-alpha/CXCR4 pathway is involved in an aggressive EC phenotype, as tumors with poor prognostic traits were less likely to show positive staining.

The third study to examine this pathway was performed by Tsukamoto et al. [108] who investigated the interaction between CXCL12 and CXCR4 on the ability of endometrial tumors to invade the muscular layer of the endometrium. Muscular infiltration is an important prognostic factor in EC; regional node metastases and distant organ metastases are significantly more likely

to occur as the depth of muscular invasion increases [105]. In this study, five human EC cell lines and EC tissues from 34 Type I EC were examined. CXCR4 protein expression was detected using IHC and scored on a 3-titered scale similar to Mizokami et al [106]. The outcome, muscular invasion, was classified on the basis of depth: invasion of more than half of the muscle layer vs. invasion of less than half of the muscle layer. CXCR4 expression was significantly higher in endometrial tumors that invaded more than half of the myometrium compared to tumors with superficial invasion. The *in vitro* assays revealed several key findings: 1.) the receptor, CXCR4 is expressed in both EC cell lines and EC tissue shown by Western blot analyses, 2.) tumor cells became migratory when cultured with uterine smooth muscle cells as measured by an *in vitro* migration assay, 3.) uterine smooth muscle cells produce the chemokine, CXCL12 as measured by an ELISA assay, 4.) CXCL12 activates the PI3K/Akt pathway as shown by Western blot analysis using a p-Akt-specific antibody, and 5.) Akt activation is required for uterine smooth muscle cell-induced EC cell migration and treatment with a PI3K inhibitor significantly impeded cell migration [108]. The findings from this study suggest that the CXCL12/CXCR4 axis plays a significant role in EC invasion.

Most recently, Gelmini et al. [105] examined mRNA and protein expression of CXCR4 and CXCL12 in 41 Type I EC. Tumor samples and adjacent non-neoplastic tissues were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and IHC for the detection of mRNA and protein, respectively. The main prognostic factor in the *ex vivo* portion of the study was histological grade. CXCR4 mRNA expression was significantly higher in ECs compared to the paired normal tissue (median mRNA expression: 10.3 vs. 2.9, $p=0.04$). Conversely, CXCL12 mRNA expression was significantly higher in normal tissues compared to corresponding EC tissues (median mRNA expression: 15.6 vs. 5.1, $p=0.002$). This relationship mirrors the paradigm

of expression noted in other cancers. Additionally, CXCR4 mRNA expression was significantly lower in grade 1 tumors compared to grades 2 and 3 tumors indicating that CXCR4 expression increases in undifferentiated cancers ($p=0.04$) [105]. CXCR4 protein expression did not significantly differ by tumor grade as all ECs showed a uniform and high expression, regardless of grade.

2.6.1.3 *In vivo* studies of CXCL12/CXCR4 and EC

In addition to exploring this pathway in human EC tissues, Gelmini et al. [105] further explored the invasive capacity of CXCR4 receptor expressing cells in an *in vivo* mouse model. Following intraperitoneal injection of the HEC1A human endometrium adenocarcinoma cell line, experimental mice developed distant metastases in the lung, liver, and peritoneum. Nude mice treated with an anti-CXCR4 antibody had complete regression of liver and lung metastases compared to the control mice. In anti-CXCR4 treated mice the metastatic index of the peritoneum was 2.5% compared to a metastatic index of 70% in the control mice, implying that treatment with chemokine antagonists may reduce distant metastases in patients where CXCR4 is highly expressed.

2.6.1.4 Mechanism of action

Although the results describing the association between CXCL12/CXCR4 expression and EC prognosis are conflicting, studies with growth and migratory assays confirm the ability of CXCL12 to stimulate cancer cell growth and invasion. Two mechanisms of action are downstream activation of two independent pathways: the PI3K/Akt pathway and the MAPK/ERK pathway [109]. Akt is a downstream target molecule of PI3K. PI3K is activated by growth factor receptor signaling cascades and once stimulated PI-3K phosphorylates and

activates Akt. Akt targets many proteins involved in cellular functions such as growth, differentiation, cell cycle progression, and cell metabolism [109]. The MAPK pathway (also known as extracellular signal-regulated kinases, ERK) is also involved in cellular processes similar to Akt [84]. When activated by upstream kinases, MAPK/ERK translocates to the nucleus of stimulated cells and phosphorylates nuclear targets [84].

EC *in vitro* studies have provided evidence that CXCL12 activates both the PI3K/Akt pathway and the MAPK/ERK pathway [109, 110]. Furthermore, activation of each pathway is correlated with PTEN and estrogen receptor expression status. Li et al. [110] used the PTEN-deficient Ishikawa cell line to examine the effect of CXCL12 on signaling transduction and cell growth. PTEN was transfected back into Ishikawa cells to examine whether proliferation differed in PTEN-deficient vs. PTEN-present cell lines. Levels of pAKT were significantly lower in the PTEN-present cells compared to the PTEN-deficient cells, however the level of pERK was not significantly different between the two cell lines [110]. The growth-promoting effects of CXCL12 on PTEN-present cells were significantly less compared to the PTEN-deficient cells; when 50 ng/ml of CXCL12 was added to 96-well plates of both lines, the optical density (measured at wavelength 490 nm) was 1.4 in the PTEN-present cells and 0.6 in the PTEN-deficient cells ($p < 0.05$). The findings from this study imply that PTEN may inhibit CXCL12 induced growth-promoting effects. As PTEN mutations are reported in 40-80% of Type I ECs, the role of CXCL12 in stimulating further growth and invasion in EC is highly relevant.

Zhao et al. [109] further investigated this mechanism by studying the role of estrogen receptor status of the tumor and the ability of CXCL12 to induce proliferation in EC cell lines. Using Ishikawa and HEC-1A EC cell lines, both of which differ with respect to ER and PTEN profiles, low concentrations of CXCL12 were able to produce cell proliferation in the Ishikawa

cell line but not the HEC-1A cell line. The Ishikawa cell line is positive for ER expression but is negative for the production of PTEN due to a mutation in the PTEN gene [110]. Conversely, the HEC-1A cell line is positive for PTEN expression and lacks ER expression [109]. A Western blot analysis showed that low concentrations of CXCL12 increased the level of ERK in HEC-1A cells without changing the level of Akt. Conversely, CXCL12 increased the level of Akt without changing the level of ERK in Ishikawa cells. When high concentrations of CXCL12 were administered, both Akt and ERK increased significantly in both cell lines. This study indicates that low concentrations of CXCL12 activate only the dominant signal transduction pathway. In EC cells that lack PTEN expression (Ishikawa), the Akt pathway is activated with CXCL12 stimulation. In EC cells that lack ER expression (HEC-1A), the ERK pathway is stimulated following administration of CXCL12 to cells.

2.6.2 The HGF/c-Met/bFGF pathway

2.6.2.1 Physiological role

Hepatocyte growth factor (HGF) (also called scatter factor, SF) is a stromal-derived growth factor with mitogenic and motogenic effects on various cell types [111]. HGF regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to its proto-oncogenic receptor, c-Met [112]. HGF acts as a multi-functional cytokine on cells of mainly epithelial origin. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorigenesis, and tissue regeneration.

In normal epithelial cells, HGF is not expressed however carcinoma cells may acquire the ability to produce HGF. Additionally, various factors secreted by carcinoma cells can induce expression of HGF. Examples of these inducers include interleukin-1 β , basic fibroblast growth

factor (bFGF), tumor necrosis factor (TNF)- α , and prostaglandin E2 [113, 114]. Similar to the CXCR4 receptor, the c-Met receptor is overexpressed in carcinoma cells. The proposed relationship of this pathway in EC is that HGF-inducers stimulate HGF secretion from stromal cells which can then bind to the c-Met receptor stimulating metastasis and invasion of the epithelial tumor in a paracrine fashion.

2.6.2.2 *In vitro* and *ex vivo* studies of HGF/c-Met and EC

In vitro studies in breast, bladder, lung, and pancreatic cancer cell lines verify the ability of HGF to stimulate invasion [115-117]. Additionally, the c-Met receptor is commonly overexpressed in these cancers indicating a paracrine relationship between HGF and c-Met. The findings published thus far on the role of this pathway in EC progression corroborate these results.

In normal endometrial tissues, Sugawara et al. [118] demonstrated that HGF had stimulatory and migratory effects. In this *in vitro* study endometrial biopsy samples were obtained from women undergoing laparoscopy for benign gynecological conditions. All women were in the proliferative phase of the menstrual cycle when samples were taken. RT-PCR and Southern blot hybridization demonstrated that c-Met mRNA was present in purified endometrial epithelial cells and the normal human endometrium, whereas HGF mRNA was not observed in the isolated epithelial cells but was present in the normal endometrium [118]. This study hypothesized the cell type of origin for HGF to be stromal fibroblasts or infiltrated blood cells such as macrophages, however this was not further explored. An MMT proliferation assay showed that HGF stimulated a two-fold increase in the number of endometrial epithelial cells compared to the control culture where no HGF was added ($p < 0.05$). Moreover, the potential motogenic effects of HGF were examined with a Boyden's chamber assay; a significant increase in the number of migrated endometrial epithelial cells occurred when HGF was added to the medium compared to

the control experiment where no HGF was added. This study is important in clarifying the role of the HGF/c-Met pathway in the non-neoplastic endometrium. In this context, HGF promotes regeneration and repair that occurs normally during the menstrual period. Dysregulation of this pathway during malignant transformation has negative consequences, namely invasion and metastasis.

Bae-Jump et al. [119] studied HGF and c-Met expression in ECs obtained from surgical specimens (N=4) and EC cell lines (KLE, RL-95, HEC-1A, and HEC-1B). After isolating endometrial stromal cells from normal human endometrial tissue, enzyme-linked immunosorbent assay (ELISA) was performed to demonstrate that stromal cells secrete the HGF protein. Additionally, EC tissues and EC cell lines showed positive staining for c-Met mRNA and protein using RT-PCR and Western blot analysis, respectively [119]. Importantly, these experiments also showed that endometrial stromal cells did not express the c-Met receptor, reinforcing the paracrine nature of this pathway. Different grade EC cell lines expressed various amounts of c-Met, however the relationship was inconsistent with findings from other cancers. The KLE cell line, which was derived from a poorly differentiated endometrial adenocarcinoma, expressed the least c-Met protein; the RL-95 cell line, derived from a moderately differentiated tumor expressed an intermediate amount of c-Met protein; and the HEC-1A and HEC-1B cell lines, isolated from patients with well-differentiated stage 1A and 1B adenocarcinoma tumors, respectively, expressed the greatest amount of c-Met protein. Assuming that c-Met overexpression is a marker of advanced ECs, expression would be expected to increase with poorly differentiated tumors. Importantly, the sample size in this study consisted of four cell lines and four endometrial carcinoma tissue specimens.

In addition to HGF and c-Met, HGF-inducers play an important role in this pathway. As their name implies, these proteins induce the transcription and expression of HGF. Using the Ishikawa and HEC-1 EC cell lines, as well as tissue from neoplastic and non-neoplastic endometria, Yoshida et al. [111] characterized staining patterns of HGF, c-Met, and several possible HGF inducers by IHC and RT-PCR. EC tissue and normal tissue from benign endometrial cases were collected from patients and the stromal and carcinoma components were isolated in order to profile the components separately.

HGF secreted from endometrial stromal cells (normal and cancer associated) promoted the proliferation and invasion of EC cells. HGF mRNA was not detected in the HEC-1 or Ishikawa EC cell lines however both normal endometrial stromal cells and cancer associated stromal cells expressed the HGF gene. HGF mRNA was six-fold higher in cancer stromal cells compared to the normal stromal cells. The effect of HGF on the invasive ability of EC cells was significant; addition of HGF to cell cultures of HEC-1 and Ishikawa cells increased the penetrance of these cells four times more than cells cultured in the absence of HGF as measured by the Matrigel invasion chamber assay ($p < 0.05$) [111]. Proliferation of cancer cells measured by an MTT assay was promoted by the addition of HGF in the HEC-1 cancer cell line but not the Ishikawa. Additionally, the c-Met gene was expressed in both EC cell lines and in 8 of the 10 cancer cases. In order to further clarify the mechanism of interaction between carcinoma cells and the cancer stroma, several HGF inducers were investigated. bFGF, interleukin-6 (IL-6), TNF- α , and prostaglandin E2 were investigated as potential carcinoma-associated inducers of HGF. bFGF significantly increased HGF transcription three-fold. The findings from this study indicate that carcinoma cells express the c-Met receptor as well as produce bFGF which acts upon stromal

cells. In turn, cancer associated stromal cells secrete HGF which then binds to the c-Met receptor activating a complex program of invasive growth [111, 112].

Two prognostic studies have examined HGF and c-Met expression in EC patients. Wagatsuma and colleagues [120] examined the association between HGF and c-Met expression, clinicopathologic factors, and OS in 93 surgically staged Type I EC patients. Protein expression was analyzed in 14 normal endometrial specimens: 5 in the proliferative phase of the menstrual cycle, 4 in the secretory phase of the menstrual cycle, and 5 atrophic specimens. IHC for HGF and c-Met was performed on paraffin-embedded blocks; staining of both proteins was dichotomized as focal (as less than one-third of the gland or cancer cells showing positive reactivity for HGF or c-Met) or diffuse (more than one-third of the gland or cancer cells showed positive staining for HGF or c-Met). In normal endometrial specimens, diffuse staining for HGF and c-Met was observed in 79% and 14% of specimens, respectively. Approximately 90% and 63% of ECs showed diffuse staining for HGF and c-Met, respectively. Diffuse staining of c-Met was significantly associated with advanced surgical stage (stages III and IV, $p=0.03$) and poorly differentiated histology ($p=0.002$) compared to focal c-Met staining. Age, myometrial invasion, and vascular involvement were not significantly associated with diffuse c-Met staining at a $p<0.05$. HGF expression was significantly higher in stage III and IV endometrial tumors compared to stage I and II endometrial tumors (Mann-Whitney U -test, $p=0.0013$). Multivariable analyses showed that OS was significantly associated with stage, differentiation, presence of myometrial invasion, and a microvessel count greater than 110; neither HGF or c-Met expression were independent predictors of prognosis.

Similarly, Bishop et al. [121] reported positive HGF and c-Met staining in 100% ($n=38$) and 87% ($n=33$) of PS EC patients, respectively. Furthermore, the level of expression was compared

between PS cases and women with atrophic endometrial tissues, low-grade Type I cases, and high-grade Type I cases. Patients with cancer had more c-Met and HGF expression than those with atrophic endometrial tissues. Neither HGF nor c-Met was significantly associated with depth of invasion, stage, or lymph node status. Compared to patients with weak HGF expression, OS was significantly worse for patients with strong HGF expression ($p=0.04$). A similar trend for c-Met expression was observed, however this comparison did not reach statistical significance ($p=0.10$).

The prognostic value of bFGF has also been examined in two small cohorts of EC cases. High expression of tumor-derived bFGF protein was significantly associated with poor differentiation, presence of tumor necrosis, and vascular invasion [122]. Similarly, Fujimoto et al. [123] showed that bFGF mRNA expression was significantly higher in poorly differentiated and advanced stage tumors. The association between bFGF expression and survival in EC has not been explored.

The *in vitro* and *ex vivo* studies presented here demonstrate the following: 1.) the HGF ligand is expressed in the normal endometrium 2.) HGF has the ability to induce migration of endometrial epithelial cells as shown by migration assays 3.) the expression of c-Met is upregulated in ECs compared to normal endometrial tissues 4.) several inducers of HGF have been examined in EC studies however bFGF is the only factor that has been shown to significantly increase the transcription of HGF 5.) the role of HGF, c-Met, and bFGF in EC prognosis is inconclusive based on the few studies that have examined these relationships.

2.6.2.3 Mechanism of action

Kanayama et al. [124] proposed that HGF stimulates anoikis resistance in EC cells by interacting with cyclooxygenase-2 (COX-2), an enzyme that has been implicated in the promotion of

carcinogenesis [124]. RL95-2 cells, a human EC cell line, were plated on tissue culture dishes in the presence and absence of HGF. A terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assay, which reports the percentage of apoptotic cells, was performed. Treatment with HGF significantly decreased the number of apoptotic cells in the RL95-2 cell line compared to the cells incubated in the absence of HGF ($23.0 \pm 2.7\%$ vs. $33.6 \pm 3.4\%$, $p < 0.05$).

Additionally, activation of the ERK or PI3K/AKT signaling pathways was examined to determine the mechanism by which HGF induces anoikis resistance. Cells from the RL95-2 cell line were pretreated with ERK and PI3K inhibitors and incubated with HGF. TUNEL was used to count apoptotic cells. Compared to untreated cells, the number of apoptotic cells was significantly increased in the cells pretreated with PI3K inhibitors compared to cells incubated with HGF ($34.9 \pm 3.4\%$ vs. $23.0 \pm 2.7\%$, $p < 0.01$), indicating this pathway is involved in HGF anoikis resistance. Conversely, cells pretreated with an ERK inhibitor failed to effectively inhibit anoikis resistance induced by HGF.

Finally, this study demonstrated that Meloxicam, a COX-2 inhibitor, significantly blocked cellular viability. In those cells treated with the highest concentration of Meloxicam, the percent of surviving RL95-2 cells was significantly decreased compared to those cells cultured in the absence of Meloxicam. Therefore, HGF may inhibit anoikis by activating the PI3K/AKT pathway which further induces COX-2 expression. Importantly, COX-2 inhibitors may play a role in the treatment of ECs which overexpress HGF as seen by the inhibition of this pathway with Meloxicam.

Choi et al. [114] studied the hormonal influence of estrogen on HGF stimulation in EC cell lines. Although estrogen is known to be an important factor in endometrial carcinogenesis, its role in promoting the invasion and metastasis of EC has not been addressed. The HEC-1A (well

differentiated) and KLE (poorly differentiated) EC cell lines were cultured either in an estrogen dominant environment, a progesterone dominant environment, or without ovarian hormones (control). Estrogen increased the invasion of both HEC-1A and KLE cells significantly compared to the control ($p < 0.05$) whereas progesterone opposed invasion [114]. Importantly, when HEC-1A cells were cultured in the presence of estrogen without stromal cells present, little invasion was detected. When HGF was added to the culture, EC cells resumed an invasive presence, even in the absence of stromal cells. Furthermore, cell cultures showed that TNF- α was a potent mediator of the estrogen-stimulated stromal HGF secretion. When NK4, an HGF antagonist was added to the cell culture, estrogen-induced invasion was completely nullified.

2.6.3 The role of estrogen signaling in tumor-stroma interactions

Although the molecular mechanisms related to estrogen signaling in EC have not been fully clarified, epidemiologic evidence supports an association between increased estrogen exposure and the risk of developing EC. Several lines of evidence support a relationship between estrogen action and the stromal pathways presented here in the context of EC. First, Choi et al. [114] reported that EC cells cultured under estrogen dominant conditions induced TNF- α expression from carcinoma cells which subsequently induced HGF expression from stromal cells [114]. Furthermore, Zhao et al. [109] reported that CXCL12 is a direct target of estrogen action and a strong inducer of cell proliferation in EC. In this study, CXCL12 induced cell growth in a dose-dependent manner in the Ishikawa cell line, a cell line known to express estrogen receptor, whereas the same effect was not achieved within the HEC-1A cell line which lacks estrogen receptor expression.

Additionally, stromal-derived pathways can directly contribute to the activation of estrogen receptor. CXCL12/CXCR4 and HGF/c-Met activate downstream kinases, notably MAPK and PI3K/Akt, which subsequently phosphorylate estrogen receptor on the transcriptional activation function domain, AF-1 [109, 114, 125, 126]. Ligand-independent stimulation of estrogen receptor by MAPK and PI3K/Akt results in conformational changes in estrogen receptor, recruitment of co-activators, and activation of target gene transcription, similar to estrogen activation of the receptor [127]. Therefore, a potent feedback loop is plausible: the targets of the HGF/c-Met and CXCL12/CXCR4 pathways activate estrogen receptor which can further stimulate production of HGF and CXCL12, binding to their cognate receptors (c-Met and CXCR4, respectively), and activation of downstream signaling events.

Furthermore, stromal cells surrounding the primary tumor cells can contribute to the biosynthesis of estrogen. Estrogen metabolizing enzymes such aromatase and the 17 β -hydroxysteroid dehydrogenases are abundantly expressed in stromal cells and convert androgen precursors and inactive estrogens into the metabolically active estradiol. Consequently, the intratumoral concentration of E2 increases which may further promote EC progression through estrogen receptor activation [128].

3.0 SUMMARY

EC is a significant public health problem. In 2010, the American Cancer Society estimated that nearly 8,000 deaths from this cancer occurred. Two subtypes are commonly described in the literature, Type I and Type II EC. These two subtypes differ with respect to etiology, carcinogenic mutations, and prognosis. Type I EC is a highly preventable cancer; estrogen exposure mediated by obesity is the strongest risk factor for development of this subtype. Furthermore, a large proportion of Type I EC patients is cured by surgery and favorable prognosis is observed. Cancer initiating mutations of this subtype include PTEN inactivation, K-Ras mutation, and MSI. Conversely, the risk factors associated with development of Type II EC are unknown; these patients are more likely to have an aggressive disease characterized by recurrence, metastasis, and high mortality. Typically, p53 and HER2/*neu* are the commonly described carcinogenic mutations in this subtype.

Current treatments for the management of aggressive EC include radiotherapy and chemotherapy, which are limited in prolonging OS despite reducing the risk of recurrence. Furthermore, toxicities associated with these treatments are substantial. In recent years, cancer control treatments have moved away from traditional cytotoxic therapies to the use of small-molecule inhibitors or antibodies, which provide a targeted treatment approach. The identification of prognostic biomarkers in epidemiologic studies is a necessary precursor to implementing such treatment.

The tumor microenvironment refers to the complex network of cells that surround and interact with the primary tumor. In normal tissue homeostasis, fibroblasts, endothelial cells, and inflammatory cells interact with tissue-specific epithelial cells to exert normal tissue function. Following endometrial carcinogenesis, the ability of the local microenvironment to regulate cell growth becomes disrupted and results in an activated stroma. Further recruitment of stromal cells to the primary tumor site supports the survival and proliferation of carcinoma cells due to abnormal paracrine signaling [97]. This altered relationship between tumor cells and stromal cells allows for an aggressive cancer phenotype, characterized by growth and invasion of the tumor. Recent studies in breast, colorectal, and lung cancers assert that tumor microenvironmental factors are associated with poor survival and can potentially serve as therapeutic targets.

The *in vitro* and *ex vivo* literature suggests a role for two pathways, CXCL12/CXCR4 and HGF/c-Met/bFGF, to enhance EC progression and metastasis. CXCL12 and CXCR4 may influence survival through directional movement of tumor cells, while HGF, c-Met, and bFGF are angiogenic factors that enhance tumor growth and metastasis. In order to study how expression of these proteins relates to the etiology and prognosis of EC, these biomarkers need to be explored in large sets of existing data and tissue bank repositories. The main goal of this research is to add to the body of knowledge regarding EC prognosis by exploring the role of the tumor microenvironment. The three specific aims of this research are:

- 1a.) Compare the characteristics of patients with Type I and Type II EC
- 1b.) Identify prognostic factors for OS among patients with low-grade Type I, high-grade Type I, and Type II EC

2.) Evaluate the association between CXCR4 and CXCL12 protein expression, prognostic factors, and survival outcomes in a sample of Type I and Type II EC cases

3.) Evaluate the association between HGF, c-Met, and bFGF protein expression, prognostic factors, and survival outcomes in a sample of Type I and Type II EC cases

4.0 ARTICLE 1: FACTORS ASSOCIATED WITH TYPE I AND TYPE II ENDOMETRIAL CANCER

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With kind permission from Springer Science+Business Media: *Cancer Causes and Control*, *Factors associated with Type I and Type II endometrial cancer*, 21, 2010, 1851, Ashley S. Felix, Joel L. Weissfeld, Roslyn A. Stone, Robert Bowser, Mamatha Chivukula, Robert P. Edwards, Faina Linkov.

4.1 ABSTRACT

Objective: We investigated risk factors for Type II (N=176) vs. Type I (N=1,576) endometrial cancer (EC) in cases treated at Magee-Womens Hospital between 1996 and 2008.

Methods: Clinical data were available from the University of Pittsburgh Medical Center (UPMC) Network Cancer Registry. Logistic regression was used to estimate the adjusted odds of having Type II EC vs. Type I EC. Risk factors of interest in this analysis were: age, race, body mass index (BMI), year of diagnosis, parity, menopausal status, and history of additional primary tumors.

Results: Relative to women with Type I EC, women with Type II EC were more likely to be older at diagnosis (OR: 1.03 per 1 year increase in age, 95% CI 1.01-1.05), of non-white race (OR: 2.95, 95% CI 1.66-5.27), have a history of additional primary tumors (OR: 1.56, 95% CI 1.05-2.32), and less likely to be obese (OR: 0.45, 95% CI 0.29-0.70).

Conclusion: In this large retrospective cohort of patients with EC, the striking difference in risk factors associated with Type II vs. Type I tumors suggests that these subtypes represent different disease entities that require different treatment modalities. Currently, Type II cases have a significantly worse prognosis compared to Type I. Further characterization of risk factors associated with developing Type II tumors is needed to prevent this aggressive malignancy.

Keywords: endometrial cancer, epidemiology, Type I, Type II

4.2 INTRODUCTION

Endometrial cancer (EC) is a common malignancy in the US and around the world. The incidence of EC exceeds the incidence of cervical, ovarian, vaginal, and vulvar cancers combined [129]. Despite being a common cancer, the mortality rates from this disease (4.1 deaths per 100,000 women) are relatively low, which is mainly attributed to early detection. Between 75% and 80% of cases are diagnosed with tumors confined to the uterus (stage 1), which are effectively treated with hysterectomy [130]. Since the 1960's, EC-related mortality has declined significantly, although recent data suggest that the number of EC deaths may be on the rise [3, 131-133].

Prior to the 1980's, EC was broadly characterized as a single disease. However, observations by Lauchlan, Hendrickson et al., and Bokhman led to the description of two distinct types based on histologic and molecular characteristics [80, 134, 135]. Type I EC, commonly referred to as the endometrioid type, comprises 80%–90% of all sporadic endometrial cancers [11]. Histologically, these tumors can be adenocarcinoma with or without squamous differentiation and often are well-differentiated [17]. Furthermore, epidemiological evidence suggests that the multi-step carcinogenic process of Type I endometrial tumors begins with simple endometrial hyperplasia, progresses to complex atypia hyperplasia, and then develops into the precursor lesion, endometrial intraepithelial neoplasia (EIN) [63-65]. Type II EC, or nonendometrioid tumors, encompasses the remaining 10%-20% of sporadic endometrial tumors [11]. The two histologies of this subtype are uterine papillary serous carcinoma (UPSC) and clear cell carcinoma. Both cancers appear to progress from an atrophic endometrium to the precursor lesion, endometrial glandular dysplasia (EmGD) [14, 90, 136].

In addition to differences in histology, the etiology and survival related to these two subtypes are vastly different. Type I tumors are the prototypical estrogen-dependent tumors; risk factors that increase women's exposure to circulating levels of estrogen are associated with increased risk of Type I EC. Similarly, factors that decrease progesterone are associated with increased risk of Type I EC. Traditionally cited risk factors for Type I EC are obesity, estrogen replacement therapy (ERT), nulliparity, and medical conditions that result in high estrogen levels, such as estrogen-secreting ovarian tumors and polycystic ovarian syndrome. In addition, Type I tumors are more common than Type II tumors in pre- and peri-menopausal women [11].

The epidemiology and biology of Type II tumors are not well characterized, although a few studies report that Type II cases are more likely to be older, of normal-weight, multiparous, and African-American compared to Type I cases [10, 15, 68, 137-141]. The tumorigenesis of Type II EC is not thought to operate through the estrogen pathway, as normal-weight and parous women have decreased estrogen exposure compared to obese and nulliparous women. Low incidence of Type II tumors makes this subtype difficult to study.

While the incidence of Type II tumors is low compared to Type I, excess mortality is associated with Type II EC. In an analysis of Surveillance, Epidemiology and End Results (SEER) data, Hamilton et al. reported that while 11% of endometrial cancers were Type II, 47% of deaths in the SEER cohort occurred in this subtype [10]. Furthermore, stage adjusted five-year overall survival rates for Type II tumors are significantly worse compared to Type I tumors [15]. Understanding the etiology of this rare, under-investigated, and deadly malignancy is important for the primary prevention of these cancers, early detection, and monitoring for relapse. Therefore, the primary goal of the present study is to compare the characteristics of Type I and Type II EC cases treated at Magee-Womens Hospital between 1996 and 2008.

4.3 METHODS

4.3.1 Data collection

All data for this study were retrieved from the University of Pittsburgh Medical Center (UPMC) Registry Information Services (RIS), a division within the UPMC Network Cancer Registry [142]. The UPMC Network Cancer Registry collects demographic, medical history, diagnostic findings, primary cancer identification, stage, grade, treatment and outcomes information on patients from all UPMC managed facilities. Certified cancer registrars abstract data from both the paper and electronic medical records into the Cancer Registry database, which is then queried by an RIS research specialist. This study includes cases with an International Classification of Diseases for Oncology (ICD-O 3rd Edition) primary site code between C54.0–C54.9 and C55.9 who were treated at Magee-Womens Hospital between 1996 and 2008. The coding scheme of this data system has varied over time and current standardized coding protocols were first used in 1996 [143]. Specific data elements include age at diagnosis, year of diagnosis, height, weight, race, history of additional cancer primaries, number of live births (parity), menopausal status, age at menopause, and tumor histology.

4.3.2 Case ascertainment

ECs treated between 1996 and 2008 were identified by the RIS research specialist. Histology subtype (Type I and Type II) was assigned by a trained gynecologic pathologist (MC) based on expertise and previously published literature [80, 144]. Type I EC histologies included adenocarcinoma, endometrioid, mucinous adenocarcinoma, and adenocarcinoma with squamous

differentiation (ICD-O-3 morphology codes: 8140, 8380, 8382, 8480, 8482, 8560, and 8570).

Type II EC histologies included clear cell carcinomas and papillary serous carcinomas (ICD-O-3 morphology codes: 8310, 8441, and 8460). Slides for nine patients with papillary adenocarcinoma (ICD-O-3 code: 8260) were reviewed by the pathologist and confirmed to be papillary serous carcinoma.

4.3.3 Statistical analysis

Unconditional logistic regression was used to estimate the crude and adjusted odds ratios (ORs) of having Type II vs. Type I EC. The factors of interest in this study were categorized as shown in Table 1: race was classified as white or non-white due to the low number of African-American, Asian, and other races. Year of diagnosis was coded as 12 indicator variables. BMI was calculated from weight and height as $(\text{weight in pounds} \times 703) / (\text{height in inches})^2$ [145]. BMI was analyzed both as a continuous variable (kg/m^2) and a categorical variable (i.e., underweight ($<18.5 \text{ kg/m}^2$), normal weight ($18.5\text{-}24.9 \text{ kg/m}^2$), overweight ($25\text{-}29.9 \text{ kg/m}^2$), and obese ($>30 \text{ kg/m}^2$)) using definitions from the Centers for Disease Control and Prevention [145]. We also incorporated an unknown category when height or weight was missing ($N=142$, 8.1%). The categorical BMI variable was used in the univariate and multivariable models. Parity was categorized as no live births, 1 live birth, 2 live births, 3 or more live births, and unknown. History of additional cancer primaries and postmenopausal status were coded as no, yes, or unknown. Age at diagnosis and age at menopause were treated as continuous variables.

Variable selection for the multivariable logistic regression model was based on the association between each potential factor and the probability of having Type II EC rather than Type I EC using the likelihood ratio test p -value from the univariate logistic regression models.

A broad significance level of $p < 0.10$ in the univariate models was used as the criterion for entry into the multivariable model. Pairwise multiplicative interactions between each of the covariates were added to the model one at a time and tested with likelihood ratio tests. Interactions between the covariates were tested using a two-sided alpha of 0.05. The Hosmer and Lemeshow goodness of fit test was performed to assess lack of fit of the final model. This study was approved by the University of Pittsburgh Institutional Review Board.

4.4 RESULTS

Between 1996 and 2008, 1,964 EC patients were diagnosed at Magee-Womens Hospital. Of these, the 1,752 cases with either Type I or Type II EC were included in the present report. Based on the pathology report, Type I tumors accounted for 90% of cases in the study group. Table 5 compares the frequency of potential risk factors between the tumor types. Approximately 11% of Type II cases were non-white compared to 4% of Type I cases. Year of diagnosis was not significantly different between the two tumor types. A large proportion of all cases were overweight or obese, however 36% of Type II cases were obese compared to 55% of Type I cases. Forty-five percent of Type II cases had three or more live births compared to 32% of Type I cases. Type II cases were also more likely to have a history of additional cancer primaries compared to Type I cases (23.9% vs. 14.2%). Breast cancer (N=125, 47%), ovarian cancer (N=32, 12%), and colorectal cancer (N=26, 10%) were the three most common additional malignancies. Breast and colorectal cancers were more common among the Type II cases, while ovarian cancer was more common among the Type I cases. Menopausal status was also significantly related to tumor type; 86% of Type II cases were postmenopausal compared to 76%

of Type I cases. Finally, Type II cases were significantly older than Type I cases (median age: 68 years vs. 60 years).

In the univariate analyses, race, BMI, parity, history of additional primaries, menopausal status, and age at diagnosis were significantly associated with type of EC (Table 6). Although year of diagnosis was not significantly associated with tumor type, this variable was retained for adjustment purposes. In the adjusted models, increasing age ($p<0.001$), non-white race ($p<0.001$), and history of additional primaries ($p=0.03$) were significantly associated with increased odds of having Type II EC, while obesity was inversely associated with the odds of Type II EC ($p<0.001$). The Hosmer and Lemeshow goodness of fit test indicated no lack of fit of this model ($\chi^2=6.35, p=0.61$). None of the interactions considered was statistically significant.

4.5 DISCUSSION

This registry-based study examines the relationship between pretreatment characteristics in Type I and Type II ECs in a large group of patients diagnosed between 1996 and 2008. Factors significantly associated with Type II EC vs. Type I EC were older age, non-white race, lower BMI, and history of additional primaries, all of which have been identified in the published literature. Soslow, Hamilton, and Cirisano have reported that Type II cases are older than their Type I counterparts, although the age differential is most pronounced for uterine papillary serous carcinomas (UPSC) compared to endometrioid tumors [10, 139, 146]. Furthermore, African-Americans make up a disproportionate number of Type II tumors in many case-series, with the widest differential being between the UPSC and endometrioid cases. In the Cirisano study, 34% of UPSC's were African-American compared to 15% of cases with endometrioid tumors

($p < 0.0001$); in the Hamilton case-series 15% of cases with UPSC tumors were African-American while 7% of cases with grade 3 endometrioid tumors were African-American ($p < 0.0001$) [10, 139]. Our study is not directly comparable to the previously mentioned studies, as we grouped all non-white cases together.

To date, obesity is the strongest risk factor for development of EC, with the underlying mechanism being increased estrogen exposure [14]. Consequently, the link between obesity and endometrial cancer is stronger for cases with Type I tumors, the prototypical estrogen-dependent tumor. In the present study, compared to normal weight cases, obese cases had an OR of 2.22 of having Type I EC rather than Type II EC. Although this finding is consistent with the estrogen hypothesis, two prospective studies have reported that increasing BMI also is a risk factor for development of Type II EC. In the 1 million Norwegian women study, Bjorge et al. reported that overweight and obese women were 1.26 and 1.94 times more likely, respectively, to develop Type II cancer compared to normal-weight women over a 25-year follow-up [147]. Likewise, McCullough and colleagues reported that a BMI of 30 kg/m² or greater was significantly associated with developing Type II tumors (RR: 2.87, 95% CI 1.59-5.16). Importantly, both studies combined grade 3 endometrioid tumors with UPSC and clear cell tumors in their analyses, which may explain the association between obesity and Type II tumors in these studies. Furthermore, both studies compared cancer cases to healthy controls. BMI may have an important role in the development of all ECs, however the effect appears to be stronger for Type I cancers compared to Type II cancers.

In this study, Type II cases had an OR of 1.56 of having a history of an additional primary compared to Type I cases ($p = 0.03$). The most common additional primary cancer in this cohort was breast cancer. Of the Type II cases with an additional primary, 59.5% had breast cancer,

compared to 44.6% for the Type I cases ($p=0.07$). Several hypotheses for an association between Type II EC and additional cancer primaries exist. First, these cancers may share similar risk factor profiles. Second, radiation treatment for proximate cancers may increase the incidence of radiation-induced ECs or vice-versa. Third, the presence of multiple cancers may be a manifestation of inherited cancer syndromes, such as hereditary nonpolyposis colorectal cancer (HNPCC) syndrome; however these genetic disorders are relatively rare in the population. Finally, multiple cancer primaries may be a result of mutations in unidentified cancer predisposing genes [68].

Potential limitations of this study include patient selection and misclassification biases. Although all cases in this study received their first course of treatment at Magee-Womens Hospital, not all cases were diagnosed at this facility. Forty-five percent of the cases in this study came to Magee-Womens Hospital after being diagnosed elsewhere; these patients could be significantly different from the patients who were diagnosed and treated at Magee-Womens Hospital. The referred cases may be more advanced or suffer from multiple co-morbidities that require specialty care at a large academic hospital such as Magee-Womens Hospital. Second, our use of registry data obtained through data abstraction from medical records allows for potential data entry errors. The UPMC Cancer RIS performs rigorous quality control on certain data elements, which enhances their reliability; we focused only on those variables in our analyses. As the UPMC Network Cancer Registry is the official source of cancer statistics for the Pennsylvania Department of Health and maintains a reputation of high quality, misclassification bias is not a major concern.

The major strengths of this study include a large cohort of patients, reliable data, and central pathology review at a single institution. Overall, this study included 1,752 EC patients, including

176 Type II cases. Compared to other single institution studies of EC, our study included the largest number of Type II cases to date. In other case-series, the numbers of Type II cases has ranged between 32 and 87, excluding a study which examined patients from the population-based SEER registry [140, 146, 148, 149]. Furthermore, we only included cases that were treated at Magee-Womens Hospital instead of including the entire pool of EC cases available from the UPMC Network Cancer Registry. The fact that all cases were centrally reviewed by gynecologic pathologists at Magee-Womens Hospital increases confidence in the validity of the tumor type definitions.

The etiology of Type II tumors remains elusive. The findings from this study verify previously published reports. In our study, BMI was inversely associated with having Type II EC, which suggests that this carcinogenic pathway is not driven by excess estrogen exposure. Importantly, a large proportion of Type II cases in this study were overweight or obese (27.3% and 36.4%, respectively). Finally, this study adds to the growing body of literature related to an association between multiple cancers and Type II EC. Future studies on the etiology of the rare yet aggressive Type II subtype should examine risk factors that are not related to estrogen exposure, in order to identify novel mechanisms of endometrial carcinogenesis.

4.6 TABLES

Table 3 Patient demographic and epidemiologic characteristics by tumor type (N=1,752)

	Type I (N=1,576)	Type II (N=176)	<i>p</i> -value [†]	
	N (%)	N (%)		
Race				
White	1511 (95.9)	157 (89.2)	<0.001	
Non-white	65 (4.1)	19 (10.8)		
Year of diagnosis				
1996	108 (6.9)	15 (8.5)	0.16	
1997	83 (5.3)	18 (10.2)		
1998	133 (8.4)	12 (6.8)		
1999	110 (7.0)	8 (4.5)		
2000	125 (7.9)	11 (6.2)		
2001	104 (6.6)	10 (5.7)		
2002	91 (5.8)	16 (9.1)		
2003	115 (7.3)	9 (5.1)		
2004	122 (7.7)	8 (4.5)		
2005	117 (7.4)	16 (9.1)		
2006	151 (9.6)	14 (7.9)		
2007	162 (10.3)	20 (11.4)		
2008	155 (9.8)	19 (10.8)		
Body Mass Index				
Underweight (<18.5 kg/m ²)	12 (0.8)	7 (4.0)		<0.001
Normal (18.5-24.9 kg/m ²)	238 (15.1)	44 (25.0)		
Overweight (25-<29.9 kg/m ²)	326 (20.7)	48 (27.3)		
Obese (>30 kg/m ²)	871 (55.3)	64 (36.4)		
Unknown	129 (8.2)	13 (7.4)		
Parity (# of live births)				
0	384 (24.4)	31 (17.6)	0.002	
1	230 (14.6)	14 (7.9)		
2	413 (26.2)	45 (25.6)		
3+	511 (32.4)	80 (45.4)		
Unknown	38 (2.4)	6 (3.4)		
History of Additional Primaries				
No	1328 (84.3)	131 (74.4)	0.006	
Yes	224 (14.2)	42 (23.9)		
Breast cancer	100 (6.3)	25 (14.2)		
Ovarian cancer	30 (1.9)	2 (1.1)		

Table 3 continued

Colorectal cancer	20 (1.3)	6 (3.4)	
Other cancers	74 (4.7)	9 (5.1)	
Unknown	24 (1.5)	2 (1.7)	
Menopausal status			
Premenopausal	289 (18.3)	15 (8.5)	0.002
Postmenopausal	1191 (75.6)	151 (85.8)	
Unknown	96 (6.1)	10 (5.7)	
	Median ± IQR	Median ± IQR	
Age at diagnosis	60.0 ± 17.0	68.0 ± 15.5	<0.001
Age at menopause	50.0 ± 5.0	50.0 ± 4.5	0.90
† Likelihood ratio <i>p</i> -value			

Table 4 Multivariable logistic regression model to predict Type II endometrial cancer (N=176) vs. Type I endometrial cancer (N=1,576)

Variable	Unadjusted OR (95% CI)	Adjusted OR (95% CI)[†]	Likelihood ratio <i>p</i>-value[‡]
Race			
Non-White	2.81 (1.65, 4.81)	2.95 (1.66, 5.27)	<0.001
Body Mass Index			
Underweight (<18.5 kg/m ²)	3.16 (1.18, 8.46)	2.59 (0.90, 7.45)	<0.001
Overweight (>25 kg/m ² and <29.9 kg/m ²)	0.80 (0.51, 1.24)	0.82 (0.52, 1.29)	
Obese (>30 kg/m ²)	0.40 (0.26, 0.60)	0.45 (0.29, 0.70)	
Unknown	0.54 (0.28, 1.04)	0.48 (0.23, 0.99)	
History of Additional Primaries			
Yes	1.90 (1.31, 2.77)	1.56 (1.05, 2.32)	0.09
Unknown	1.27 (0.38, 4.26)	1.30 (0.34, 4.94)	
Age at diagnosis[*]	1.04 (1.03, 1.06)	1.03 (1.01, 1.05)	<0.001
† Adjusted for year of diagnosis, race, BMI, history of additional primaries, age at diagnosis, parity, and menopausal status ‡ From the adjusted model * Centered at 62 years Reference groups: white race, normal weight, no history of additional primaries, nulliparous, and premenopausal			

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5.0 ARTICLE 2: PROGNOSTIC FACTORS FOR SURVIVAL IN SUBTYPES OF ENDOMETRIAL CANCER

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5.1 ABSTRACT

Objective: The goal of this study is to identify prognostic factors for overall survival (OS) among low-grade Type I, high-grade Type I, and Type II endometrial cancer (EC) patients.

Methods: We conducted a retrospective cohort study of cases treated at Magee-Womens Hospital between 1996 and 2008. Clinical and follow-up data were available from the University of Pittsburgh Medical Center Network Cancer Registry. Histology-specific Cox regression models were used to compare the relative importance of prognostic factors for OS among low-grade Type I (N=1,309), high-grade Type I (N=252), and Type II cases (N=219). We also examined if OS differed between the subtypes in a combined model.

Results: The prevalence of obesity, advanced stage tumors, and positive lymph node involvement significantly differed between all three subtypes. Increasing age and advanced stage were significant prognostic factors common to all three subtypes. In low-grade Type I EC cases, surgery plus chemotherapy was associated with a significantly increased risk of death (HR: 3.65, 95% CI 1.33, 9.99). In high-grade Type I EC cases, surgery plus radiotherapy was significantly associated with reduced mortality (HR: 0.43, 95% CI 0.23, 0.81) as was surgery plus radiotherapy and chemotherapy (HR: 0.28, 95% CI 0.08, 0.95). In the combined model, OS did not differ significantly between high-grade Type I and Type II cases (HR: 1.12, 95% CI 0.79, 1.57).

Conclusions: Despite clinical and epidemiologic differences, high-grade Type I and Type II patients share similar prognostic factors and survival outcomes. Due to the higher mortality associated with these highly aggressive subtypes, a concerted effort to include greater numbers of patients with these tumors in clinical trials focusing on treatment options is warranted.

Keywords: high-grade Type I; Type II; mortality

5.2 INTRODUCTION

Endometrial cancer (EC) is a heterogeneous disease. The traditional classification system, originally proposed by Bokhman [80] broadly groups EC based on clinicopathologic characteristics. Type I ECs are estrogen-dependent, arise from endometrial hyperplasia, and are of adenocarcinoma histology [61]. Furthermore, Type I endometrial tumors can be further classified as low-grade or high-grade. Type II ECs are estrogen-independent, develop in the setting of an atrophic endometrium, and are either clear cell (CC) or papillary serous (PS) [95]. Furthermore, the survival experience between Type I and Type II ECs differs significantly. Five-year overall survival (OS) for early stage Type I cancers ranges from 80% to 90%, whereas five-year OS for early stage Type II cancers is approximately 60% [15, 150].

Despite the known poor prognosis of patients with Type II cancers, prognostic factors for this type of malignancy are poorly characterized. Survival analyses are hindered by small numbers of patients with Type II EC in single institution studies. Additionally, many studies exclude CC from the Type II classification, which limits the generalizability of results. Recently, Fader et al. [138] investigated clinicopathologic factors for OS in 206 stage I-II PS cases. Age, tumor depth, and treatment with platinum/taxane-based chemotherapy were significantly associated with all-cause mortality. Furthermore, tumors with just 5% PS histology had patterns of recurrence and death similar to patients with pure PS tumors, indicating the aggressiveness of this histology. In a single institution review of 129 PS cases, Slomovitz et al. [151] reported that deep myometrial invasion and nodal involvement were significantly associated with mortality. Additionally, the high proportion of cases with no myometrial invasion that developed abdominal metastases suggests that PS cases are at increased risk of extra-uterine disease even in the absence of myometrial invasion. Sagar et al. [141] analyzed the association between stage,

myometrial invasion, lymphovascular space invasion, adjuvant treatment modality (radiotherapy and/or chemotherapy) and OS in 45 PS patients. Only higher stage was significantly associated with increased mortality in this small case series. The prognostic studies that have been performed for the CC histology subtype were small. In the largest series of CC cases (N=181), disease stage and age were significantly associated with recurrence [152].

Several studies have estimated the survival difference between high-grade Type I and Type II cancers. Some authors have reported significant differences in survival between these high-grade subtypes [10, 148, 149], while others have not [140, 146, 153, 154]. These studies are difficult to compare because the definitions for the subtypes were not uniform and different statistical methods were employed. Additionally, the number of Type II cases in the natural history studies ranged significantly; most included between 30 and 80 cases [140, 146, 149, 153], two studies included 139 and 207 cases [148, 154], and one included 1,864 Type II cases from the Surveillance Epidemiology and End Results database [10].

As few studies have examined the differences in prognostic factors between high-grade Type I and Type II cancers (both CC and PS), the primary goal of this study was to identify prognostic factors for OS among low-grade Type I, high-grade Type I, and Type II ECs using separate Cox proportional hazards models. We also sought to assess whether OS differed between the three subtypes. Understanding differences in the prognostic factors between the distinct histologies may improve the understanding of the underlying biology of EC and fill an important gap in existing research.

5.3 METHODS

5.3.1 Study population

This is a retrospective cohort study of EC cases (N=1,780) diagnosed at Magee-Womens Hospital between 1996 and 2008. Primary treatment included surgical intervention (hysterectomy with or without removal of the fallopian tubes and ovaries) in 92% (N=1,638) of cases. Non-surgical cases (N=142, 8%) differed significantly from surgical cases with respect to stage (chi-square $p < 0.001$); 33% of non-surgical cases had late stage tumors compared to 16% of surgical cases.

Clinical, pathology, and follow-up data were retrieved from the University of Pittsburgh Medical Center (UPMC) Network Cancer Registry using an honest broker. The UPMC Network Cancer Registry gathers demographic, medical history, diagnostic findings, and treatment and outcomes information on cancer patients treated within the network. EC cases with an International Classification of Diseases for Oncology (ICD-O 3rd Edition) primary site code between C54.0–C54.9 and C55.9 were included in this analysis. Information on age, race, weight, height, stage, tumor size, post-operative therapy, tumor histology, grade (where applicable), date of diagnosis, and date of death was collected from electronic medical records. Information on the cause and date of death are supplied to the UPMC Network Cancer Registry with daily notifications from oncologist offices. This study was approved by the University of Pittsburgh Institutional Review Board.

Tumor histology types were classified into one of three, distinct groups: low-grade Type I, high-grade Type I, and Type II. Adenocarcinoma with or without squamous differentiation, mucinous adenocarcinoma, or endometrioid tumors were classified as Type I. Well or

moderately differentiated tumors were grouped as low-grade Type I while poorly differentiated tumors were classified as high-grade Type I. CC and PS tumors were classified as Type II. These classifications were based on the body of literature reporting different etiologies and outcomes between these subtypes.

5.3.2 Statistical analysis

Demographic and clinical characteristics were compared between the three histology subtypes with chi-square tests for categorical variables and one-way ANOVAs for continuous variables. Variables with a significant overall *p*-value were further compared using contrasts to determine which subtypes significantly differed. Non-surgical cases were excluded from survival analyses. Stage-specific Kaplan-Meier curves and log-rank tests were generated to compare OS between the three histology subtypes. Separate Cox proportional hazards regression models were used to model survival for each of the three histology subtypes. Age, race, body mass index (BMI), stage, tumor size, and post-operative therapy were included in each of these histology-specific models. BMI categories were based on the Centers for Disease Control and Prevention definitions: normal weight ($< 24.9 \text{ kg/m}^2$), overweight ($25\text{-}29.9 \text{ kg/m}^2$), and obese ($> 30 \text{ kg/m}^2$) [145]. Year of diagnosis was entered into all models as a categorical adjustment factor. OS was defined as the number of days between the date of diagnosis and the date of death from all causes. Patients lost to follow-up were censored on the last date of contact and those patients alive at the end of follow-up (December 31, 2008) were censored at that time.

A combined Cox model including all cases was run in order to ascertain if OS was significantly different between low-grade Type I, high-grade Type I, and Type II cases adjusted for prognostic factors. In this model, we assessed the assumption of proportional hazards for the

histology types with Grambsch and Therneau's method [155]. Interactions between histology subtypes and all covariates were tested using a two-sided alpha of 0.05; given that the interactions were non-significant the hazard ratios are based on the main-effects only model.

5.4 RESULTS

Of the identified 1,964 EC cases diagnosed at Magee-Womens Hospital between 1996 and 2008, 1,864 had a diagnosis code pertaining to the three histologic cell types under investigation. Mixed mullerian tumors (N=82) and endometrial sarcomas (N=18) were excluded. We also excluded cases who were younger than 34 years of age or older than 92 years of age at the time of diagnosis (N=14). This age range reflects the overlap of ages for the Type I and Type II subtypes in our cohort; excluding cases outside of this age range was performed in an effort to reduce confounding by age. Cases with no information on stage (N=43), grade (N=26), or follow-up time (N=1) also were excluded, resulting in a final sample size of 1,780 cases.

Distributions of demographic and clinical characteristics are shown in Table 5 by histologic category. Patients with low-grade Type I tumors were significantly younger than high-grade Type I and Type II EC cases (61 years vs. 65 and 67, respectively; $p < 0.001$). Non-white race was significantly more common among Type II EC cases (10%) compared to low-grade Type I (4%) and high-grade Type I EC cases (6%). BMI varied significantly across subtypes, with relatively more low-grade Type I EC cases being obese (58%) compared to the high-grade Type I (48%) and Type II EC cases (40%). Stage at diagnosis significantly differed between low-grade Type I, high-grade Type I, and Type II EC cases; 91% vs. 65% vs. 53%, respectively, had early stage tumors. Positive lymph node involvement was significantly more common among Type II EC

cases (20%) compared to high-grade Type I EC cases (13%) which was significantly greater than positive lymph node involvement in low-grade Type I EC cases (5%). Tumor size significantly differed by subtype, although data were missing for approximately 60% of cases. Type II EC cases had a significantly larger proportion of large tumors (> 2 cm) compared to low-grade Type I EC cases (39% vs. 29%). Type of post-operative therapy differed significantly between the three subtypes, with approximately 50% of low-grade Type I EC cases treated with surgery only, compared to 18% of the high-grade Type I and 16% of the Type II EC cases. One-third of the Type II EC cases were treated with surgery plus chemotherapy compared to 3% of the low-grade Type I and 12% of the high-grade Type I EC cases. Surgery plus radiotherapy was most common among the high-grade Type I EC cases (48%) compared to 38% of low-grade Type I and 24% of Type II EC cases. Surgery, radiotherapy, and chemotherapy use was most common among Type II EC cases (17%) compared to high-grade Type I (7%) and low-grade Type I EC cases (3%). Finally, median follow-up time was significantly longer for the low-grade Type I EC cases vs. the high-grade Type I and Type II EC cases (47 months vs. 28 and 26 months, respectively).

Table 6 shows the distribution of post-operative treatments according to stage and histology type. In low-grade Type I cases, surgery only was more prevalent among early stage cases (53%) compared to late stage cases (11%). Approximately 78% of late stage low-grade Type I cases had chemotherapy and/or radiotherapy. Among high-grade Type I cases, 64% of early stage cases had radiotherapy compared to 20% of late stage cases. Chemotherapy with or without radiotherapy was more prevalent among late stage high-grade Type I cases (47%) compared to early stage high-grade Type I cases (4%). In early stage Type II cases, 25% of patients were

treated with surgery plus chemotherapy compared to 43% of late stage cases. Surgery plus radiotherapy was used in 34% of early stage cases compared to 13% of late stage cases.

Cases that did not have surgery were excluded from survival analyses which reduced the sample size to 1,638 cases. Stage specific Kaplan-Meier OS curves for the three histology subtypes are shown in Figures 4 and 5. In early and late stage cases, low-grade Type I cases had significantly better OS compared to high-grade Type I and Type II cases ($p < 0.001$), while OS was similar for the high-grade Type I and Type II cases (early stage: $p = 0.62$, late stage: $p = 0.28$).

OS was modeled separately for cases of each histologic subtype to assess the relative importance of each prognostic factor (Table 7). For low-grade Type I cases, increasing age (HR: 1.09, 95% CI: 1.07, 1.10) advanced stage (HR: 2.86, 95% CI: 1.59, 5.16), and surgery plus chemotherapy treatment (HR: 3.65, 95% CI 1.33, 9.99) were significantly associated with higher mortality. Similarly, among high-grade Type I cases increasing age (HR: 1.04 95% CI: 1.02, 1.06) and advanced stage (HR: 2.98 95% CI: 1.56, 5.68) were significantly associated with worse OS. Post-operative treatment was significantly associated with better OS in high-grade Type I cases; surgery plus radiotherapy was associated with an HR of 0.43 (95% CI 0.23, 0.81) and surgery plus radiotherapy and chemotherapy was associated with an HR of 0.28 (95% CI 0.08, 0.95) compared to surgery only. Among Type II cases increasing age (HR: 1.03 95% CI: 1.00, 1.05) and late stage (HR: 4.54 95% CI: 2.48, 8.32) were significantly associated with OS.

Finally, in an adjusted Cox model including all EC cases (Table 8), low-grade Type I cases had significantly reduced hazards of all-cause mortality relative to the high-grade Type I (HR: 0.48, 95% CI 0.35, 0.65) and Type II cases (HR: 0.53, 95% CI: 0.38, 0.74) adjusted for age, race, BMI, stage, tumor size, post-operative therapy, and year of diagnosis. High-grade Type I cases

did not have significantly different OS compared to Type II cases (HR: 1.12, 95% CI: 0.79, 1.57).

5.5 DISCUSSION

This study examined prognostic factors associated with OS in low-grade Type I, high-grade Type I, and Type II ECs. Prognostic indicators for all three subtypes were generally similar; OS was negatively associated with increasing age and advanced stage, which is consistent with the findings from other EC case series [141, 146, 149]. Post-operative treatment had different effects for low-grade Type I vs. high-grade Type I cases; surgery plus chemotherapy was associated with a significantly higher risk of death in low-grade Type I EC cases while surgery plus radiotherapy with or without chemotherapy was associated with better survival.

The second goal of this study was to analyze survival differences among the high-risk subtypes, high-grade Type I and Type II ECs. Kaplan-Meier graphs did not show a significant difference in survival between high-grade Type I and Type II cases in early or late stage cases. We ran a Cox model including all cases to directly test whether the Type II subtype is associated with a higher risk of all-cause mortality than the high-grade Type I subtype. Adjusted for age, race, BMI, stage, post-operative treatment, tumor size, and year of diagnosis, Type II cases did not have a significantly higher risk of death compared to high-grade Type I cases.

Previous studies have examined the relative survival of Type II cancers compared to high-grade Type I. Soslow et al. [146] reported no difference in the hazard of dying comparing high-grade Type I, PS, and CC cases despite significant differences in age, race, stage, and sites of metastasis among the three subtypes. Likewise, Alektiar et al. [153] compared the outcome of 42

Type II cases (PS and CC combined) to 41 high-grade Type I cases and reported no significant difference in 5-year survival rates adjusting for age, race, myometrial invasion, and cervical involvement. Finally, Cirisano et al. [149] reported that high-grade Type I cases had a similar risk of death compared to PS and CC cases combined.

Others have reported significant survival differences between the subtypes. Hamilton et al. [10] compared high-grade Type I tumors vs. PS vs. CC and reported a statistically significant difference in OS adjusted for stage, age, race, and adjuvant radiotherapy. Similarly, Boruta and colleagues [148] reported OS to be significantly better in high-grade Type I cases compared to UPSC cases with > 50% serous histology in the tumor, adjusted for age, race, lymph vascular space invasion, myometrial invasion, and treatment.

The demographic and clinical characteristics of the Magee-Womens Hospital cohort are similar to previously published reports [10, 139, 146]. Low-grade Type I cases were younger, more likely to be white, and have early stage tumors compared to the high-grade Type I and Type II groups. Race was not a significant predictor of mortality in the Type II subgroup. Several studies have reported that when diagnosed with EC, African-American women are significantly more likely to die compared to white women [156-160]. This difference has largely been attributed to the more frequent occurrence of Type II cancers in African-American women [156, 157]. Due to small numbers of African-Americans and ‘other’ races in the UPMC Network Cancer Registry, all non-white races were grouped together. Therefore, this hypothesis cannot be tested in this dataset. Post-operative treatment also differed significantly between the three subtypes. Low-grade Type I tumors were less likely to be treated with radiotherapy and/or chemotherapy compared to high-grade Type I and Type II tumors. Disease stage may confound

this apparent relationship, as high-grade tumors were more likely to be diagnosed in late stages compared to the low-grade Type I tumors.

The major strengths of this study include reliable data from a reputable cancer registry, a mean follow-up of 4.3 years, and a relatively large sample size with numerous events. Other case-series that have explored prognosis in high-grade subtypes have generally suffered from small numbers of events, which limits the statistical modeling. Limitations of this study include the retrospective study design, lack of specific information on the post-operative therapy, and potential misclassification of histologic subtype. Detailed information regarding chemotherapy regimens (single agent vs. multimodal agents and type of agents used) would have allowed for interesting sub-analyses, especially in the Type II group where chemotherapy use was prevalent. Additionally, misclassification bias of the histology type may have occurred as the criteria for classification of Type II tumors has varied over time. The Gynecologic Oncology Group asserts that in the case of mixed histology subtypes, UPSC or CCC must comprise 50% or more of the tumor in order to be classified as such, which is the standard at Magee-Womens Hospital [138].

In conclusion, high-grade Type I and Type II ECs differ from each other clinically, although survival following diagnosis does not significantly differ. The International Federation of Gynecology and Obstetrics has clearly defined disease stage, age, depth of myometrial invasion, and grade to be significantly associated with OS in endometrioid-type EC [161]. Whether these factors are prognostically relevant for high risk subtypes (high-grade Type I and Type II tumors) is less clear, as these tumor types make up a small proportion of all diagnosed EC cases. Due to the high mortality associated with these highly aggressive subtypes, an understanding of the key determinants will improve the current state of knowledge.

5.6 TABLES

Table 5 Demographic and clinical characteristics by histology type (N=1,780)

	Low-grade Type I (N=1,309)	High-grade Type I (N=252)	Type II (N=219)	p-value ¶
Age, mean (SD)	61 (12) [†]	65 (12)	67 (11)	<0.001
Race				
White	1,261 (96%) [‡]	237(94%)	197 (90%)	<0.001
Non-white	48 (4%)	15 (6%)	22 (10%)	
Body Mass Index				
Normal (<24.9 kg/m ²)	201 (15%)*	38 (15%)	57 (26%)	<0.001
Overweight (25-29.9 kg/m ²)	250 (19%)	72 (29%)	58 (26%)	
Obese (>30 kg/m ²)	757 (58%)	120 (48%)	88 (40%)	
Unknown	101 (8%)	22 (9%)	16 (8%)	
Stage				
Early (I & II)	1191 (91%)*	165 (65%)	116 (53%)	<0.001
Late (III & IV)	118 (9%)	87 (35%)	103 (47%)	
Lymph node involvement				
No nodal exam	474 (36%)*	88 (35%)	60 (27%)	<0.001
Negative	716 (55%)	123 (49%)	107 (49%)	
Positive	61 (5%)	32 (13%)	44 (20%)	
Unknown	58 (4%)	9 (4%)	8 (4%)	
Tumor size				
< 2 cm	153 (12%) [‡]	20 (8%)	21 (10%)	0.01
> 2 cm	379 (29%)	88 (35%)	86 (39%)	
Unknown	777 (59%)	144 (57%)	112 (51%)	
Post-operative therapy				
No surgery	86 (7%) [§]	37 (15%)	19 (9%)	<0.001
Surgery only	642 (49%)	45 (18%)	36 (16%)	
Surgery + CT	34 (3%)	31 (12%)	73 (33%)	
Surgery + RT	504 (38%)	122 (48%)	53 (24%)	
Surgery + CT + RT	43 (3%)	17 (7%)	38 (17%)	
Follow-up (months), median (IQR)	47 (19-82) [†]	28 (15-55)	26 (12-51)	<0.001

[†] Low-grade Type I is significantly different from high-grade Type I and Type II ($p<0.05$)
[‡] Low-grade Type I is significantly different from Type II ($p<0.05$)
* All three histologies are significantly different from one another ($p<0.05$)
[§] Type II is significantly different from low-grade Type I and high-grade Type I ($p<0.05$)
¶ Likelihood ratio p -value for categorical variables; ANOVA p -value for continuous variables
SD = standard deviation, CT = chemotherapy, RT = radiotherapy, IQR = Interquartile range

Table 6 Post-operative therapy stratified by disease stage and histology type

N=1,780	Early stage (N=1,191)	Late stage (N=118)	<i>p</i>-value[†]
Low-grade Type I (N=1,309)			<0.001
No surgery	73 (6%)	13 (11%)	
Surgery only	629 (53%)	13 (11%)	
Surgery + CT	15 (1%)	19 (16%)	
Surgery + RT	468 (39%)	36 (31%)	
Surgery + CT + RT	6 (1%)	37 (31%)	
High-grade Type I (N=252)			<0.001
No surgery	17 (10%)	20 (23%)	
Surgery only	36 (22%)	9 (10%)	
Surgery + CT	4 (2%)	27 (31%)	
Surgery + RT	105 (64%)	17 (20%)	
Surgery + CT + RT	3 (2%)	14 (16%)	
Type II (N=219)			<0.001
No surgery	5 (4%)	14 (14%)	
Surgery only	23 (20%)	13 (13%)	
Surgery + CT	29 (25%)	44 (43%)	
Surgery + RT	40 (34%)	13 (13%)	
Surgery + CT + RT	19 (16%)	19 (18%)	
† Likelihood ratio <i>p</i> -value			

Table 7 Subtype-specific analyses of overall survival for low-grade Type I, high-grade Type I, and Type II ECs[†]

Variable	Low-grade Type I N=1223, deaths=168		High-grade Type I N=215, deaths=75		Type II N=200, deaths=76	
	HR (95% CI)	<i>p</i> -value [‡]	HR (95% CI)	<i>p</i> -value [‡]	HR (95% CI)	<i>p</i> -value [‡]
Age (years)	1.09 (1.07, 1.10)	<0.001	1.04 (1.02, 1.06)	<0.001	1.03 (1.00, 1.05)	0.03
Race						
White	Reference	0.22	Reference	0.13	Reference	0.99
Non-white	1.62 (0.75, 3.50)		2.13 (0.81, 5.62)		1.00 (0.38, 2.68)	
Body Mass Index						
Normal weight	Reference	0.20* (3)	Reference	0.31* (3)	Reference	0.16* (3)
Overweight	0.69 (0.42, 1.15)	0.15	1.00 (0.47, 2.15)	0.99	0.45 (0.22, 0.91)	0.03
Obese	1.05 (0.70, 1.58)	0.80	1.64 (0.82, 3.30)	0.16	0.68 (0.36, 1.29)	0.24
Unknown	1.26 (0.67, 2.35)	0.47	1.14 (0.28, 4.62)	0.86	0.84 (0.25, 2.85)	0.78
Stage						
Early (I & II)	Reference	<0.001	Reference	<0.001	Reference	<0.001
Late (III & IV)	2.86 (1.59, 5.16)		2.98 (1.56, 5.68)		4.54 (2.48, 8.32)	
Tumor size						
< 2 cm	Reference	0.44* (2)	Reference	0.09* (2)	Reference	0.46* (2)
> 2 cm	1.25 (0.63, 2.47)	0.52	3.72 (0.98, 14.10)	0.05	2.17 (0.60, 7.82)	0.24
Unknown	0.92 (0.49, 1.74)	0.80	2.23 (0.61, 8.19)	0.23	2.21 (0.61, 7.99)	0.23
Post-operative therapy						
Surgery only	Reference	0.03* (3)	Reference	0.03* (3)	Reference	0.15* (3)
Surgery + CT	3.65 (1.33, 9.99)	0.01	0.66 (0.29, 1.51)	0.33	0.62 (0.31, 1.22)	0.17
Surgery + RT	0.92 (0.66, 1.30)	0.65	0.43 (0.23, 0.81)	0.008	0.56 (0.25, 1.26)	0.16
Surgery + CT + RT	0.72 (0.27, 1.93)	0.51	0.28 (0.08, 0.95)	0.04	0.27 (0.08, 0.88)	0.03
[†] Adjusted for year of diagnosis [‡] Wald <i>p</i> -value * Wald <i>p</i> -value for multi-parameter tests, degrees of freedom are noted in parentheses						

Table 8 Combined overall survival model including all EC cases (N=1,638)[†]

Variable	HR (95% CI)	<i>p</i>-value[‡]
Low-grade Type I vs. High-grade Type I	0.48 (0.35, 0.65)	<0.001
Low-grade Type I vs. Type II	0.53 (0.38, 0.74)	<0.001
High-grade Type I vs. Type II	1.12 (0.79, 1.57)	0.53
Age (years)	1.06 (1.05, 1.07)	<0.001
Race		
White	Reference	0.37
Non-white	1.25 (0.76, 2.05)	
Body Mass Index		
Normal weight (18.5-24.9 kg/m ²)	Reference	0.17 (3)
Overweight (25-29.9 kg/m ²)	0.75 (0.53, 1.05)	0.09
Obese (>30 kg/m ²)	1.03 (0.78, 1.37)	0.83
Unknown	1.04 (0.64, 1.71)	0.86
Stage		
Early (I & II)	Reference	<0.001
Late (III & IV)	3.44 (2.53, 4.68)	
Tumor size		
< 2 cm	Reference	0.18 (2)
> 2 cm	1.52 (0.90, 2.56)	0.12
Unknown	1.20 (0.72, 2.00)	0.49
Post-operative therapy		
Surgery only	Reference	0.02 (3)
Surgery + CT	1.11 (0.73, 1.70)	0.61
Surgery + RT	0.79 (0.60, 1.03)	0.08
Surgery + CT + RT	0.52 (0.29, 0.94)	0.03
[†] Adjusted for year of diagnosis [‡] Individual Wald <i>p</i> -value * Wald <i>p</i> -value for multi-parameter tests, degrees of freedom is noted in parentheses		

5.7 FIGURES

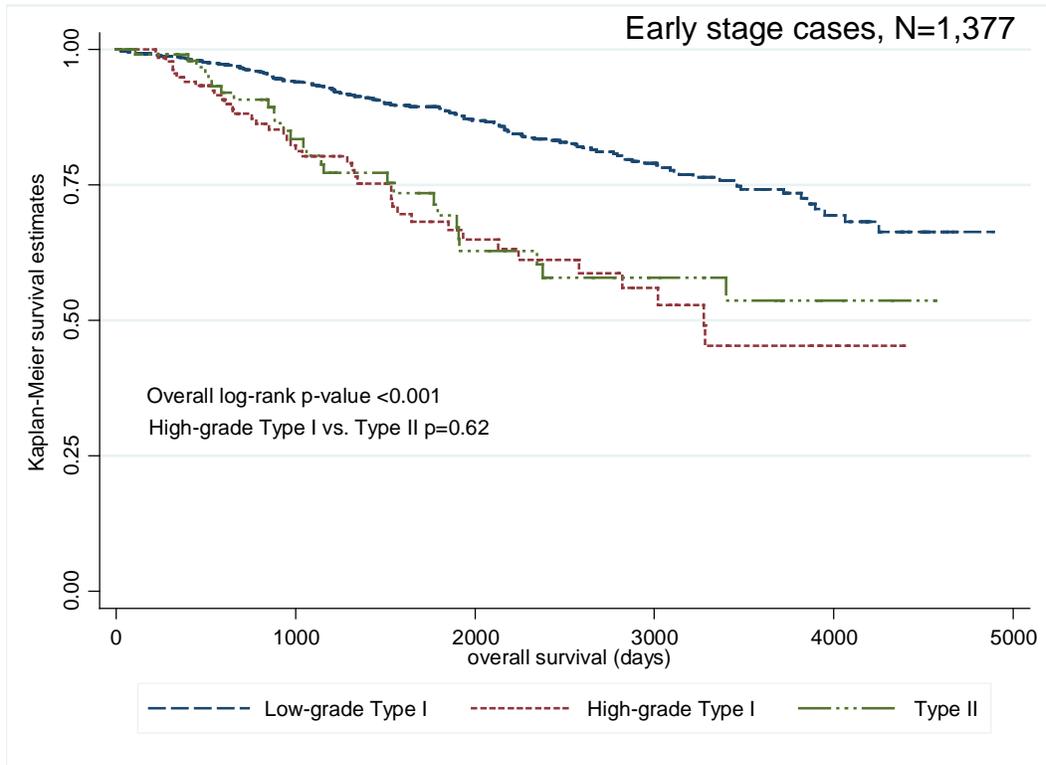


Figure 4 Kaplan-Meier overall survival curves for low-grade Type I, high-grade Type I, and Type II cases: early stage (I&II)

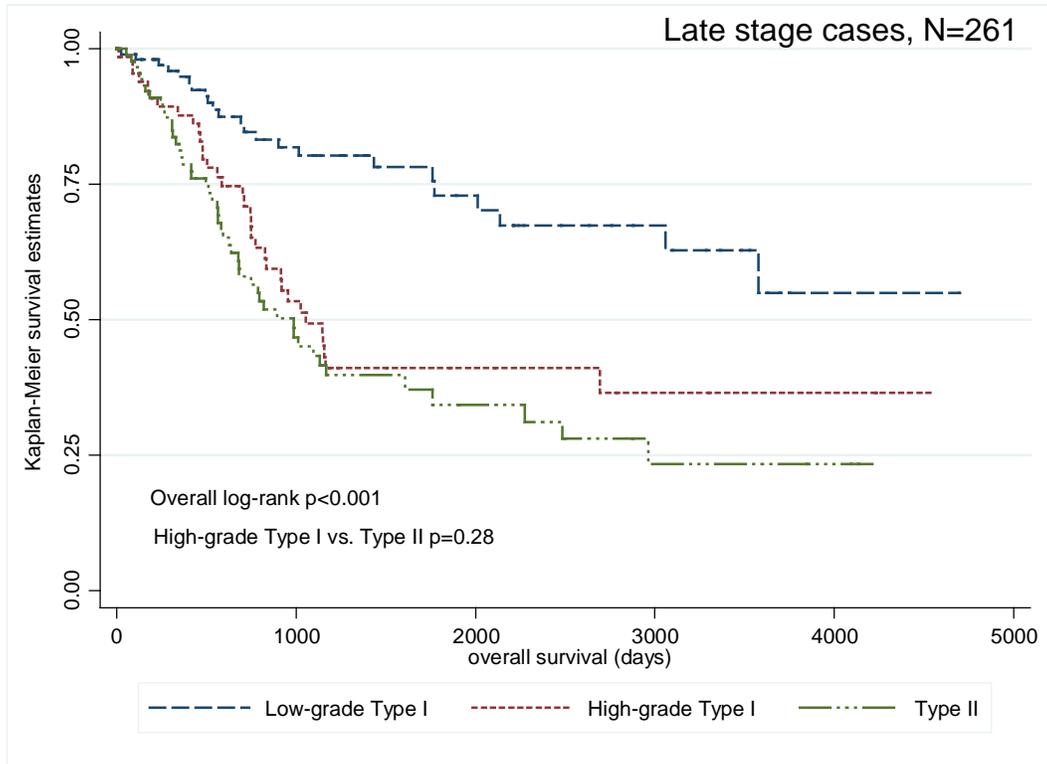


Figure 5 Kaplan-Meier overall survival curves for low-grade Type I, high-grade Type I, and Type II cases: late stage (III&IV)

5.8 ACKNOWLEDGMENTS

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**6.0 ARTICLE 3: PROGNOSTIC VALUE OF CXCL12 IN ENDOMETRIAL CANCER
PATIENTS**

To be submitted for publication

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6.1 ABSTRACT

Introduction: CXCL12 is a chemotactic cytokine that plays an important role in the invasion and metastasis in several malignancies by interacting with its receptor CXCR4. Few studies examine the prognostic role of this pathway in endometrial cancer (EC), specifically in high-grade Type I, clear cell (CC), and papillary serous (PS) ECs, all of which show a tendency for regional and distant spread. The goal of this study is to evaluate the association between CXCR4 and CXCL12 expression, prognostic factors, overall survival (OS), and recurrence-free survival (RFS) in a cohort of low-grade Type I, high-grade Type I, CC, and PS EC cases.

Methods: Demographic, clinical, treatment, and survival information on each EC case was available from the University of Pittsburgh Medical Center Network Cancer Registry. Tumor blocks were retrieved from the Pathology Department at Magee-Womens Hospital and sectioned into slides (N=199). CXCR4 and CXCL12 protein expression was measured using immunohistochemistry (IHC). The association between expression and clinicopathologic factors was assessed with chi-square tests. As CXCR4 expression did not vary substantially in the sample, we examined associations between CXCL12 and OS and RFS with Kaplan-Meier graphs, log rank tests, and Cox proportional hazards models.

Results: CXCR4 was expressed in all ECs; 30% (n=59) and 70% (n=140) of cases showed moderately and strongly positive CXCR4 expression, respectively. CXCL12 expression was absent in 63 (32%), weakly positive in 24 (12%), moderately positive in 84 (42%), and strongly positive in 28 (14%) cases. Neither CXCL12 nor CXCR4 expression was significantly associated with race, body mass index, histology subtype, stage, lymph node involvement or tumor size. In multivariable analysis, we observed a significant association between CXCL12 expression, OS, and RFS among high-grade Type I cases; positive CXCL12 expression was associated with

significantly improved OS (HR: 0.17, 95% CI 0.05, 0.59) and RFS (HR: 0.10, 95% CI 0.02, 0.57).

Discussion: This is the first study to examine the role of CXCL12 in a histologically diverse cohort of EC cases. We demonstrated that CXCL12 was an independent prognostic marker associated with better OS and RFS in an aggressive subtype of EC. In addition to pathological characteristics of the primary tumor, IHC expression of CXCL12 may be clinically useful for assigning adjuvant treatment to EC cases.

6.2 INTRODUCTION

Endometrial cancer (EC) is a heterogeneous cancer with two broad subtypes commonly described in the literature, Type I and Type II. Type I EC represents 80%-90% of all sporadic tumors and can be further subdivided by differentiation or grade of the tumor [15, 150]. Low-grade Type I tumors (grades 1 and 2) are indolent and five-year survival is approximately 90% [162]. Conversely, high-grade Type I tumors have five-year survival rates ranging between 45% and 77% [10, 140, 146, 153]. Advanced disease stage is present in 50% of cases with high-grade Type I tumors [146]. The remaining 10%-20% of ECs, Type II, are characterized by invasive disease spread at the time of diagnosis, with survival rates ranging between 50 and 60% [15]. Clear cell (CC) and papillary serous (PS) are the two histology subtypes that comprise the Type II category. Most studies examining molecular alterations involved in EC tumorigenesis, invasion, and progression focus on the more prevalent Type I cases. Despite being relatively rare, high-grade Type I and Type II ECs are important to study due to the disproportionate mortality associated with these subtypes.

As high-grade Type I, CC, and PS endometrial tumors are characterized by extensive extra-uterine disease at the time of diagnosis, molecular mechanisms related to disease spread are strongly implicated in these subtypes. Previous research suggests that soluble factors secreted by cells of the tumor microenvironment contribute to dissemination of cancer cells from the primary tumor site to local and distant sites [98, 163-167]. Chemokines are a family of chemotactic cytokines that direct the movement of target cells that express the appropriate chemokine receptor. This movement occurs along a chemical gradient of the chemokine (i.e. the chemokine gradient) allowing cells to move towards high local concentrations of chemokines [100]. When chemokine ligands bind to cancer cells that aberrantly express chemokine receptors, signal

transduction pathways are activated which can result in several consequences, namely invasion and metastasis [168].

An important and well-studied chemokine ligand-receptor pair, CXCL12 and CXCR4, is associated with proliferation, adhesion, and most notably chemotaxis in several malignancies, including breast [169], colon [170], ovarian [171], and pancreatic cancers [172]. Within the breast cancer literature, the roles of CXCL12 and CXCR4 on metastasis and ultimately survival have been examined. Muller et al. [169] demonstrated that common sites of metastasis in primary breast cancer patients (bone, lung, and brain) overexpress CXCL12 while the primary breast tumor is characterized by high levels of CXCR4. For breast cancer survival, the cumulative evidence points to an improved survival associated with high expression of CXCL12 at the primary site [173-175]. Mechanistically, high levels of CXCL12 at the primary tumor site may saturate the receptor and inhibit progression of the tumor beyond the primary site.

The biology of CXCR4 and CXCL12 in normal endometrial tissues and EC has been described. In the normal endometrium, CXCR4 gene expression is significantly higher in epithelial cells compared to stromal cells while CXCL12 is highly expressed in stromal cells, yet undetectable in normal epithelial cells [176]. Compared to normal endometrial tissues, CXCR4 expression is significantly higher in EC cell lines and human EC tissues while CXCL12 mRNA is significantly higher in normal endometrial tissues compared to EC [105]. Mizokami [106] and Zhao [109] provided evidence that CXCL12 stimulates growth of EC cells *in vitro*, while Tsukamoto [108] demonstrated the migratory ability of CXCR4-expressing EC cells into the myometrial layer of the uterus which is a CXCL12-rich environment. Finally, in a murine model where primary EC and metastases were induced, metastatic cancer in the liver and lung were completely inhibited in mice treated with anti-CXCR4 antibodies compared to isotype control

treated mice. Despite the biological evidence supporting a role for CXCR4 and CXCL12 in EC, only one study has examined the prognostic value of CXCR4 and overall survival (OS) in EC. In Kaplan-Meier analyses, CXCR4-positive tumors had a significantly improved OS compared to those with CXCR4-negative tumors [107].

The previous research strongly suggests a potential prognostic role for CXCL12 and CXCR4 in EC. As epidemiologic studies of this association are lacking, we investigated expression of CXCL12 and CXCR4 and its relationship with clinicopathologic factors and survival. Our primary hypothesis was that higher expression of CXCL12 would be associated with better survival due to saturation of CXCR4 at the primary site, thus inhibiting migration beyond the uterus.

6.3 METHODS

6.3.1 Study sample

The study sample was identified through an honest broker system at the University of Pittsburgh Medical Center Cancer Registry. The original database included 1,964 EC cases that were either diagnosed and/or received all or part of the first course of treatment at Magee-Womens Hospital between 1996 and 2008. Demographic, clinical, treatment, and survival information was retrieved for each patient in the original cohort by an honest broker. In this study, Type I (endometrioid or adenocarcinoma) and Type II (CC or PS) EC patients comprised the target population. Additional exclusion criteria were applied to generate the sampling frame of 1,486 cases from which the study sample was drawn. Information on the availability of tumor blocks

was obtained from the Pathology Department at Magee-Womens Hospital; of the 1,486 cases, 1,003 (67%) were indicated as having tumor blocks available. Appendix figures 33 and 34 show the generation of the sampling frame (N=1,486) and the reduced sampling frame based on presumed tumor block availability (N=1,003), respectively. Characteristics of the sampling frame are available in Appendix Table 28.

Case selection from the reduced sampling frame was achieved with a stratified random sampling design. Histology type (Type I and Type II) and stage (stage I, stage II, stage III, and stage IV) were the two variables used to construct the sampling strata. Within each of the eight strata, a simple random sample was drawn using the ‘*surveyselect*’ procedure in SAS. A flow chart for case selection for this study is shown in Figure 6. Two hundred cases (25 from each stratum) were originally requested; however 216 cases were provided by the Pathology Department at Magee-Womens Hospital. Within strata where the number of available cases was less than 25, we made an effort to include all cases; however inadequate tissue limited our ability to achieve this goal. Furthermore, following slide sectioning of the selected blocks, 17 slides did not have evaluable tumor present, which reduced the analytic cohort to 199 EC cases. This study was approved by the University of Pittsburgh Institutional Review Board.

6.3.2 Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks for the 216 selected cases were retrieved from the Pathology Department at Magee-Womens Hospital. Four μm thick slides were cut at the time of block retrieval and stored in a refrigerator at 4°C in order to prevent loss of antigenicity as we anticipated a time delay between cutting and staining of slides. The immunohistochemistry (IHC) protocol began with deparaffinization and hydration of the slides with xylenes and washes

with progressively decreasing alcohol concentrations. Endogenous peroxidase activity was blocked with 3% methanol peroxide. Next, antigen retrieval in 0.01 M boiling citrate buffer (pH 6.0) in a microwave was performed followed by blocking of non-specific staining with Protein Block (Dako North America, Inc, Carpinteria, California). Whole tissue sections were then incubated for one hour at room temperature with 100 microliters of polyclonal CXCR4 antibody (Abcam Inc., Cambridge, MA, dilution 1:50) and polyclonal CXCL12 antibody (R&D Systems, Minneapolis, MN, dilution 1:200). Slides were subsequently rinsed in PBS/Tween solution for five minutes followed by incubation with a polymer (ImmPRESS Universal reagent, Vector Laboratories). Slides were again rinsed in the PBS/Tween solution followed by development with diaminobenzidine for detection. Counterstaining with Shandon Hematoxylin (Thermo Scientific), rinsing in several concentrations of alcohol solution, mounting, and viewing of the slides was performed. Positive and negative controls were run with each batch of slides which consisted of approximately 40 slides per batch. Normal heart and normal tonsil tissue were the positive controls for CXCR4 and CXCL12, respectively. The primary antibody was omitted from the negative control slides. We observed nuclear and cytoplasmic staining for CXCR4 and cytoplasmic and membranous staining for CXCL12.

6.3.3 Evaluation of staining

Expression of CXCL12 and CXCR4 was scored semi-quantitatively by incorporating the intensity of staining (IS) and the proportion of positive-staining cells (PS). The carcinoma and stromal compartments were scored separately for each case. The PS was interpreted as 1=1-5%, 2=6-20%, 3=21-80%, and 4=>80%, while the IS was interpreted as (1=weak, 2=moderate, 3=strong). Slides with no positive-staining cells were coded as 0. A cumulative score was

calculated by summing the PS and IS. Acceptable values of the cumulative score are 0, 2, 3, 4, 5, 6, and 7. Interpretation of the cumulative score for a given marker is as follows: 0 (negative), 2 (weakly positive) 3-5 (moderately positive), and 6-7 (strongly positive) [177]. Stromal expression of CXCL12 was absent in all cases while CXCR4 stromal expression was absent in 98% of cases. Additionally, CXCR4 was expressed in all EC cases.

6.3.4 Statistical analyses

6.3.4.1 Sampling weights

Sampling fractions (f_i), the probability of case selection, were calculated as follows: stratum-specific fraction of available tumor blocks with non-missing IHC data (A_i) * estimated case-specific probability of tumor block availability (B_i). Calculation of A_i for the OS and RFS models are shown in Tables 9 and 15, respectively. The predicted probability that tumor blocks were available for a given case (B_i) was computed in the total target population ($N=1,486$) using a logistic regression model (Appendix Table 31). A_i and B_i were multiplied to calculate the probability that a case was sampled, i.e. the sampling fraction, f_i . The sampling weight was derived by taking the inverse of the sampling fraction. All analyses applied sampling weights that allowed for proportions and other effect sizes to be applicable to the target population. In STATA 11, the *svy* family of commands was used to implement features of the study design into the analysis (StataCorp LP, College Station TX).

6.3.4.2 Independent variables

We hypothesized that differences in negative expression vs. positive expression at the primary tumor site would be clinically relevant. Therefore, we collapsed the categories of CXCL12

expression to represent positive expression (weak + moderate + strong cumulative scores) vs. negative expression. Other clinicopathologic variables of interest were age, race (white vs. non-white), body mass index (BMI: normal, overweight, obese), additional cancer primaries (no, yes, unknown), histology subtype (low-grade Type I, high-grade Type I, CC, PS), stage (I & II vs. III & IV), lymph node involvement (no nodal involvement, positive nodal involvement, unknown nodal involvement, no nodal examination), tumor size (< 2 cm in the largest dimension, > 2 cm in the largest dimension, unknown size), post-operative treatment (surgery only, surgery + chemotherapy (CT), surgery + radiotherapy (RT), surgery + CT + RT), and year of diagnosis.

6.3.4.3 Descriptive statistics

All subsequent statistical analyses were performed using the *svy* family of commands in STATA 11 (StataCorp LP, College Station TX). Survey commands take into account the stratified random sampling design and variance of estimators is obtained using the Taylor-linearized variance estimator method. For contingency tables the difference between observed and expected weighted cell frequencies were compared using an adjusted Wald F statistic which approximately follows a chi-square distribution.

The distribution of clinicopathologic characteristics by histology subtype (low-grade Type I, high-grade Type I, CC, and PS) was examined with chi-square tests (*svy: tabulate*) for categorical variables. For continuous variables (i.e. age) the population mean was calculated by histology subtype (*svy: mean*) and a test command was used to test the null hypothesis that the means were equal across histology subtypes. The association between CXCL12 and CXCR4 expression and clinicopathologic variables was similarly examined. All tables provide counts for the sample, the sample proportion, and the weighted proportion. When describing proportions in

the text, the weighted proportion (i.e. the proportion attributable to the target population) is referenced.

6.3.4.4 Survival analysis

OS and recurrence-free survival (RFS) were measured as the number of days between the date of diagnosis and the date of death from all causes or date of recurrence, respectively. Patients that did not experience either outcome were censored at the last date of contact or the date of last follow-up (December 31, 2008). For RFS analyses we only included cases that were known to become disease-free following the primary surgery (see Appendix Table 34 for an enumeration of disease recurrence in this population).

Kaplan-Meier estimates of OS and RFS and log-rank tests were used to compare survival distributions by CXCL12 and CXCR4 expression status. Due to the lack of meaningful variation in CXCR4, only CXCL12 was examined in the multivariable survival analysis. The effect of CXCL12 on survival outcomes adjusted for histology subtype, stage, and age was investigated. A multiplicative interaction between CXCL12 and histology subtype was examined to assess effect modification and a $p < 0.05$ was considered statistically significant to enter the final model. When the interaction was significant, hazard ratios comparing the presence vs. absence of CXCL12 for each histology subtype were estimated by exponentiating the following expression: $\beta_{\text{CXCL12}} + \beta_{\text{CXCL12} * \text{histology subtype}}$. Stratification by year of diagnosis was performed in order to control for year of diagnosis without estimating coefficients for these variables.

6.4 RESULTS

6.4.1 Characteristics of sample

Table 10 shows demographic and clinical characteristics of the study sample and target population by histology subtype. Mean age differed significantly among the four histology subtypes. Low-grade Type I and high-grade Type I cases were younger (mean age: 61) compared to CC (mean age: 67) and PS cases (mean age: 66). Race and BMI were borderline significantly different across the subtypes. Non-white race was more common among PS (17%) and CC (11%) cases compared to low-grade Type I (5%) and high-grade Type I cases (4%). The proportion of normal weight cases was lowest among high-grade Type I cases (5%), followed by PS (23%), low-grade Type I (26%), and CC (31%). Additional cancer primaries did not vary significantly by histology subtype ($p=0.58$). Stage was significantly different among the four histology types; PS cases were commonly late stage (51%), followed by high-grade Type I (38%), CC (29%), and low-grade Type I tumors (7%). Positive lymph node involvement was significantly higher in PS and high-grade Type I cases (20%) than in CC (9%) or low-grade Type I cases (2%).

Finally, post-operative treatment significantly varied by histology subtype ($p<0.001$). Low-grade Type I cases were most likely to have surgery only (68%) and surgery plus RT (29%). Among high-grade Type I cases, surgery only was most common (41%), followed by surgery plus RT (36%), and surgery plus RT and CT (24%). In CC cases, surgery plus RT was employed in almost half of the cases (47%), while PS cases had the highest proportion of a surgery plus CT regimen (41%).

6.4.2 CXCL12 and CXCR4 expression in EC patients

CXCR4 was expressed in all ECs in the study sample. Fifty-nine of 199 (30%) cases showed moderately positive CXCR4 expression while 140 (70%) cases were classified as strongly positive CXCR4 expressers (Figures 7-8). CXCL12 expression was negative in 63 cases (32%), weakly positive in 24 cases (12%), moderately positive in 84 cases (42%), and strongly positive in 28 cases (14%) (Figures 9-12). Reclassifying CXCL12 expression as negative vs. positive resulted in 63 (32%) negative-expressers and 136 (68%) positive-expressers. CXCR4 and CXCL12 expression were not significantly associated with each other ($p=0.74$, Table 11). The association between CXCR4, CXCL12, and clinicopathologic factors is presented in Table 12. Neither CXCR4 nor CXCL12 were significantly associated with race, BMI, histology subtype, stage, lymph node involvement, or tumor size.

6.4.3 CXCL12/CXCR4 expression and OS

Kaplan-Meier survival curves were produced to graphically illustrate the association between CXCL12 and CXCR4 expression and OS (Figure 13). Neither CXCL12 ($p=0.33$) nor CXCR4 ($p=0.86$) were significantly associated with OS. As CXCR4 lacked variability, we only examined the independent effect of CXCL12 expression on OS using Cox proportional hazards models. In all EC patients, CXCL12 expression was not significantly associated with OS (HR: 0.72, 95% CI 0.30, 1.72, Table 13); however we did observe a statistically significant interaction between CXCL12 and histology subtype ($p=0.05$, Table 14). The effect of CXCL12 among the histology subtypes is shown in Table 14. Using linear combinations to compare categories of expression, we observed that high-grade Type I cases with positive CXCL12 expression had a significantly

better OS compared to high-grade Type I cases with negative CXCL12 expression (HR: 0.17, 95% CI 0.05, 0.59, data not shown). CXCL12 expression was not significantly associated with OS in low-grade Type I, CC, or PS cases.

6.4.4 CXCL12/CXCR4 expression and RFS

For the Kaplan-Meier analyses and the Cox proportional hazards modeling, the sampling weights were re-estimated to reflect the number of cases that were disease-free following the primary surgery (N=163, Table 15). CXCL12 and CXCR4 expression were not significantly associated with RFS based on the log-rank test (Figure 14). The main effect of CXCL12 adjusted for histology subtype, stage, and age is shown in Table 16. Although CXCL12 was not significantly associated with RFS in all EC cases (HR: 0.52, 95% CI 0.20, 1.34), we did observe a significant interaction between CXCL12 and histology subtype ($p=0.01$, Table 17). Among high-grade Type I cases, positive CXCL12 expression was significantly associated with a reduced risk of recurrence compared to negative CXCL12 expression (HR: 0.10, 95% CI 0.02, 0.57, data not shown). Among low-grade Type I cases, RFS was borderline significantly associated with CXCL12 expression. Risk of recurrence was reduced in low-grade Type I patients with positive CXCL12 expression compared to negative expression (HR: 0.23, 95% CI 0.05, 1.05).

6.5 DISCUSSION

In normal tissues, homeostasis is maintained through controlled interactions between epithelial and stromal cells. Chemokines are an important class of stromal-derived signaling proteins

which contribute to tissue maintenance and repair by binding specific receptors expressed by target cells. For a particular chemokine ligand-receptor pair, high concentrations of the ligand “chemoattract” cells that express the receptor through a chemokine gradient. CXCR4 is typically expressed by immune cells, such as hematopoietic cells, T-lymphocytes, B-lymphocytes, monocytes and macrophages, as well as cells of the liver, kidney, brain, lung, and colon [178]. Upon binding, downstream processes such as chemotaxis, cell survival and proliferation, increases in intracellular calcium, and gene transcription are triggered [178]. Several chemokine ligand-receptor pairs have been examined in the cancer literature, among which, the CXCL12/CXCR4 axis is the most frequently studied. During carcinogenesis, tumor cells may acquire CXCR4 expression as a result of hypoxic tissue conditions or activating mutations. Consequently, CXCR4-expressing cancer cells have the potential to metastasize to tissues which abundantly express CXCL12 via a chemokine gradient. By analogy, positive expression of CXCL12 at the primary tumor site would presumably suppress the metastasis of CXCR4-expressing cells, which has been shown in colon [179] and breast cancer models [180].

We examined the role of CXCR4 and CXCL12 in a diverse sample of EC cases. CXCR4 was highly and uniformly expressed by all cases in this study. Positive expression of CXCL12 at the primary tumor site was significantly associated with better OS and RFS in high-grade Type I cases. Furthermore, positive CXCL12 expression was borderline associated with better RFS in low-grade Type I cases. To our knowledge, this is the first study to demonstrate the prognostic significance of CXCL12 in a cohort of diverse EC subtypes. Our findings highlight additional molecular and prognostic differences between Type I (low-grade and high-grade) and Type II (CC and PS) ECs.

The differences in prognosis between EC subtypes associated with CXCL12 expression may reflect the wide-ranging effects induced by CXCL12 expression. Although high concentrations of CXCL12 at the primary tumor site may suppress the metastasis of tumors, mechanisms other than cell migration, including proliferation, immune evasion, and adhesion, may be stimulated. For example, in a murine melanoma model, Vianello et al. [181] reported high levels of CXCL12 tumor expression were associated with “chemorepulsion” of effector T cells that would normally infiltrate the tumor and kill neoplastic cells. Consequently, these tumors were capable of evading the immune response. In epithelial ovarian cancer cells, expression of CXCL12 at the primary tumor site was associated with intraperitoneal metastasis through CXCL12-mediated adhesion to human peritoneal cells [171].

Previous EC studies have investigated the association between CXCR4 and/or CXCL12 and tumor grade [105, 106], myometrial invasion [108], and survival [107]. Using IHC, Mizokami et al. [106] reported CXCR4 and CXCL12 protein expression were significantly higher in low-grade Type I tumors (N=27) compared to high-grade Type I tumors (N=15). Conversely, Gelmini et al. [105] reported that IHC expression of CXCR4 showed high and uniform staining among 41 Type I EC tumors with different grades of differentiation [105]. In this case-series, CXCR4 was moderately or strongly expressed by all ECs, which agrees with the Gelmini series [105]. Another analysis showed that CXCR4 expression was significantly higher in endometrial tumors that invaded more than half of the myometrial layer compared to endometrial tumors with superficial invasion [108].

Kodama and colleagues [107] investigated the prognostic association of CXCR4 expression on OS. Positive CXCR4 expression (defined as staining in 50% or more of cells) was associated with significantly better OS (log-rank $p=0.04$) compared to no CXCR4 expression (defined as

less than 50% of cells stained positive), however this association was not significant in an adjusted regression model. In our Kaplan-Meier analysis comparing OS and RFS between moderately positive and strongly positive CXCR4 expressers, we did not observe a statistically significant association.

Relatively few EC studies have examined CXCL12 expression. CXCL12 was highly variable in our sample; 32% of cases had no CXCL12 expression while the remaining 68% of cases had varying levels of positive CXCL12 expression. We did not observe significant associations between CXCL12 expression and demographic or clinical variables. A trend in decreasing CXCL12 expression and increasing BMI was noted, however the *p*-value for this association was 0.21. Although non-significant, this finding is particularly interesting because BMI is typically used as a surrogate indicator for circulating estrogen exposure. As BMI increases, estrogen levels are also thought to increase due to the enhanced conversion of androstenedione to estrogen [19]. The downstream effects of estrogen action are regulated through direct interaction with estrogen receptors alpha and beta [182]. In normal human endometrial cell lines, Ruiz et al. [176] showed that CXCL12 gene expression was significantly downregulated by estrogen treatments, however a concurrent downregulation in CXCL12 protein expression was not observed. Conversely, Tsutsumi et al. [183] reported that estrogen was a potent inducer of CXCL12 mRNA and protein in endometrial stromal cells purified from patients with benign gynecologic diseases, supporting the role of a positive interaction between estrogen and CXCL12. The relationship between estrogen and CXCL12 expression in EC remains indefinite.

Interestingly, stromal expression of CXCL12 was absent in this study. Based on the biology of CXCL12 in normal tissues [176], we anticipated that CXCL12 would be expressed by infiltrating stromal cells, which was not the case. However, other studies have reported CXCL12

and CXCR4 production in primary tumors which supports the hypothesis of autocrine signaling to enhance tumor proliferation [179, 180].

Our prognostic results are in accordance with large studies performed in breast cancer patients. Mirisola [173], Hassan [175], and Kobayashi [174] reported that CXCL12 expression was an independent and significant prognostic factor for better OS and RFS. These studies proposed that the CXCL12/CXCR4 axis mediates survival through inhibition of metastatic spread. Interestingly, studies of other cancer sites report that CXCL12 expression is a poor prognostic factor for RFS and OS. In stage II pancreatic ductal adenocarcinomas [184] high tumoral CXCL12 expression was associated with poor OS adjusted for tumor size, differentiation, and lymph node status. In rectal cancer patients treated with preoperative chemoradiotherapy, strong stromal expression of CXCL12 and CXCR4 was associated with shorter RFS and OS [185]. Akishima-Fukasawa et al. [186] reported that high CXCL12 expression was significantly associated with worse OS and RFS adjusted for tumor depth, lymph node metastasis, and blood vessel invasion in colorectal cancer patients. These prognostic studies further emphasize the conflicting role CXCL12 plays in various cancers; in some cancers, a protective effect is observed while in others, poor outcomes are apparent. We propose that survival outcomes in various cancers are dependent on the dominant CXCL12 mechanism observed for specific cancer types.

Several limitations with the current study should be mentioned. First, as we selected cases for this study from a larger population of EC cases, selection bias may play a role in the findings. Tumor blocks were unavailable for 33% of the target population; cases without available tumor blocks differed from included cases with respect to stage and histology type, two important prognostic factors. Second, our use of IHC as the method for protein detection is a potential

limitation. IHC is a subjective and semi-quantitative method used for the detection of protein expression. Despite the known limitations of IHC [187], it can be argued that while IHC remains the gold standard in routine pathology, translational studies should utilize the molecular methods commonly used in clinical practice. Finally, this study lacks generalizability to EC patients of other races, as our study population was mostly white.

The major strength of this clinicopathologic study was the inclusion of a large sample of high-grade Type I, CC, and PS tumors compared to other published case-series; it was particularly relevant to study this pathway in a group of aggressive ECs, as higher stage, poor differentiation, and metastasis are characteristic of these tumors. Furthermore, the large sample size allowed us to investigate the independent prognostic significance of CXCL12 in subtypes of EC. Other strengths include the high quality pathology data, information on potential confounders, and use of archived tissue specimens.

In conclusion, the results of this study suggest that CXCL12 expression is associated with better OS and RFS in a subset of EC cases. In addition to the usual pathological prognostic factors (stage and histology subtype), this biomarker may be relevant for identifying a subset of patients who will have a better outcome, and can potentially avoid adjuvant therapy. Future EC studies of this pathway should examine a larger sample of patients to confirm or challenge the findings presented in this study. Furthermore, basic science studies which examine mechanisms associated with CXCL12 expression in EC will add to the body of knowledge regarding this pathway. Future studies planned by our group include an evaluation of concurrent CXCL12 and estrogen receptor expression in EC cases. As CXCL12 is an estrogen receptor related target and estrogen exposure is etiologically the most important factor for EC development, an

understanding of the relationship between these two proteins in a diverse group of EC cases may provide useful insights.

6.6 TABLES

Table 9 Probability of sampling given that tumor blocks were available

Stratum	Type I				Type II				Total
	Stage I	Stage II	Stage III	Stage IV	Stage I	Stage II	Stage III	Stage IV	
n with tumor blocks available	691	65	85	21	62	16	34	29	1,003
n sampled	30	28	36	16	36	9	23	21	199
pr (sampled tumor blocks available)= (sampled/ tumor blocks)	0.0434	0.4308	0.4235	0.7619	0.5806	0.5625	0.6765	0.7241	

Table 10 Demographic and clinical characteristics of the EC sample by histology subtype

Characteristics N=199	Low-grade Type I n=76		High-grade Type I n=34		Clear cell n=29		Papillary serous n=60		<i>p</i> *
	n [§] (%) [†]	% [‡]							
Mean age (sd)	62 (12)	61 (1.67)	62 (12)	61 (3.71)	67 (11)	67 (2.00)	66 (11)	66 (1.37)	0.04
Race									
White	71 (93)	95	32 (94)	96	26 (90)	89	50 (83)	83	0.10
Non-White	5 (7)	5	2 (6)	4	3 (10)	11	10 (17)	17	
Body Mass Index									
Normal (BMI < 25 kg/m ²)	19 (25)	26	4 (12)	5	9 (32)	31	16 (27)	23	0.08
Overweight (BMI 25-30 kg/m ²)	20 (26)	18	10 (29)	48	10 (34)	34	18 (30)	23	
Obese (BMI > 30 kg/m ²)	36 (47)	56	20 (59)	47	10 (34)	35	26 (43)	54	
Additional cancer primaries									
No	66 (87)	83	27 (79)	73	23 (79)	80	44 (74)	81	0.58
Yes	8 (10)	14	7 (21)	27	6 (21)	20	13 (23)	16	
Unknown	2 (3)	3	0 (0)	0	0 (0)	0	2 (3)	3	
FIGO stage									
Early (I & II)	52 (68)	93	6 (18)	62	19 (66)	71	26 (43)	49	<0.001
Late (III & IV)	24 (32)	7	28 (82)	38	10 (34)	29	34 (57)	51	
Lymph node involvement									
No nodes examined	18 (24)	39	9 (26)	27	10 (34)	34	12 (20)	20	<0.001
None	44 (58)	57	12 (35)	53	16 (55)	57	30 (50)	52	
Positive	8 (10)	2	13 (38)	20	3 (10)	9	13 (22)	20	
Unknown	6 (8)	2	0 (0)	0	0 (0)	0	5 (8)	9	
Tumor size									
< 2 cm	11 (14)	21	4 (12)	40	3 (10)	11	8 (13)	15	0.16
> 2 cm	34 (45)	27	20 (59)	46	14 (48)	50	27 (45)	44	
Unknown	31 (41)	52	10 (29)	14	12 (42)	39	25 (42)	41	
Post-operative treatment									
Surgery only	30 (39)	68	4 (12)	41	6 (21)	20	12 (20)	20	<0.001
Surgery + CT	8 (11)	2	11 (32)	13	9 (31)	28	26 (43)	41	
Surgery + RT	34 (45)	29	11 (32)	36	13 (45)	47	11 (18)	20	

Table10 continued

Surgery + CT + RT	4 (5)	1	8 (24)	10	1 (3)	4	11 (18)	20	
§ sample count † proportion or mean in the study sample ‡ proportion or mean in the target population * Adjusted Wald <i>p</i> -value									

Table 11 Association between CXCR4 and CXCL12 expression

CXCL12 expression	CXCR4 expression				p^{\ddagger}
	Moderately positive n=59 [§]		Strongly positive n=140 [§]		
	n [§] (%) [*]	% [†]	n [§] (%) [*]	% [†]	
Negative n=63 [§]	24 (41)	36	39 (28)	31	0.74
Positive n=136 [§]	35 (59)	64	101 (72)	69	

[§] sample count
^{*} proportion in the study sample
[†] proportion in the target population
[‡] Adjusted Wald p -value

Table 12 Association of biomarker expression with clinicopathologic characteristics

Characteristic N=199	Sample counts	% Strong expression [†]	% Positive expression [‡]
		CXCR4 [§]	CXCL12 [§]
Race			
White	179	69	67
Non-White	20	76	79
<i>p</i> -value [*]		0.69	0.50
Body Mass Index			
Normal	49	69	84
Overweight	58	59	71
Obese	92	71	59
<i>p</i> -value [*]		0.92	0.21
Histology type			
Low-grade Type I	76	66	68
High-grade Type I	34	86	64
Clear cell	29	73	60
Papillary serous	60	76	71
<i>p</i> -value [*]		0.49	0.81
FIGO stage			
Early stage (I & II)	103	70	67
Late stage (III & IV)	96	64	69
<i>p</i> -value [*]		0.49	0.81
Lymph node involvement			
None	102	70	75
Positive	37	68	70
No nodal exam	49	70	55
Unknown	11	64	83
<i>p</i> -value [*]		0.99	0.42
Tumor size			
< 2 cm	26	84	55
> 2 cm	95	70	62
Unknown	78	62	77
<i>p</i> -value [*]		0.36	0.35
[†] Strong expression vs. moderate expression [‡] Positive expression vs. negative expression [§] Weighted percentages [*] Adjusted Wald <i>p</i> -value			

Table 13 Cox proportional hazards model for OS: main effect of CXCL12

N=199	deaths/N	Multivariable*	
		HR (95% CI)	<i>p</i> [†]
CXCL12			
Negative	28/63	1.00 (Reference)	0.46
Positive	58/136	0.72 (0.30, 1.72)	

* Adjusted for stage (early stage, late stage), histology subtype (low-grade type I, high-grade type I, clear cell, papillary serous), and age at diagnosis (continuous, range 34-93 years)
 Stratified by year of diagnosis (single year, 1996 through 2008)
[†] Adjusted Wald *p*-value

Table 14 Cox proportional hazards model* for OS: interaction between CXCL12 and histology subtype

N=199	deaths/N	HR (95% CI)	<i>p</i> [†]
Histology subtype and CXCL12 expression			0.05[‡]
Low-grade Type I and negative CXCL12	6/19	1.00 (Reference)	
Low-grade Type I and positive CXCL12	15/57	0.91 (0.20, 4.22)	0.90
High-grade Type I and negative CXCL12	8/14	10.78 (1.53, 76.09)	0.02
High-grade Type I and positive CXCL12	11/20	1.80 (0.25, 12.87)	0.55
Clear cell and negative CXCL12	6/12	2.92 (0.37, 23.12)	0.31
Clear cell and positive CXCL12	7/17	2.43 (0.57, 10.40)	0.23
Papillary serous and negative CXCL12	8/18	2.35 (0.38, 14.69)	0.36
Papillary serous and positive CXCL12	25/42	4.72 (0.70, 31.55)	0.11

* Adjusted for stage (early stage, late stage) and age at diagnosis (continuous, range 34-93 years)
 Stratified by year of diagnosis (single year, 1996 through 2008)
[†] Adjusted Wald *p*-value
[‡] *p*-value for interaction

Table 15 Probability of sampling given that tumor blocks were available in disease-free cases

	Type I				Type II				
Stratum	Stage I	Stage II	Stage III	Stage IV	Stage I	Stage II	Stage III	Stage IV	Total
n with tissue available	691	65	85	21	62	16	34	29	1,003
n sampled	28	24	30	13	30	7	19	12	163
pr (sampled tissue available)= (sampled/tissue available)	0.0405	0.3692	0.3529	0.6190	0.4839	0.4375	0.5588	0.4138	

Table 16 Cox proportional hazards model for RFS: main effect of CXCL12, N=163

N=163	Multivariable*		
	recurrences/N	HR (95% CI)	<i>p</i> [†]
CXCL12			
Negative	20/55	1.00 (Reference)	0.17
Positive	22/108	0.52 (0.20, 1.34)	

* Adjusted for stage (early stage, late stage), histology subtype (low-grade type I, high-grade type I, clear cell, papillary serous), and age at diagnosis (continuous, range 34-93 years)
 Stratified by year of diagnosis (single year, 1996 through 2008)
[†] Adjusted Wald *p*-value

Table 17 Cox proportional hazards model* for RFS: interaction between CXCL12 and histology subtype

N=163	recurrences/N	HR (95% CI)	<i>p</i> [†]
Histology subtype and CXCL12 expression			0.01[‡]
Low-grade Type I and negative CXCL12	4/17	1.00 (Reference)	
Low-grade Type I and positive CXCL12	2/50	0.23 (0.05, 1.05)	0.06
High-grade Type I and negative CXCL12	7/12	1.66 (0.33, 8.26)	0.53
High-grade Type I and positive CXCL12	8/16	0.16 (0.02, 1.46)	0.10
Clear cell and negative CXCL12	2/10	0.29 (0.02, 4.30)	0.37
Clear cell and positive CXCL12	3/13	2.84 (0.44, 18.16)	0.27
Papillary serous and negative CXCL12	7/16	1.03 (0.18, 5.73)	0.98
Papillary serous and positive CXCL12	9/29	1.83 (0.36, 9.17)	0.46

* Adjusted for stage (early stage, late stage) and age at diagnosis (continuous, range 34-93 years)
 Stratified by year of diagnosis (single year, 1996 through 2008)
[†] Adjusted Wald *p*-value
[‡] *p*-value for interaction

6.7 FIGURES

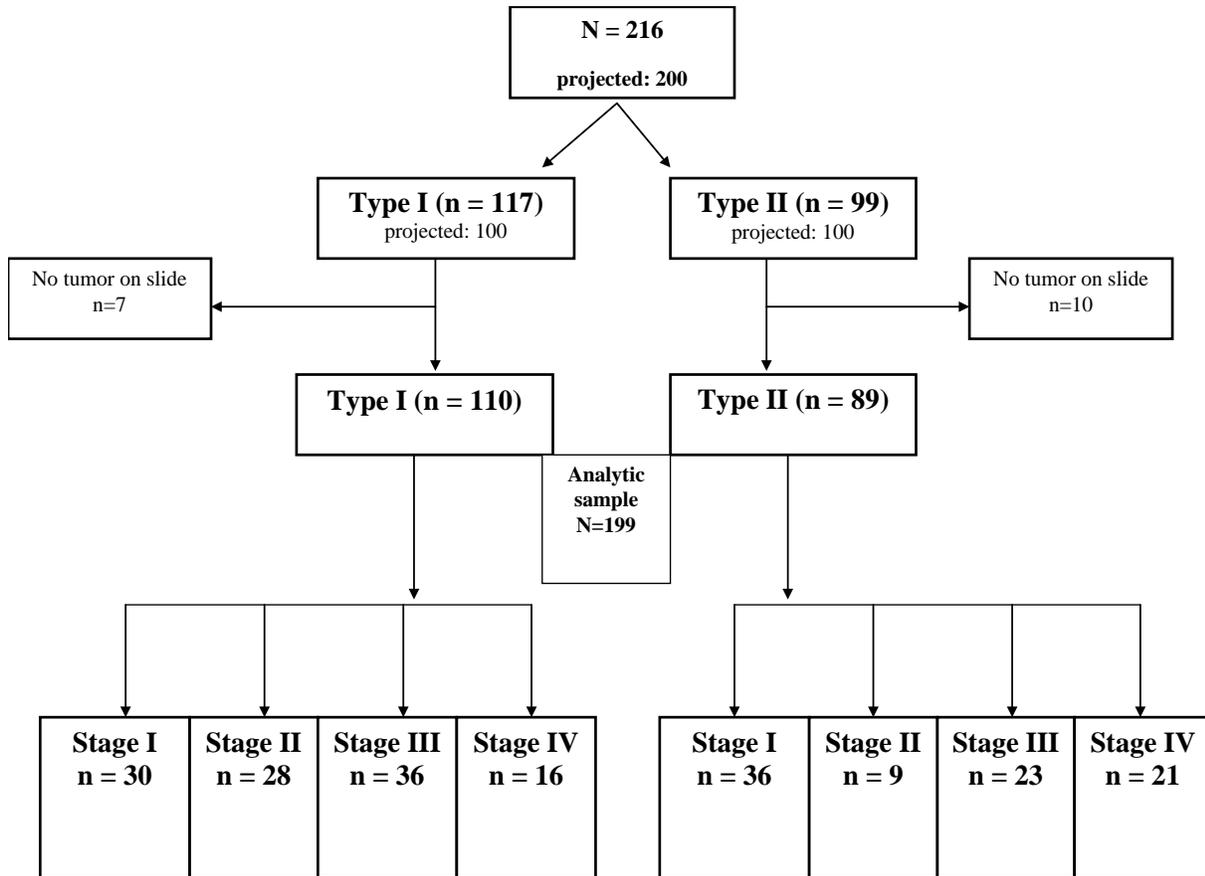


Figure 6 Sampling strategy for CXCL12/CXCR4 expression study

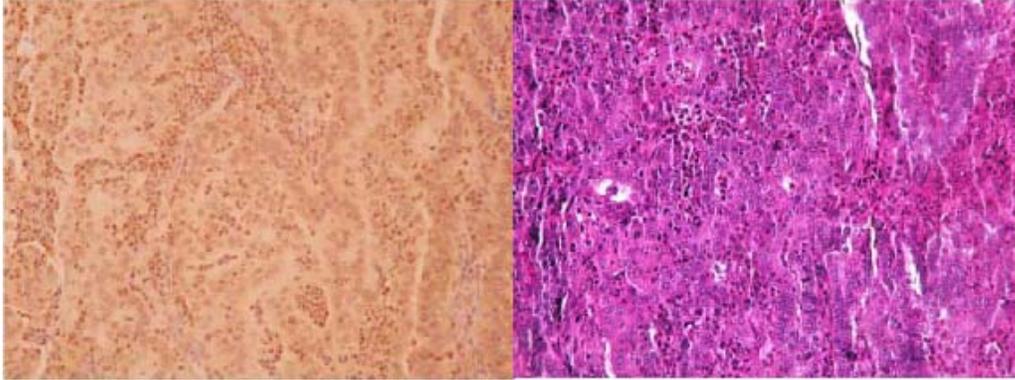


Figure 7 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Moderate CXCR4 expression

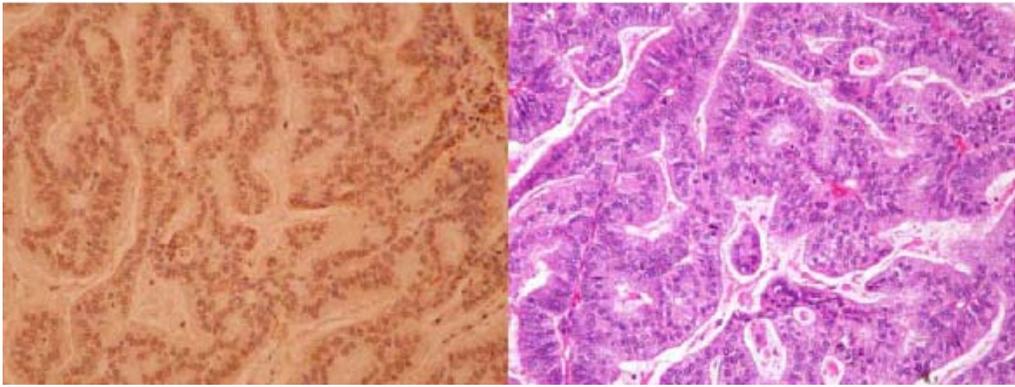


Figure 8 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Strong CXCR4 expression

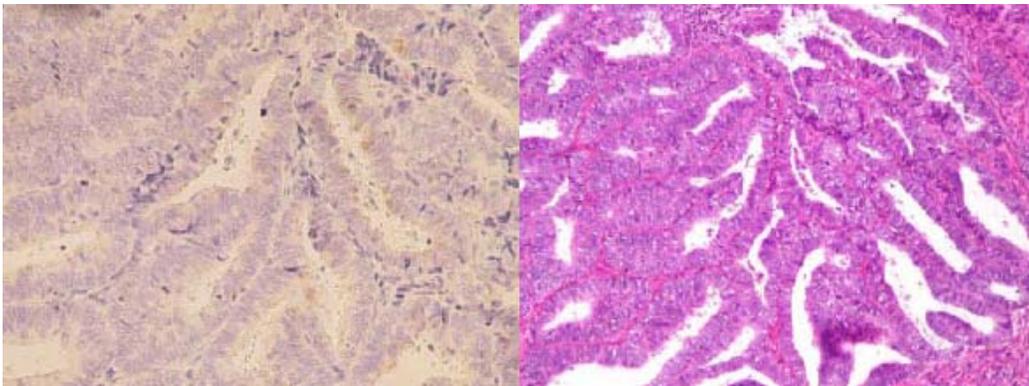


Figure 9 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Negative CXCL12 expression

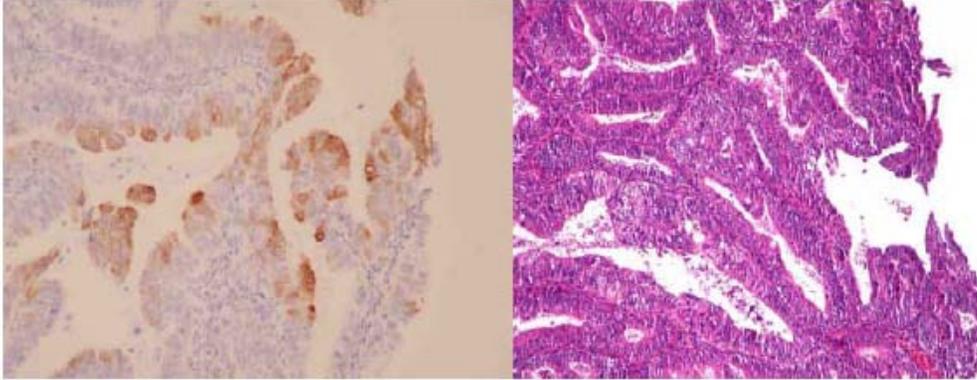


Figure 10 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Weak CXCL12 expression

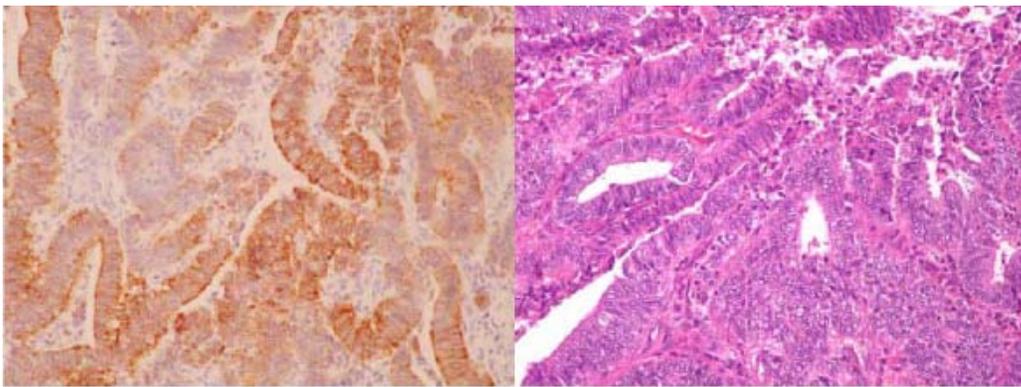


Figure 11 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Moderate CXCL12 expression

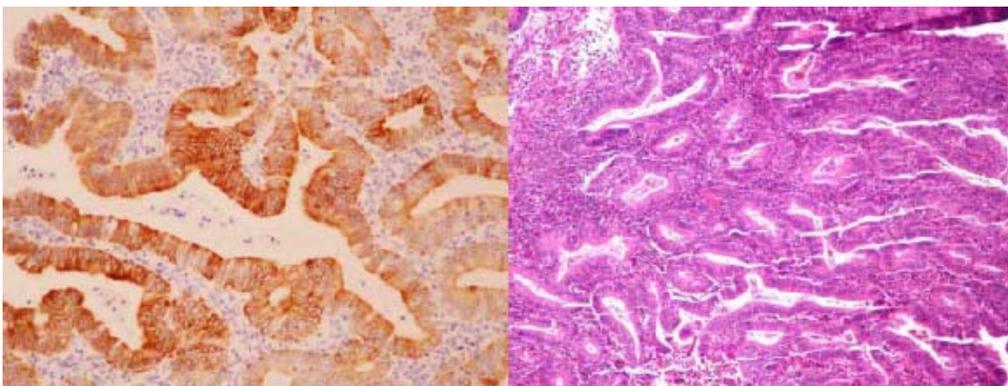


Figure 12 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Strong CXCL12 expression

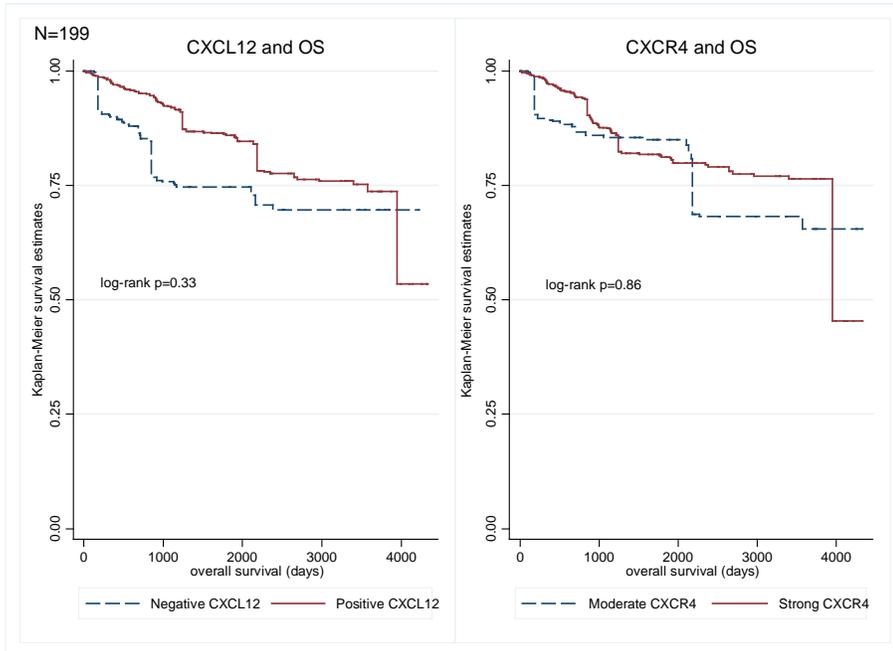


Figure 13 Kaplan-Meier overall survival curves for CXCL12 and CXCR4

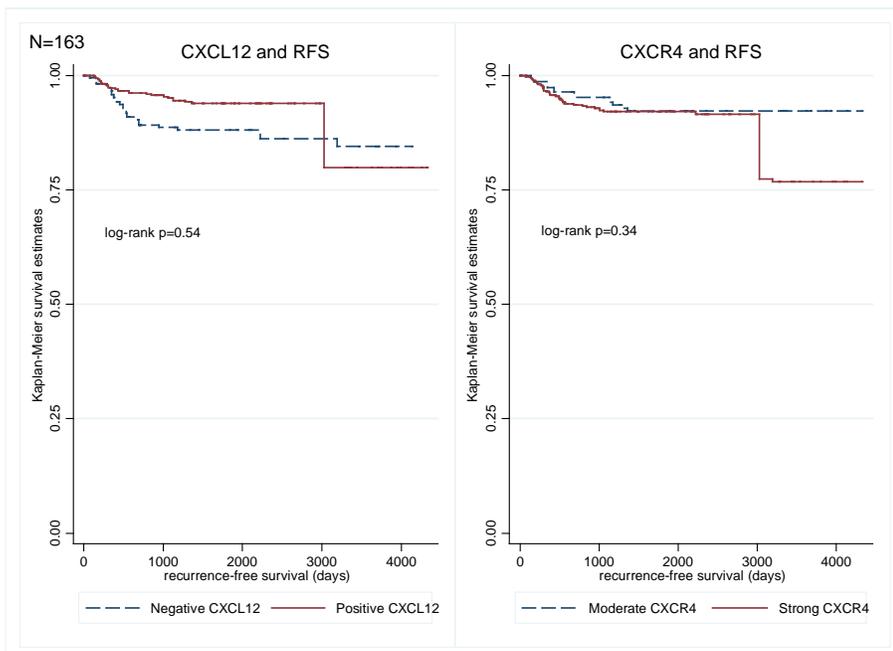


Figure 14 Kaplan-Meier recurrence-free survival curves for CXCL12 and CXCR4

6.8 ACKNOWLEDGEMENTS

We wish to thank the University of Pittsburgh Medical Center (UPMC) Registry Information Services (RIS) team, especially Louise Mazur, for her help with procuring the data for this study. We also would like to thank Lindsay Mock (Magee Womens Hospital, Tissue Bank) for procuring the tissue blocks and sectioning slides and Kim Fuhrer (Molecular Anatomic Pathology laboratory) for the immunohistochemical staining of all slides.

7.0 ARTICLE 4: PROGNOSTIC IMPACT OF HEPATOCYTE GROWTH FACTOR, C-MET, AND BASIC FIBROBLAST GROWTH FACTOR IN ENDOMETRIAL CANCER PATIENTS

To be submitted for publication

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7.1 ABSTRACT

Introduction: Angiogenesis plays a central role in the invasion and metastasis of many common cancers. Hepatocyte growth factor (HGF) is a stromal-derived, multifunctional protein that acts on epithelial cells through activation of the proto-oncogenic receptor, c-Met. Activation of the HGF/c-Met pathway is typically associated with poor cancer outcomes. Additionally, basic fibroblast growth factor (bFGF) is an angiogenic cytokine which induces expression of HGF and promotes cell proliferation and angiogenesis. The goal of this study is to determine the prognostic significance of HGF, c-Met, and bFGF in patients with endometrial cancer (EC).

Methods: Tumor blocks were available for 211 EC cases diagnosed at Magee-Womens Hospital. Immunohistochemistry was used to detect stromal and tumor protein expression of the three biomarkers. The association between protein expression and clinicopathologic factors was examined with chi-square tests. Kaplan-Meier curves, log-rank tests, and Cox regression were used to investigate the association between biomarker expression, overall survival (OS), and recurrence-free survival (RFS).

Results: Positive expression in the sample was as follows: tumor HGF (15%), tumor c-Met (27%), tumor bFGF (17%), and stromal bFGF (55%). Tumor bFGF was significantly associated with histology subtype ($p<0.001$), stage ($p=0.008$), lymph node involvement ($p=0.002$), and age ($p=0.003$). Positive tumor bFGF expression was significantly associated with worse OS (log-rank $p=0.009$) and RFS (log-rank $p<0.001$). In the adjusted OS Cox model, a reduced risk of death was observed in tumor HGF positive, stromal bFGF positive patients compared to tumor HGF positive, stromal bFGF negative patients (HR: 0.14, 95% CI 0.03, 0.60). Tumor HGF positive, tumor bFGF positive patients had significantly worse RFS compared to patients with negative expression of both markers (HR: 9.88, 95% CI 2.63, 37.16).

Discussion: The present study demonstrates that interactions between tumor-derived and stromal-derived proteins influence survival outcomes in EC patients. Interactions between HGF and bFGF played an important role in OS and RFS, however the localization of bFGF immunostaining modified the association. Pending further investigations, tumor-derived bFGF may be an attractive target in EC therapy.

7.2 INTRODUCTION

Angiogenesis, the formation of new vessels from preexisting parent vessels, is a critical factor in growth and dissemination of primary tumors [188]. Endometrial stromal cells are a highly active source of angiogenic cytokines, which regulate endothelial cell proliferation, migration, and vessel density during the proliferative and secretory phases of the menstrual cycle [189]. During carcinogenesis, neoplastic cells exploit the pathways controlled by angiogenic growth factors and their receptors, leading to cell scattering, proliferation, evasion of apoptosis, and ultimately metastasis [190].

Hepatocyte growth factor (HGF), also known as scatter factor, has mitogenic and motogenic effects on epithelial and endothelial cells via its receptor c-Met [111]. c-Met activation by HGF is associated with stimulation of various signaling cascades including the ras-mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), and the signal transducers and activators of transcription (STAT) signaling pathways; aberrant activation of these pathways can result in motility, protection from apoptosis, and proliferation [191]. These consequences support the hypothesis that HGF/c-Met interactions are associated with invasion and metastasis of human malignancies. c-Met is reportedly overexpressed in many cancers compared to adjacent normal tissue which typically correlates with worse prognosis [192]. Additionally, other angiogenic growth factors are known to interact with this pathway; specifically, basic fibroblast growth factor (bFGF) upregulates expression of HGF in EC tissues [113] and mediates angiogenesis by enhancing vascular permeability and proliferation of endothelial cells [193].

The biology of HGF, c-Met, and bFGF has been examined in normal human endometrial tissues, EC cell lines, and human EC specimens. Sugawara et al. [118] demonstrated that HGF and c-Met were present in the normal endometrium; furthermore, HGF was a potent stimulator

of endometrial cell growth and motility. *In vitro* studies using EC cell lines demonstrate the presence of c-Met; well-differentiated EC cell lines (HEC-1A and HEC-1B) showed higher c-Met expression compared to moderately differentiated (RL-95) and poorly differentiated EC cell lines (KLE) [119]. Although HGF was not expressed by EC cell lines, addition of HGF to cell cultures significantly induced the invasion of EC cells, suggesting that surrounding stromal cells and not neoplastic cells produce the growth factor. Dai and colleagues [194] reported bFGF mRNA and protein expression in normal endometrial tissues, however expression was significantly higher in complex hyperplasia and EC specimens. In EC cell lines, tumor-derived bFGF was shown to increase HGF transcription, which resulted in an increased invasive and proliferative capacity of EC cells [111].

Two prognostic studies have examined HGF and c-Met expression in Type I and papillary serous (PS) EC patients [120, 121]. Wagatsuma et al. [120] reported positive HGF and c-Met expression in 90% and 63% of Type I EC patients, respectively which was higher than expression in normal endometrial specimens (HGF=79%; c-Met=14%). Positive c-Met expression was significantly associated with advanced stage, poor differentiation, and worse overall survival (OS) in univariate analysis, whereas HGF was significantly associated with advanced stage. Additionally, neither c-Met nor HGF expression were independent prognostic factors for survival after adjusting for stage, grade, vascular involvement, and microvessel count [120]. Bishop et al. [121] reported positive HGF and c-Met staining in 100% and 87% of PS EC patients, respectively. OS in cases with strong HGF or strong c-Met expression was significantly worse compared to cases with weak expression of either marker as evidenced by Kaplan-Meier graphs.

In two reports, high expression of tumor-derived bFGF protein was significantly associated with poor differentiation, presence of tumor necrosis, and vascular invasion [122]. Similarly, Fujimoto et al. [123] showed that bFGF mRNA expression was significantly higher in poorly differentiated and advanced stage tumors. To our knowledge, the association between bFGF expression and survival in EC has not been explored. The goal of this study is to expand the findings from the previous *in vitro* and prognostic studies by examining the co-expression of HGF, c-Met, and bFGF expression in a histologically diverse sample of EC cases. Furthermore, we used immunohistochemistry (IHC) to distinguish tumor and stromal expression. We hypothesized that positive expression of HGF, c-Met, and bFGF would be associated with worse survival outcomes independent of known EC prognostic factors, such as stage, histology subtype, and age.

7.3 METHODS

7.3.1 Study sample

EC cases in this study were treated at Magee-Womens Hospital between 1996 and 2008. The sampling frame and sample selection methods were previously described (section 6.3.1); briefly, 1,486 cases were eligible for inclusion in the current IHC study. Patients were classified as having Type I or Type II EC based on the surgical pathology report. Endometrioid or adenocarcinoma tumors were classified as Type I while clear cell (CC) and PS were classified as Type II. Tumor blocks were presumed to be available for 67% (N=1,003) of cases. Case selection from this reduced sampling frame was achieved using a stratified random sampling

design. Within the eight combinations of histology subtype and stage, we aimed to include 25 patients from each stratum, resulting in 200 cases; however tumor blocks for 216 cases were retrieved. The breakdown of cases in each stratum is shown in Figure 15. Within strata where the number of available cases was less than 25, we made an effort to include all cases; however inadequate tissue limited our ability to achieve this goal. Furthermore, following slide sectioning of the selected blocks, 5 slides did not have evaluable tumor present, which reduced the analytic cohort to 211 EC cases. This study was approved by the University of Pittsburgh Institutional Review Board.

7.3.2 Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks for the 216 selected cases were retrieved from the Pathology Department at Magee-Womens Hospital. Four μm thick slides were cut at the time of block retrieval. Deparaffinization and hydration of the slides with xylenes and washes with progressively decreasing alcohol concentrations was performed. Endogenous peroxidase activity was blocked with 3% methanol peroxide. Antigen retrieval in 0.01 M boiling citrate buffer (pH 6.0) in a microwave was performed followed by blocking of non-specific staining with Protein Block (Dako North America, Inc, Carpinteria, California). Whole tissue sections were then incubated for one hour at room temperature with 100 microliters of polyclonal HGF antibody (R&D Systems, Minneapolis, MN, dilution 1:30), polyclonal c-Met antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, dilution 1:75) and polyclonal basic fibroblast growth factor antibody (Millipore, Billerica, MA, dilution 1:1000). Slides were subsequently rinsed in PBS/Tween solution for five minutes followed by incubation with a polymer (ImmPRESS Universal reagent, Vector Laboratories). Slides were rinsed in the PBS/Tween solution followed

by development with diaminobenzidine for detection. Slides were counterstained with Shandon Hematoxylin for 2 minutes, rinsed in several concentrations of alcohol solution, mounted, and viewed. Positive and negative controls were run with each batch of slides, which consisted of approximately 40 slides per batch. Placenta, fibroadenoma, and granulation tissue were the positive controls for HGF, c-Met, and bFGF, respectively. The primary antibody was omitted from the negative control slides. Cytoplasmic and nuclear immunostaining was observed for HGF and bFGF; cytoplasmic staining was observed for c-Met.

7.3.3 Evaluation of staining

Calculation of the cumulative score was previously described (section 6.3.3). Tumor and stromal expression was quantified by summing the proportionality score (PS) and the intensity score (IS). The PS represents the approximate proportion of positive-staining cells (1=1-5%, 2=6-20%, 3=21-80%, and 4=>80%) while the IS represents the average intensity of positive-staining cells (0=none, 1=weak, 2=moderate, 3=strong). Slides with no positive-staining cells were coded as 0. Acceptable values of the cumulative score are 0, 2, 3, 4, 5, 6, and 7. Interpretation of the cumulative score for a given marker is as follows: 0 (none), 2 (weak), 3-5 (moderate), and 6-7 (strong) [177]. Stromal expression of HGF and c-Met were negative in all cases while bFGF showed tumor and stromal expression.

7.3.4 Statistical analyses

7.3.4.1 Sampling weights

Sampling fractions (f_i), the probability of case selection, were calculated as follows: stratum-specific fraction of available tumor blocks with non-missing IHC data (A_i) * estimated case-specific probability of tumor block availability (B_i). Calculation of A_i for the OS and RFS models are shown in Tables 18 and 25, respectively. The predicted probability that tumor blocks were available for a given case (B_i) was computed in the total target population ($N=1,486$) using a logistic regression model. A_i and B_i were multiplied to calculate the probability that a case was sampled, i.e. the sampling fraction, f_i . The sampling weight was derived by taking the inverse of the sampling fraction. All analyses applied sampling weights that allowed for proportions and other effect sizes to be applicable to the target population. In STATA 11, the *svy* family of commands was used to implement features of the study design into the analysis (StataCorp LP, College Station TX).

7.3.4.2 Independent variables

As mentioned, tumor HGF, tumor c-Met, tumor bFGF, and stromal bFGF were coded as none, weak, moderate, or strong. These categories were further collapsed to represent positive expression (weak + moderate + strong cumulative scores) vs. negative expression.

Additionally, information on age, race (white vs. non-white), body mass index (BMI: normal, overweight, obese), additional cancer primaries (no, yes, unknown), histology type (low-grade Type I, high-grade Type I, CC, PS), stage (I & II vs. III & IV), lymph node involvement (no nodal involvement, positive nodal involvement, unknown nodal involvement, no nodal examination), tumor size (< 2 cm in the largest dimension, > 2 cm in the largest dimension,

unknown size), post-operative treatment (surgery only, surgery + chemotherapy (CT), surgery + radiotherapy (RT), surgery + CT + RT), and year of diagnosis was available.

7.3.4.3 Descriptive statistics

Contingency tables were used to examine the association between clinicopathologic factors and histology subtype using Pearson chi-square tests for categorical data (*svy: tabulate*). Means of continuous variables (i.e. age) by histology subtype were estimated and a test comparing the equality of means was performed (*svy: mean*). The association between tumor HGF, tumor c-Met, tumor bFGF, stromal bFGF expression, and clinicopathologic variables was similarly assessed. Proportions and means for the study sample and the target population are presented, however statistics related to the target population are described in the text. We also examined the association between pairs of biomarkers using logistic regression (*svy: logistic*).

7.3.4.4 Multivariable models

The two survival outcomes of interest were OS and RFS, which were estimated as the number of days between the date of diagnosis and the date of death from any cause and the date of recurrence, respectively. Patients who did not experience either outcome were censored at the last date of contact or the date of last follow-up (December 31, 2008). Only cases known to be disease-free following the primary surgery were included in the RFS analysis (see Appendix Table 39 for an enumeration of disease recurrence in this sample).

Kaplan-Meier curves were compared using the log-rank test to examine the equality of OS and RFS among expressers and non-expressers of the proteins of interest. Cox proportional hazard regression modeling was used to analyze the effect of tumor HGF, tumor c-Met, tumor bFGF, and stromal bFGF expression on OS and RFS controlling for histology subtype, stage,

and age. Year of diagnosis was included as a stratification variable. In addition to the main effects model, we examined pairwise interactions between the biomarkers.

7.4 RESULTS

7.4.1 Characteristics of study sample

Distributions of demographic and clinical data by histology subtype are shown in Table 19. Mean age was significantly different between the histology subtypes; CC (67 years) and PS cases (66 years) were significantly older than low and high-grade Type I cases (mean age: 61 years). Non-white race was significantly more common among PS cases (19%) compared to CC (10%), low-grade Type I (4%), and high-grade Type I cases (4%). BMI and history of additional cancer primaries did not significantly vary across histology subtypes. Stage varied significantly between the four subtypes; advanced stage tumors (III & IV) were most common in PS cases (51%) compared to high-grade Type I (41%), CC (28%), and low-grade Type I cases (6%). Positive lymph node involvement was more common among high-grade Type I (22%) and PS cases (20%) than in CC (8%) or low-grade Type I cases (2%). Post-operative treatment was significantly different between the four subtypes. Surgery only was most common among low-grade Type I cases (61%). Among high-grade Type I cases, surgery only (39%) and surgery plus RT (34%) were prevalent. In CC cases, surgery plus RT (51%) and surgery plus CT (27%) were the most common treatments received. Surgery plus CT was used in 40% of PS cases, followed by 21% of patients with surgery plus RT, and 21% of patients with both RT and CT.

7.4.2 HGF/c-Met/bFGF expression in EC patients

Figures 16-28 show representative IHC stains for tumor HGF, tumor c-Met, tumor bFGF, and stromal bFGF expression. Tumor HGF expression was absent in 180 cases (85%); 16 (8%) and 15 cases (7%) showed weakly and moderately positive tumor HGF expression, respectively. Tumor c-Met expression was negative in 155 (73%), weakly positive in 14 (7%), moderately positive in 34 (16%), and strongly positive in 8 cases (4%). bFGF expression was present in both the tumor and stromal compartments. Tumor bFGF expression was absent in 176 (83%), moderately positive in 22 (10%), and strongly positive in 13 cases (6%). Stromal bFGF expression was absent in 95 (45%), weakly positive in 1 (1%), moderately positive in 74 (35%), and strongly positive in 41 (19%) cases. As mentioned, categories of weak, moderate, and strong were further collapsed into positive expression vs. negative expression categories.

Joint expression of tumor and stromal bFGF was as follows: 82 cases (39%) showed negative tumor bFGF and negative stromal bFGF; 13 (6%) showed positive tumor bFGF and negative stromal bFGF; 94 (45%) showed negative tumor bFGF and positive stromal bFGF; and 22 (10%) showed positive expression of both tumor and stromal bFGF expression (data not shown in tabular format).

The association between pairs of biomarkers was assessed using logistic regression (Table 20). Only tumor HGF and tumor c-Met were significantly associated (OR: 9.83, 95% CI 2.28, 42.28). Tables 21-22 show the relationship between tumor HGF, tumor c-Met, tumor bFGF, stromal bFGF expression and categorical and continuous factors. Positive tumor HGF expression was not significantly associated with race, BMI, histology subtype, stage, lymph node involvement, or age. A borderline significant association between tumor HGF expression and tumor size was observed ($p=0.07$); positive tumor HGF expression was more common among

cases with unknown tumor size (20%) compared to cases with large tumors (14%) or small tumors (2%). Tumor c-Met expression was not significantly associated with any clinicopathologic variable.

Tumor bFGF was significantly associated with histology subtype, stage, lymph node involvement, and age. Compared to low-grade Type I cases (1%), positive tumor bFGF expression was significantly more common in PS (16%), high-grade Type I (32%), and CC cases (32%). Advanced stage cases had a significantly higher proportion of positive tumor bFGF expression than early stage cases (18% vs. 5%). Additionally, tumor bFGF expression was higher in cases with positive lymph node involvement (33%) compared to no nodal involvement (8%) or no nodal examination (2%). Cases with positive tumor bFGF expression had a mean age of 69 compared to 61 in cases with negative tumor bFGF expression (Table 22). Stromal bFGF was borderline significantly associated with histology subtype ($p=0.06$). Stromal bFGF was most common among CC cases (70%) compared to high-grade Type I (61%), low-grade Type I (59%), and PS cases (44%).

7.4.3 HGF/c-Met/bFGF expression and OS

Kaplan-Meier curves for the association between tumor HGF, tumor c-Met, tumor bFGF, stromal bFGF and OS are shown in Figure 29. Positive tumor bFGF expression was significantly associated with worse OS compared to negative expression ($p=0.009$). Tumor HGF, tumor c-Met, and stromal bFGF were not associated with OS in univariate analysis. The adjusted association between tumor HGF, tumor c-Met, tumor bFGF, stromal bFGF expression and OS were examined using Cox proportional hazards models (Table 23). The independent effect of each biomarker in the absence (Model 1) and presence (Model 2) of the other biomarkers is

reported. The main effects of the biomarkers were not significantly associated with OS in either model independent of histology subtype, stage, and age.

Pairwise interactions between the biomarkers were explored and a significant interaction between tumor HGF and stromal bFGF was observed ($p=0.02$); hazard ratios associated with this model are shown in Table 24. In this table all comparisons are being made to the reference category of tumor HGF negative, stromal bFGF negative cases. Using linear combinations to compare categories of expression, we observed that tumor HGF positive, stromal bFGF positive cases had significantly reduced risk of death compared to tumor HGF positive, stromal bFGF negative cases (HR: 0.14, 95% CI 0.03, 0.60, data not shown). A Kaplan-Meier graph showing the joint expression of these two biomarkers confirmed this relationship; tumor HGF positive, stromal bFGF negative cases (deaths=7, at risk=13) had a median OS time of approximately 1,000 days (~3 years) compared to 4,000 days (~11 years) for tumor HGF negative, stromal bFGF positive cases (deaths=40, at risk=98). Median survival was not estimable for cases with negative expression of both markers or for those with positive expression of both markers (Figure 30).

7.4.4 HGF/c-Met/bFGF expression and RFS

The sampling weights for RFS were recalculated to include only disease-free cases (Table 25). RFS Kaplan-Meier curves for tumor HGF, tumor c-Met, tumor bFGF, and stromal bFGF are shown in Figure 31. Positive tumor bFGF expression was significantly associated with worse RFS ($p<0.001$), however tumor HGF, tumor c-Met, and stromal bFGF expression were not significantly associated with RFS.

Two main effects models are shown in Table 26; the role of each biomarker in the absence (Model 1) and presence (Model 2) of the other biomarkers is shown. Tumor bFGF expression had a borderline significant association with RFS in both models (Model 1: $p=0.08$ and Model 2: $p=0.10$). Pairwise interactions between the biomarkers were investigated and a significant interaction between tumor HGF and tumor bFGF expression was observed ($p=0.007$, Table 27). Tumor HGF positive, tumor bFGF positive patients had a ten times higher risk of recurrence compared to patients with negative expression of both markers (HR: 9.88, 95% CI 2.63, 37.16). A Kaplan-Meier graph of these two biomarkers confirmed this relationship; tumor HGF positive, tumor bFGF positive patients had the shortest median RFS time (Figure 32). Additionally, tumor HGF positive, tumor bFGF negative patients had an improved RFS compared to the reference group (HR: 0.07, 95% CI 0.00, 0.81).

7.5 DISCUSSION

We examined immunohistochemical expression of HGF, c-Met, and bFGF in a large sample of EC cases and correlated expression with clinicopathologic factors and patient outcomes. OS was significantly associated with stromal bFGF expression in the presence of tumor HGF expression. Tumor HGF positive, stromal bFGF positive patients had significantly better OS compared to tumor HGF positive, stromal bFGF negative patients. Additionally, a significant association between tumor bFGF and RFS was observed, and tumor HGF expression modified this association. Compared to tumor HGF negative, tumor bFGF negative patients, patients who were HGF positive, tumor bFGF positive had a ten times higher risk of recurrence. Additionally, patients who were tumor HGF positive and tumor bFGF negative had a significantly reduced risk

of recurrence compared to tumor HGF negative, tumor bFGF negative patients. To our knowledge, this is the first study to demonstrate the prognostic significance of this angiogenic pathway in EC patients.

Interactions between tumor cells and tumor-associated stromal cells are hypothesized to play a role in aggressive cancer phenotypes. The host stromal response is initially activated to eliminate tumor cells; however tumor cells co-opt the functions of recruited stromal cells to enhance the growth and invasion of the tumor, resulting in a tumor-supportive stroma [163, 167]. Furthermore, as the metabolic demand for nutrients increases, tumor cells activate angiogenesis through secretion of various cytokines. Therefore, understanding the role of tumor and stromal-derived angiogenic pathways is important for targeted therapy strategies [195-197].

The underlying mechanism describing the protective role of stromal-derived bFGF is unknown. Based on the hypotheses regarding tumor-stromal interactions, one might assume that the presence of angiogenic ligands in the stroma would stimulate tumor cell proliferation and invasion. This study, along with others, has established that angiogenic proteins expressed in the stroma have a divergent prognostic role compared to tumoral expression of these proteins. In two independent non-small cell lung cancer (NSCLC) cohorts, stromal bFGF expression was inversely associated with lymph node metastasis, advanced stage, and disease-specific mortality, indicating a protective role of this cytokine [198, 199]. Similarly, Donnem et al. [200] showed that expression of several vascular endothelial growth factor (VEGF) ligands were related to disease-specific survival, however tumor expression was associated with worse prognosis while stromal expression of the same ligands was associated with better survival in NSCLC patients. Our findings also agree with the body of literature reporting an association between tumor bFGF expression, aggressive clinicopathologic characteristics, and poor prognosis [201-205]. In this

study, tumor bFGF was significantly associated with aggressive histology subtypes, advanced stage, and positive lymph node involvement, in addition to worse RFS. We propose that a delicate balance between tumor-inhibiting and tumor-promoting effects of bFGF exists; while the host stroma controls expression of bFGF, anti-cancer effects are dominant, however when the tumor becomes independent of stromal paracrine factors through the establishment of autocrine bFGF stimulation, poor outcomes are more likely to occur.

We explored this particular pathway as the *in vitro* literature indicated that HGF, c-Met, and bFGF are overexpressed in human EC and EC cell lines compared to normal endometrial tissues. Prognostic EC studies have observed that HGF, c-Met, and bFGF are significantly associated with poor survival in univariate analyses, yet the independent prognostic role of these biomarkers and their interactions were undetermined by these studies. Wagatsuma et al. [120] reported strong IHC expression of c-Met and HGF in Type I EC cases. In models adjusted for stage, differentiation, myometrial invasion, and microvessel count, an independent prognostic role of these biomarkers was not observed, which was similar to our study. By examining interactions between biomarkers, we were able to detect a significant relationship between HGF and bFGF co-expression and survival. This finding is further validated by an EC cell line study which showed that tumor-derived bFGF expression significantly increased the transcription of HGF in EC cells which resulted in an invasive phenotype [111].

We also observed a statistically significant association between tumor HGF and tumor c-Met expression. The biological effects exerted by HGF are mediated by the c-Met receptor which is frequently overexpressed in many human cancers [192]. The most common mechanism of c-Met overexpression is through HGF-dependent autocrine/paracrine mechanisms. Upon HGF binding, c-Met activates a number of cellular responses, including motility or scattering of

epithelial cells, proliferation, and invasion [192]. Despite the importance of HGF in c-Met activation, most prognostic studies focus solely on the aberrant expression of c-Met. In fact, development of HGF antagonists lags behind the surfeit of c-Met inhibitors currently in use in clinical trials [206]. Evaluation of the co-expression of HGF and c-Met may lead to a better understanding of the overall impact this signaling pathway has in cancer prognosis. In this study, c-Met expression did not have a significant and independent effect on survival which may indicate that the presence of c-Met is a necessary but not sufficient factor in EC survival outcomes.

The prevalence of positive biomarker expression in this study differs from previous EC studies. We observed positive HGF and c-Met expression in 15% and 22% of cases compared to 90% and 63% of the tumors in the Wagatsuma series and 100% and 87% of PS EC cases in the Bishop series [120, 121]. The low positive prevalence of biomarkers in our study may reflect differences in commercial antibodies and IHC procedures. Regardless of the prevalence, we were able to identify associations between biomarker expression, clinicopathologic factors, and survival.

The major limitation of this study was the sample size. Despite including 211 EC patients, the number of events and number of patients at risk in each stratum for the multivariable survival models was low. As a result, our observed significant findings may reflect false positive results. Additionally, we did not have a sufficient number of cases to explore biomarker interactions within histology subtypes. Different associations between biomarker expression and survival outcomes may be apparent by histology subtype which we could not assess. The strengths of this study include high quality pathology data, discrimination between tumor and stromal expression

of bFGF, and an evaluation of the prognostic role of this biomarker pathway using multivariable methods.

In addition to validating the results of this study, future examinations of bFGF should examine the mechanisms that may explain the protective role observed with stromal expression. Functional studies that can provide a biological rationale for the associations seen here would be useful in recommending molecularly-targeted therapies. Recent evidence supports a role for therapeutic targeting of the tumor microenvironment. Compared to neoplastic cells which are characterized by genetic mutations, cells of the microenvironment are genetically stable which may provide a more attractive therapeutic target. In summary, this study revealed a significant interaction between HGF and bFGF expression in EC survival outcomes. The cellular localization of bFGF expression modified survival outcomes, which implies that angiogenic pathways may be differentially important in EC prognosis based on localization of expression.

7.6 TABLES

Table 18 Probability of sampling given that tumor blocks were available

Stratum	Type I				Type II				Total
	Stage I	Stage II	Stage III	Stage IV	Stage I	Stage II	Stage III	Stage IV	
n with tumor blocks available	691	65	85	21	62	16	34	29	1,003
n sampled	33	29	37	15	40	9	26	22	211
pr (sampled tumor blocks available)= (sampled/ tumor blocks)	0.0478	0.4462	0.4353	0.7143	0.6452	0.5625	0.7647	0.7586	

Table 19 Demographic and clinical characteristics of the EC sample by histology type

Characteristics N=211	Low-grade Type I n=80		High-grade Type I n=34		Clear cell n=31		Papillary serous n=66		<i>p</i> *
	n [§] (%) [†]	% [‡]							
Mean age (sd)	61 (12)	61 (1.61)	62 (12)	61 (3.56)	67 (11)	67 (1.89)	66 (11)	66 (1.34)	0.02
Race									
White	75 (94)	96	32 (94)	96	28 (90)	90	54 (82)	81	0.05
Non-White	5 (6)	4	2 (6)	4	3 (10)	10	12 (18)	19	
Body Mass Index									
Normal (BMI< 25 kg/m ²)	22 (28)	27	5 (15)	7	9 (30)	29	19 (29)	29	0.19
Overweight (BMI 25-30 kg/m ²)	21 (26)	19	10 (29)	47	11 (35)	35	18 (27)	27	
Obese (BMI> 30 kg/m ²)	37 (46)	54	19 (56)	46	11 (35)	36	29 (44)	44	
Additional cancer primaries									
No	68 (85)	79	27 (79)	73	25 (81)	81	50 (76)	76	0.76
Yes	10 (13)	18	7 (21)	27	6 (19)	19	14 (21)	21	
Unknown	2 (2)	3	0 (0)	0	0 (0)	0	2 (3)	3	
FIGO stage									
Early (I & II)	56 (70)	94	6 (18)	59	21 (68)	72	28 (42)	49	<0.001
Late (III & IV)	24 (30)	6	28 (82)	41	10 (32)	28	38 (58)	51	
Lymph node involvement									
No nodes examined	20 (25)	41	8 (24)	26	11 (35)	35	12 (18)	19	<0.001
Negative	48 (58)	55	12 (35)	52	17 (55)	57	34 (51)	53	
Positive	8 (10)	2	14 (41)	22	3 (10)	8	15 (23)	20	
Unknown	6 (7)	2	0 (0)	0	0 (0)	0	3 (8)	8	
Tumor size									
< 2 cm	11 (14)	19	4 (12)	38	4 (13)	13	9 (14)	15	0.09
> 2 cm	34 (42)	25	20 (59)	46	14 (45)	46	31 (47)	47	
Unknown	35 (44)	57	10 (29)	15	13 (42)	40	26 (39)	38	
Post-operative treatment									
Surgery only	30 (37)	61	4 (12)	39	6 (19)	19	11 (17)	17	<0.001
Surgery + CT	8 (10)	2	12 (35)	16	9 (29)	27	28 (42)	40	
Surgery + RT	38 (48)	36	10 (29)	34	15 (49)	51	13 (20)	21	

Table 19 continued

Surgery + CT + RT	4 (5)	1	8 (24)	11	1	3	14 (21)	21	
§ sample count † proportion or mean in the study sample ‡ proportion or mean in the target population * Adjusted Wald <i>p</i> -value									

Table 20 Positive biomarker expression in the study sample and target population and odds of association between biomarkers

	Positive expression		OR (95% CI)		
	Study Sample (N=211)	Target population (N=1,486)	Tumor c-Met	Tumor bFGF	Stromal bFGF
Tumor HGF	15%	15%	9.83 (2.28, 42.28)*	0.65 (0.16, 2.60)	2.35 (0.53, 10.49)
Tumor c-Met	27%	22%	---	1.00 (0.29, 3.52)	3.38 (0.97, 11.80)
Tumor bFGF	17%	7%	---	---	2.09 (0.63, 6.98)
Stromal bFGF	55%	59%	---	---	---
Unadjusted logistic regression model					
* Adjusted Wald $p=0.002$					

Table 21 Association of biomarker expression with clinicopathologic characteristics

Characteristic	Sample counts	% Positive expression			
		Tumor HGF	Tumor c-Met	Tumor bFGF	Stromal bFGF
Race					
White	189	15	23	7	61
Non-White	22	4	13	8	32
<i>p</i> -value*		0.18	0.45	0.88	0.29
Body Mass Index					
Normal	55	24	33	5	49
Overweight	60	14	28	8	52
Obese	96	10	14	8	67
<i>p</i> -value*		0.57	0.29	0.63	0.43
Histology type					
Low-grade Type I	80	16	19	1	59
High-grade Type I	34	2	35	32	61
Clear cell	31	18	26	32	70
Papillary serous	66	13	37	16	44
<i>p</i> -value*		0.12	0.20	<0.001	0.06
FIGO stage					
Early stage (I & II)	111	15	21	5	59
Late stage (III & IV)	100	14	28	18	59
<i>p</i> -value*		0.97	0.39	0.008	0.99
Lymph node involvement					
None	109	20	22	8	59
Positive	40	6	28	33	70
No nodal exam	51	7	21	2	58
Unknown	11	35	24	0	32
<i>p</i> -value*		0.19	0.92	0.002	0.25
Tumor size					
< 2 cm	28	2	26	2	51
> 2 cm	99	14	19	17	64
Unknown	84	20	22	3	59
<i>p</i> -value*		0.07	0.90	0.14	0.77
* Adjusted Wald <i>p</i> -value					

Table 22 Association between biomarker expression and age

Mean age (standard error*)	Tumor HGF	Tumor c-Met	Tumor bFGF	Stromal bFGF
Negative expression	60 (1.42)	61 (1.50)	61 (1.39)	61 (1.72)
Positive expression	66 (3.50)	61 (2.97)	69 (2.38)	61 (1.93)
<i>p</i> -value	0.14	0.97	0.003	0.92
* Linearized standard error (Taylor series)				

Table 23 Cox proportional hazards models for OS: main effects of HGF, c-Met, tumor bFGF, and stromal bFGF

N=211	Model 1			Model 2	
	deaths/N	HR (95% CI)	p^\dagger	HR (95% CI)	p^\dagger
HGF expression					
Negative	76/180	1.00 (reference)	0.97	1.00 (reference)	0.94
Positive	11/31	0.98 (0.37, 2.62)		1.04 (0.39, 2.76)	
c-Met expression					
Negative	61/155	1.00 (reference)	0.53	1.00 (reference)	0.48
Positive	26/56	0.75 (0.30, 1.86)		0.72 (0.29, 1.79)	
tumor bFGF expression					
Negative	67/176	1.00 (reference)	0.25	1.00 (reference)	0.20
Positive	20/35	1.53 (0.73, 3.21)		1.59 (0.78, 3.24)	
stromal bFGF expression					
Negative	43/95	1.00 (reference)	0.44	1.00 (reference)	0.39
Positive	44/116	0.75 (0.35, 1.58)		0.73 (0.35, 1.51)	
Model 1: Adjusted for stage (early stage, late stage), histology subtype (low-grade type I, high-grade type I, clear cell, papillary serous), and age at diagnosis (continuous, range 34-93 years)					
Model 2: Adjusted for other biomarkers, in addition to Model 1 factors					
Stratified by year of diagnosis					
† Adjusted Wald p -value					

Table 24 Cox proportional hazards model* for OS: interaction between HGF and stromal bFGF

N=211	deaths/N	HR (95% CI)	p^\dagger
HGF and stromal bFGF expression			
			0.02‡
Negative HGF and negative stromal bFGF	36/82	1.00 (Reference)	
Positive HGF and negative stromal bFGF	7/13	2.09 (0.83, 5.25)	0.12
Negative HGF and positive stromal bFGF	40/98	1.00 (0.44, 2.28)	1.00
Positive HGF and positive stromal bFGF	4/18	0.29 (0.06, 1.33)	0.11
c-Met			
Negative	61/155	1.00 (Reference)	0.48
Positive	26/56	0.73 (0.31, 1.72)	
Tumor bFGF			
Negative	67/176	1.00 (Reference)	0.27
Positive	20/35	1.52 (0.72, 3.25)	
* Adjusted for stage (early stage, late stage), histology subtype (low-grade type I, high-grade type I, clear cell, papillary serous), and age at diagnosis (continuous, range 34-93 years)			
Stratified by year of diagnosis (single year, 1996 through 2008)			
† Adjusted Wald p -value			
‡ p -value for interaction			

Table 25 Probability of sampling given that tumor blocks were available in disease-free cases

	Type I				Type II				
Stratum	Stage I	Stage II	Stage III	Stage IV	Stage I	Stage II	Stage III	Stage IV	Total
n with tissue available	691	65	85	21	62	16	34	29	1,003
n sampled	31	25	31	12	33	7	22	13	174
pr (sampled tissue available)= (sampled/tissue available)	0.0448	0.3846	0.3647	0.5714	0.5322	0.4375	0.6470	0.4482	

Table 26 Cox proportional hazards models for RFS: main effects of HGF, c-Met, tumor bFGF, and stromal bFGF

N=174	Model 1		Model 2		
	recurrences/N	HR (95% CI)	p^{\dagger}	HR (95% CI)	p^{\dagger}
HGF expression					
Negative	38/150	1.00 (reference)	0.20	1.00 (reference)	0.21
Positive	4/24	0.40 (0.10, 1.64)		0.44 (0.12, 1.59)	
c-Met expression					
Negative	28/130	1.00 (reference)	0.78	1.00 (reference)	0.68
Positive	14/44	0.90 (0.43, 1.89)		0.84 (0.36, 1.94)	
tumor bFGF expression					
Negative	30/147	1.00 (reference)	0.08	1.00 (reference)	0.10
Positive	12/27	2.55 (0.90, 7.24)		2.54 (0.83, 7.82)	
stromal bFGF expression					
Negative	19/75	1.00 (reference)	0.38	1.00 (reference)	0.44
Positive	23/99	0.68 (0.29, 1.62)		0.70 (0.29, 1.73)	
Model 1: Adjusted for stage (early stage, late stage), histology subtype (low-grade type I, high-grade type I, clear cell, papillary serous), and age at diagnosis (continuous, range 34-93 years)					
Model 2: Adjusted for other biomarkers, in addition to Model 1 factors					
Stratified by year of diagnosis					
† Adjusted Wald p -value					

Table 27 Cox proportional hazards model* for RFS: significant interaction between HGF and tumor bFGF

N=174	recurrences/N	HR (95% CI)	p^{\dagger}
HGF and tumor bFGF expression			
			0.007[‡]
Negative HGF and negative tumor bFGF	29/128	1.00 (Reference)	
Positive HGF and negative tumor bFGF	1/19	0.07 (0.00, 0.81)	0.03
Negative HGF and positive tumor bFGF	9/22	1.56 (0.44, 5.53)	0.49
Positive HGF and positive tumor bFGF	3/5	9.88 (2.63, 37.16)	0.001
c-Met			
Negative	28/130	1.00 (Reference)	0.99
Positive	14/44	1.01 (0.43, 2.33)	
Stromal bFGF			
Negative	19/75	1.00 (Reference)	0.71
Positive	23/99	0.85 (0.35, 2.03)	
* Adjusted for stage (early stage, late stage), histology subtype (low-grade type I, high-grade type I, clear cell, papillary serous), and age at diagnosis (continuous, range 34-93 years)			
Stratified by year of diagnosis (single year, 1996 through 2008)			
† Adjusted Wald p -value			
‡ p -value for interaction			

7.7 FIGURES

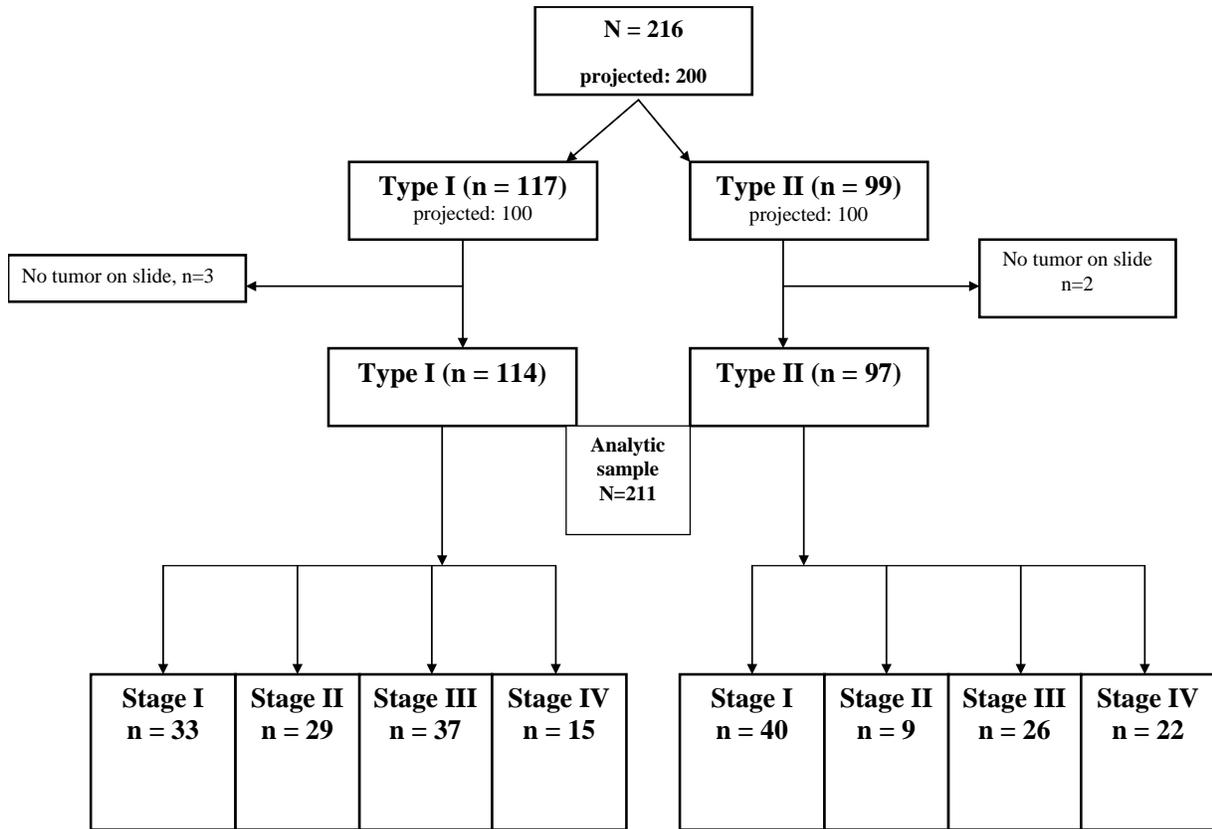
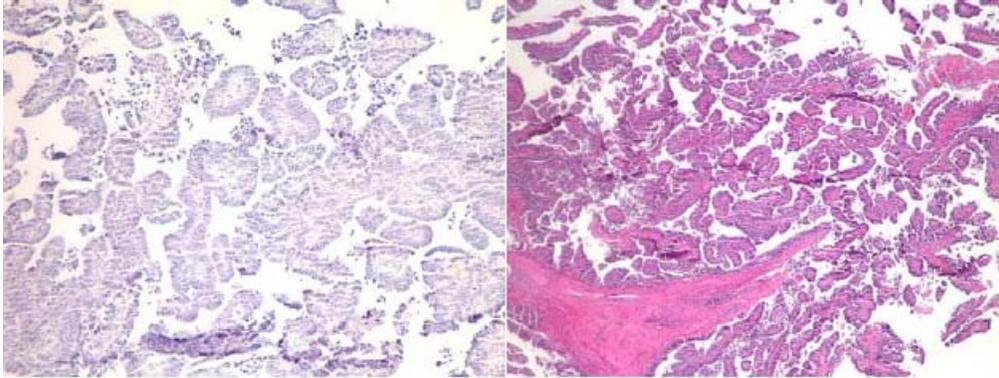
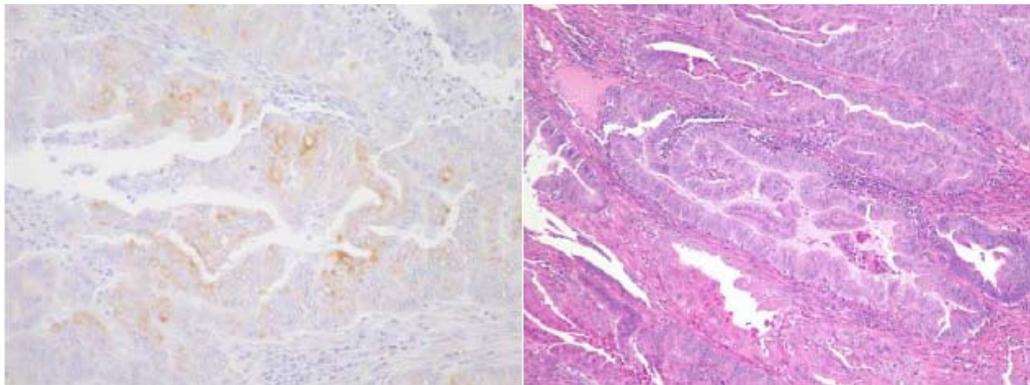


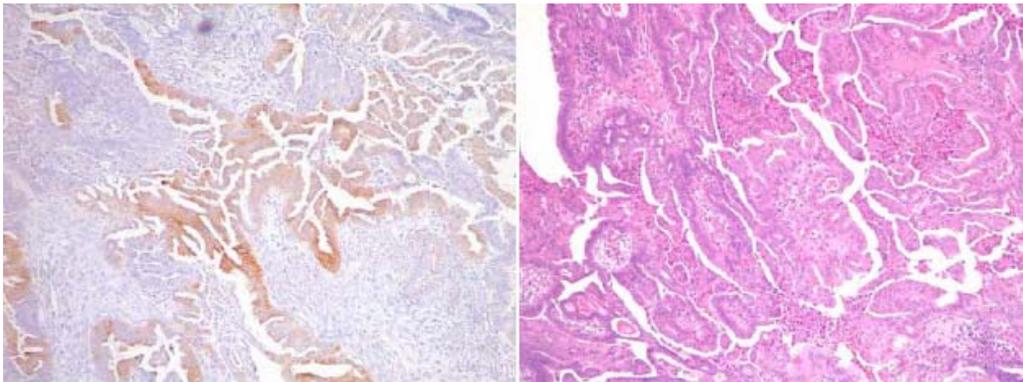
Figure 15 Sampling strategy for HGF/c-Met/bFGF expression study



**Figure 16 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification:
Negative HGF expression**



**Figure 17 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification:
Weak HGF expression**



**Figure 18 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification:
Moderate HGF expression**

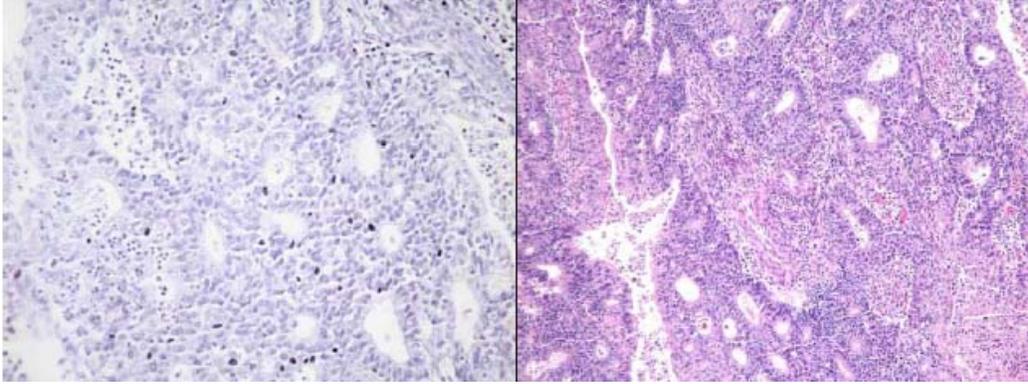


Figure 19 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Negative c-Met expression

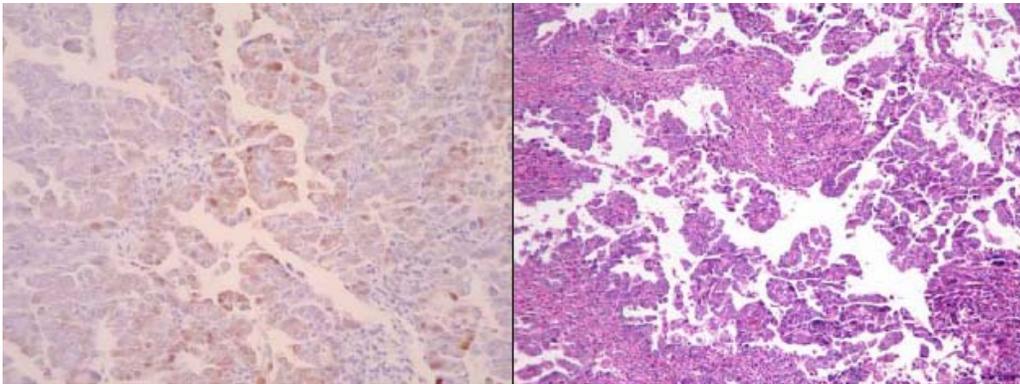


Figure 20 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Weak c-Met expression

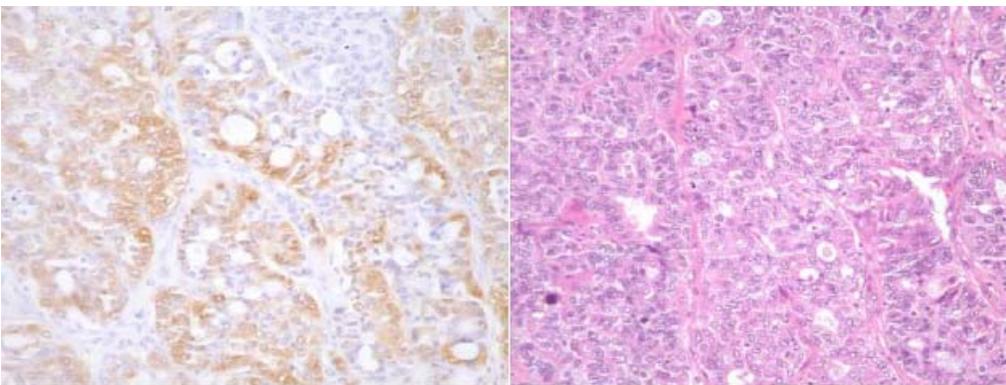
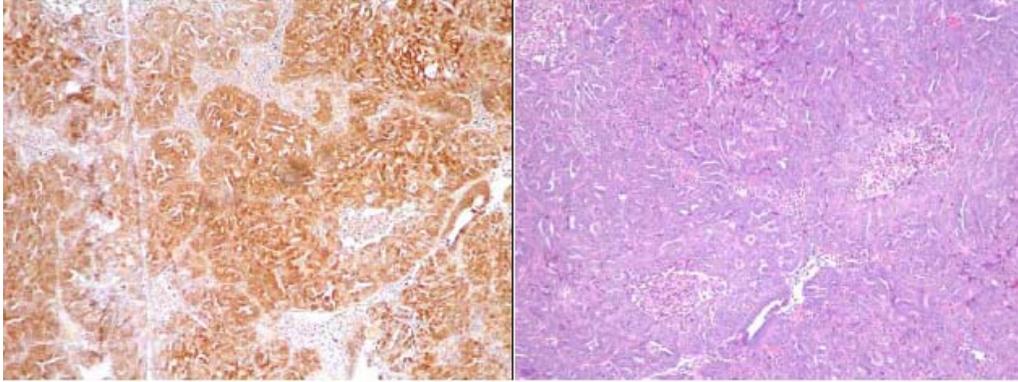
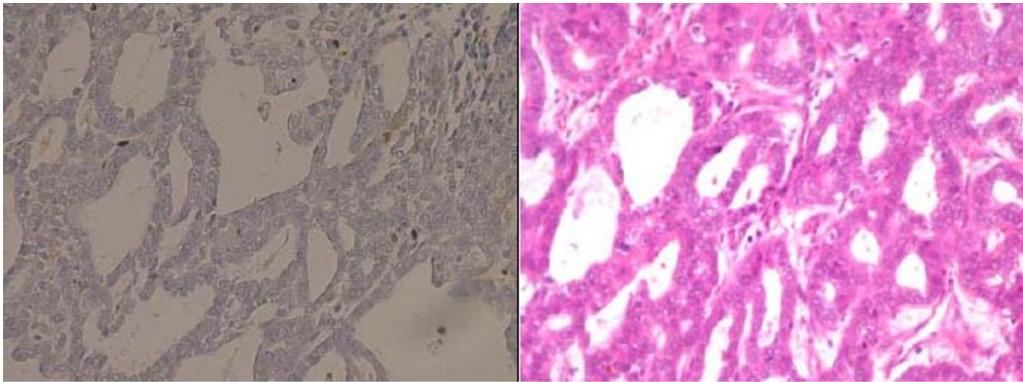


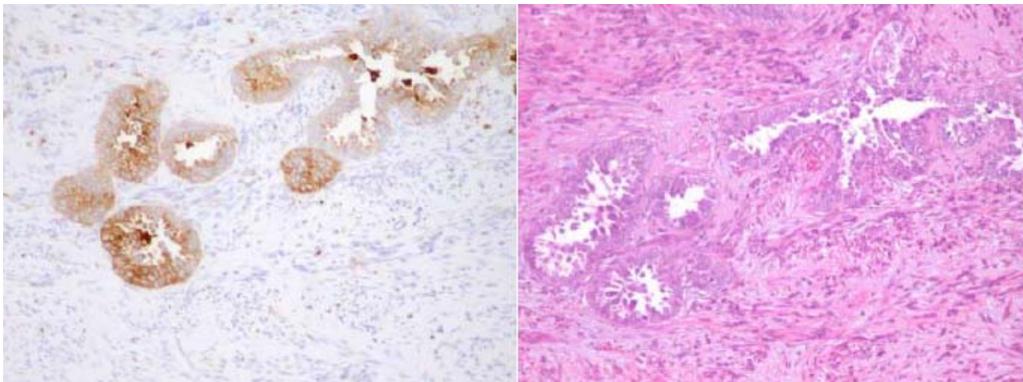
Figure 21 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Moderate c-Met expression



**Figure 22 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification:
Strong c-Met expression**



**Figure 23 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification:
Negative tumor or stromal bFGF expression**



**Figure 24 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification:
Moderate tumor bFGF expression**

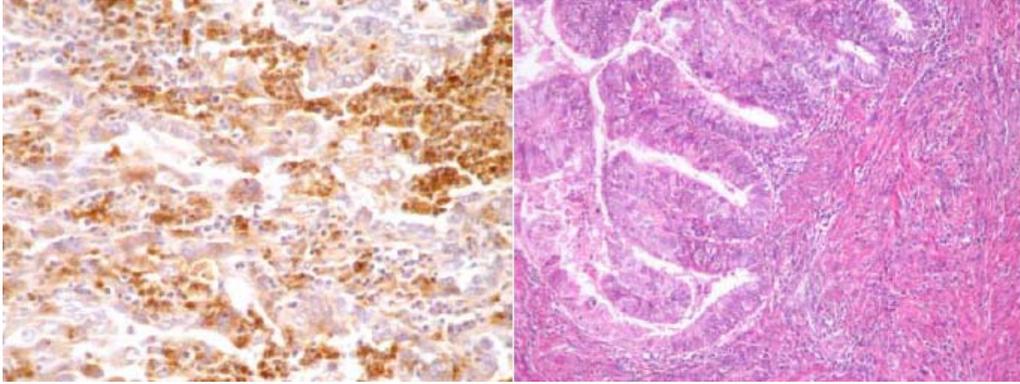


Figure 25 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Strong tumor bFGF expression

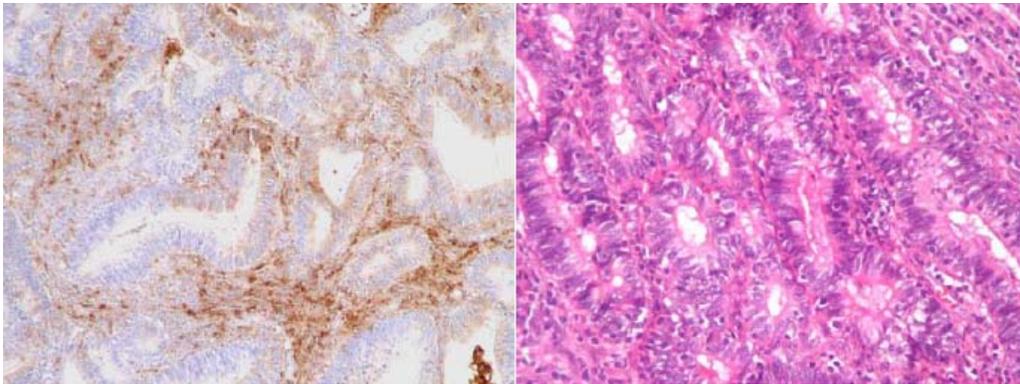


Figure 26 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Weak stromal bFGF expression

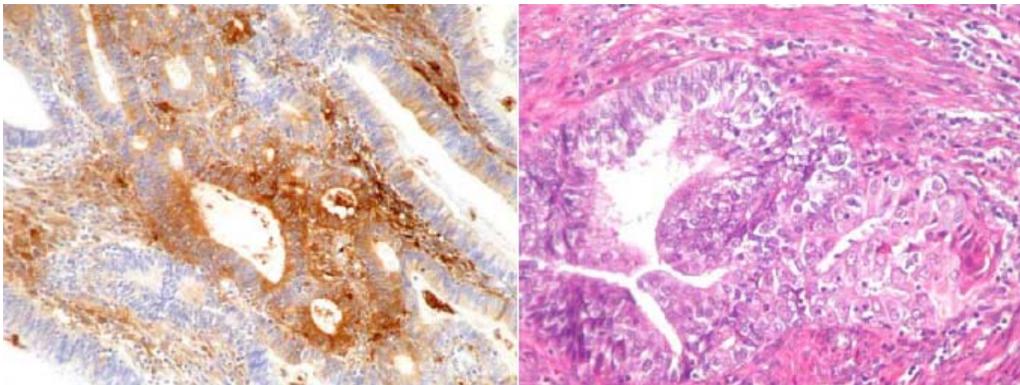


Figure 27 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Moderate stromal bFGF expression

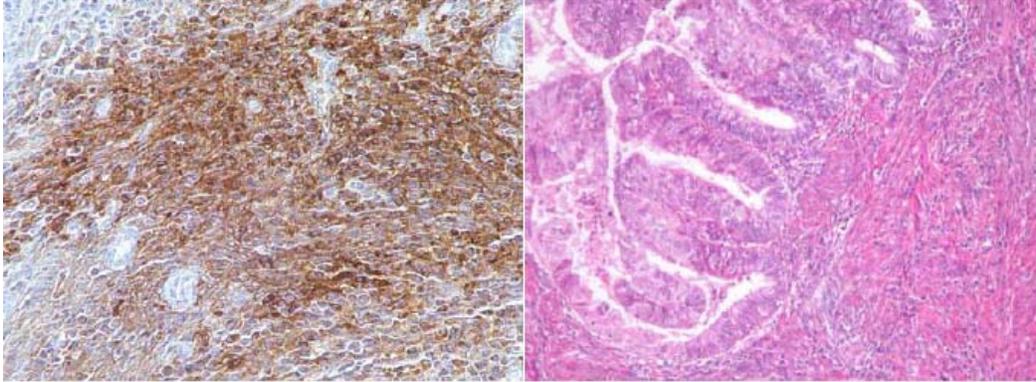


Figure 28 Representative IHC stain and corresponding hematoxylin and eosin stain, 40X magnification: Strong stromal bFGF expression

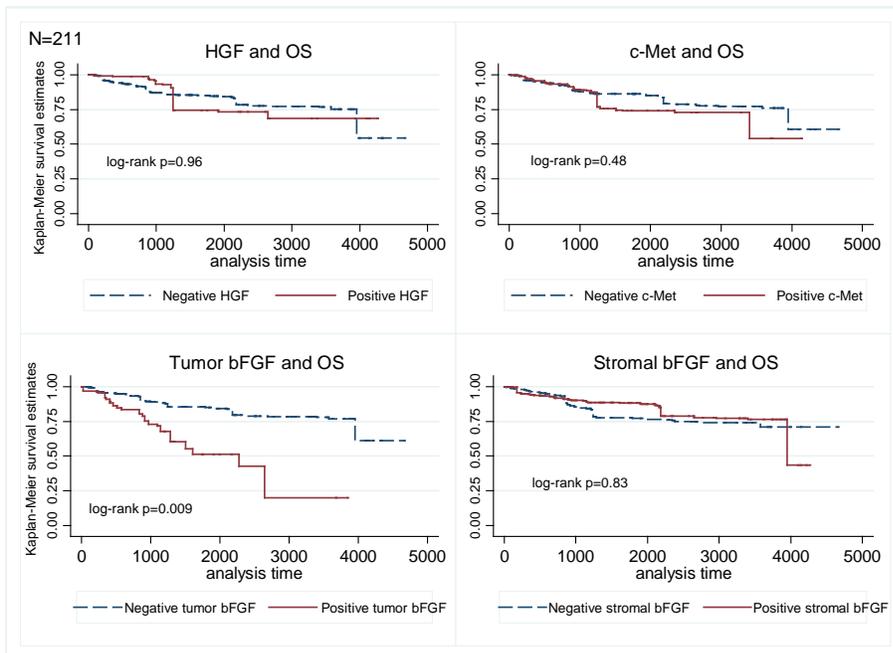


Figure 29 Kaplan-Meier overall survival curves for HGF, c-Met, tumor bFGF, and stromal bFGF

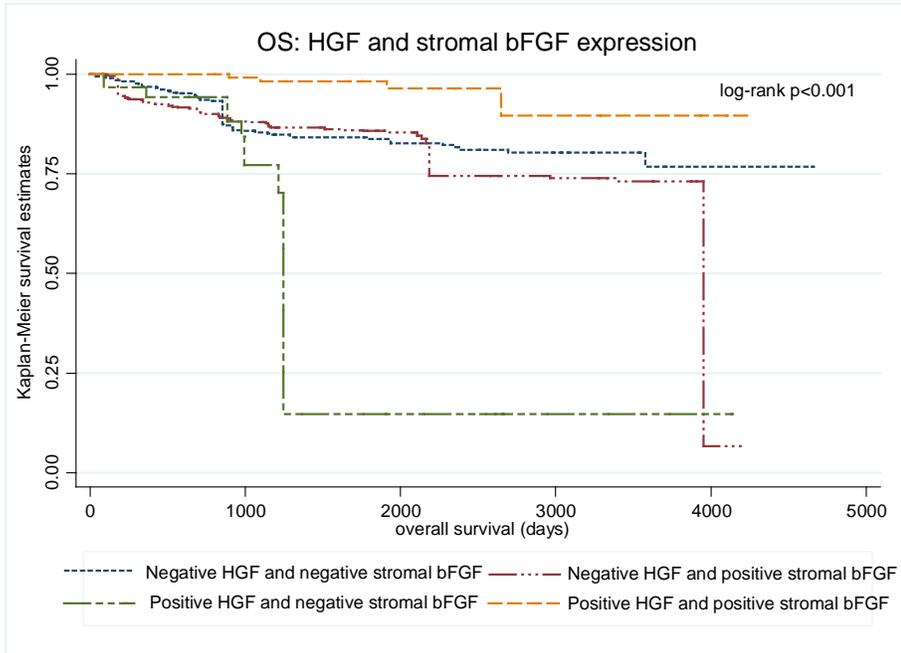


Figure 30 Kaplan-Meier overall survival curves for HGF and stromal bFGF

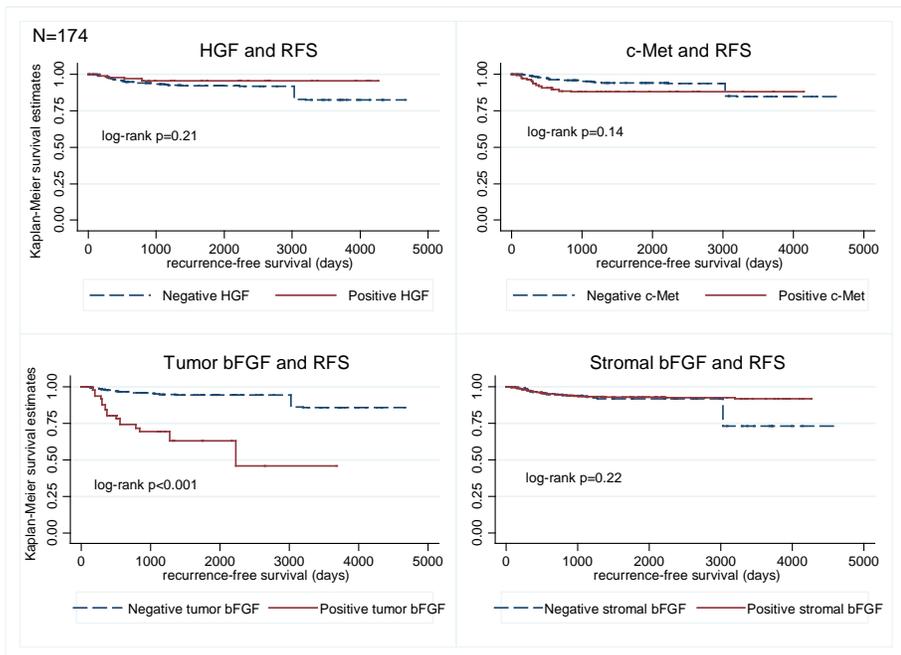


Figure 31 Kaplan-Meier recurrence-free survival curves for HGF, c-Met, tumor bFGF, and stromal bFGF

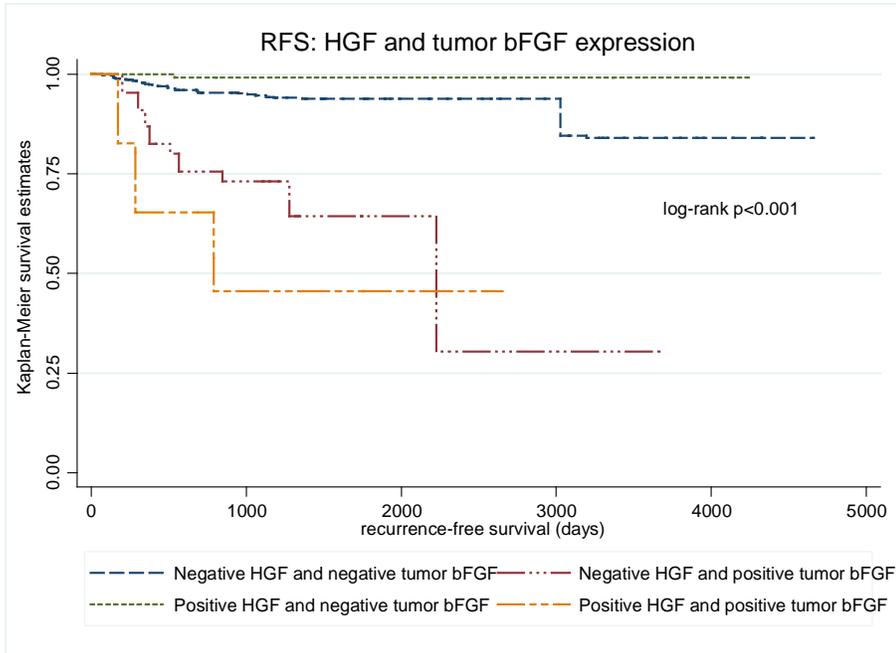


Figure 32 Kaplan-Meier recurrence-free survival curves for HGF and tumor bFGF

7.8 ACKNOWLEDGEMENTS

We wish to thank the University of Pittsburgh Medical Center (UPMC) Registry Information Services (RIS) team, especially Louise Mazur, for her help with procuring the data for this study. We also would like to thank Lindsay Mock (Magee Womens Hospital, Tissue Bank) for procuring the tissue blocks and sectioning slides and Kim Fuhrer (Molecular Anatomic Pathology laboratory) for the immunohistochemical staining of all slides.

8.0 CONCLUSIONS AND PUBLIC HEALTH SIGNIFICANCE

Endometrial cancer (EC) is the most common gynecologic malignancy in the United States. In 2010, 43,470 cases were newly diagnosed and 7,950 deaths occurred. Several histology subtypes of EC are clinically important; low-grade Type I tumors represent approximately 72% of ECs and have an indolent behavior, accounting for only 26% of EC deaths each year [10]. Conversely, high-grade Type I, clear cell (CC), and papillary serous (PS) tumors account for less than 30% of EC diagnoses each year, yet collectively these subtypes are responsible for approximately three-quarters of deaths that occur in EC patients each year [10]. The disproportionate incidence and mortality associated with these subtypes emphasizes the need for better cancer prevention and treatment options.

Invasion, metastasis, and sustained angiogenesis are hallmarks of aggressive cancers [190]. The majority of cancer-related deaths are attributable to metastases of the primary tumor; the ability of cancer cells to complete the metastatic process (i.e. breaking away from the primary tumor, invading the local stromal environment, penetrating lymph or blood vessels, surviving in the circulation, locating new tissues, and adapting to the new microenvironment) is dependent on characteristics of the primary tumor and the tumor microenvironment [207]. In recent years, the tumor microenvironment has come to forefront of cancer investigations which seek to examine the mechanisms by which tumor cells are able to achieve each step of the metastatic cascade [208].

The tumor microenvironment refers to the surrounding non-malignant cells that interact with the primary tumor mass. During carcinogenesis, stromal cells react to the presence of genetically transformed cells by supplying paracrine factors that typically function in wound repair. However, unlike wounds, cancer cells are not self-limiting and respond inappropriately to microenvironmental signals; in fact, cancer cells exploit these signaling pathways to enhance growth, survival, and dissemination of the tumor. Consequently, the tumor microenvironment is an attractive target for therapeutic interventions in cancer control [195].

The goals of this dissertation project were two-fold: the first aim of this dissertation sought to provide an evaluation of risk factors and survival outcomes in a sample of EC patients. Although other studies have described these facets in EC patients, we wanted to confirm these findings and characterize our target population for the subsequent molecular aims of this dissertation. The second goal of this study was to evaluate the role of two stromal-related biomarker pathways, CXCL12/CXCR4 and HGF/c-Met/bFGF, as potential prognostic biomarkers in EC. These pathways have been explored in other common cancers, such as breast, lung, and colon cancers. Furthermore, the *in vitro* and *in vivo* EC literature supported a role for examining the prognostic significance of these biomarkers. We sought to understand the association between biomarkers, the relationship between biomarker expression and clinicopathologic factors (race, BMI, stage, and histology subtype), and the independent association between biomarker expression and survival outcomes in EC patients. The ultimate goal of this dissertation was to add to the body of knowledge regarding the tumor microenvironment and EC progression.

The first aim of this study confirms that estrogen-related characteristics are important characteristics of Type I EC patients. Furthermore, we demonstrated similar survival outcomes

for high-grade Type I and Type II EC cases, despite differing etiologies for these two subtypes. Future directions for research related to this aim include an examination of novel EC risk factors, especially for CC and PS endometrial tumors. Effective cancer prevention strategies rely on an understanding of the putative risk factors involved in carcinogenesis, which is lacking for CC and PS endometrial tumors. Specifically, future studies should explore risk factors unrelated to hormonal carcinogenesis. Chronic inflammation is known to play a role in the carcinogenesis of many human cancers; factors such as infections, infertility, pelvic inflammatory disease, and other benign gynecologic conditions may contribute to an inflammatory local environment and should therefore be explored. Due to the rarity of these tumors, datasets with detailed information regarding exposures and large numbers of cases will be necessary. Moreover, a case-control study design with a non-diseased control population will give a better estimation of risk factors associated with development of Type II tumors. Our study was limited as we compared the probability of developing one subtype of EC as opposed to another EC subtype.

The molecular analyses in the second and third aims of this project revealed interesting associations between biomarker expression, clinical characteristics, and survival outcomes. CXCL12 was an independent prognostic marker for better overall survival (OS) and recurrence-free survival (RFS) in high-grade Type I EC cases, however CXCL12 expression was not associated with prognostic factors of EC, such as stage, lymph node involvement, or histology subtype. We also showed that the CXCL12 receptor, CXCR4, was expressed in all EC tumors. Future studies regarding the role of this pathway in EC should compare a series of tissues including normal endometrium, hyperplasia, and precursor lesions to discern when CXCR4 overexpression occurs in the malignant process. Additionally, the relationship between estrogen receptor and CXCL12 should be explored to elucidate the role of estrogen in this pathway.

CXCL12 is an estrogen-regulated gene in estrogen receptor-positive ovarian and breast cancer cells, indicating a pathway by which estrogen can induce CXCL12 production. Additional studies should examine larger numbers of cases with high-grade Type I, CC, and PS subtypes to perform well powered subgroup analyses.

Interactions between HGF and bFGF expression had significant implications for survival outcomes in EC patients (Aim 3). HGF was significantly associated with OS and RFS, however the cellular localization of bFGF expression was an important effect modifier. Stromal expression of bFGF was associated with better OS, while tumoral expression of bFGF was associated with worse RFS. Additional studies examining the role of HGF, c-Met, and bFGF in EC will need to validate of the results presented in this dissertation. Specifically, functional studies are needed to ascertain the mechanisms related to a positive outcome for stromal bFGF. The eventual goal is to provide high-quality epidemiologic data for the purpose of identifying the subset of biomarkers that are best targeted for patient survival.

The public health implications of the current dissertation project include an enhanced understanding of EC biology and molecular epidemiology, which ultimately can inform prevention efforts, early detection, and treatment strategies. Discovery of additional tumor markers could positively impact public health by improving survival outcomes for patients with aggressive EC.

APPENDIX A: DETAILED INFORMATION REGARDING THE SAMPLING FRAME

A.1 SCHEMATICS FOR SAMPLING FRAME

The molecular aims of this dissertation project required sampling from a larger source population. One thousand nine-hundred and sixty-four cases were diagnosed at Magee-Womens hospital between 1996 and 2008. The target population of interest in this dissertation was Type I and Type II EC cases (N=1,772). In order to generate a relatively homogenous population for study, we applied several exclusion criteria, including overlapping age distributions between Type I and Type II cases, invasive cancers, surgery at Magee-Womens hospital, and known stage, resulting in a sampling frame of 1,486 cases (Figure 33). Characteristics of this frame are shown in Table 28. The distributions shown here are relatively similar to the distributions reported in the first aim of this dissertation.

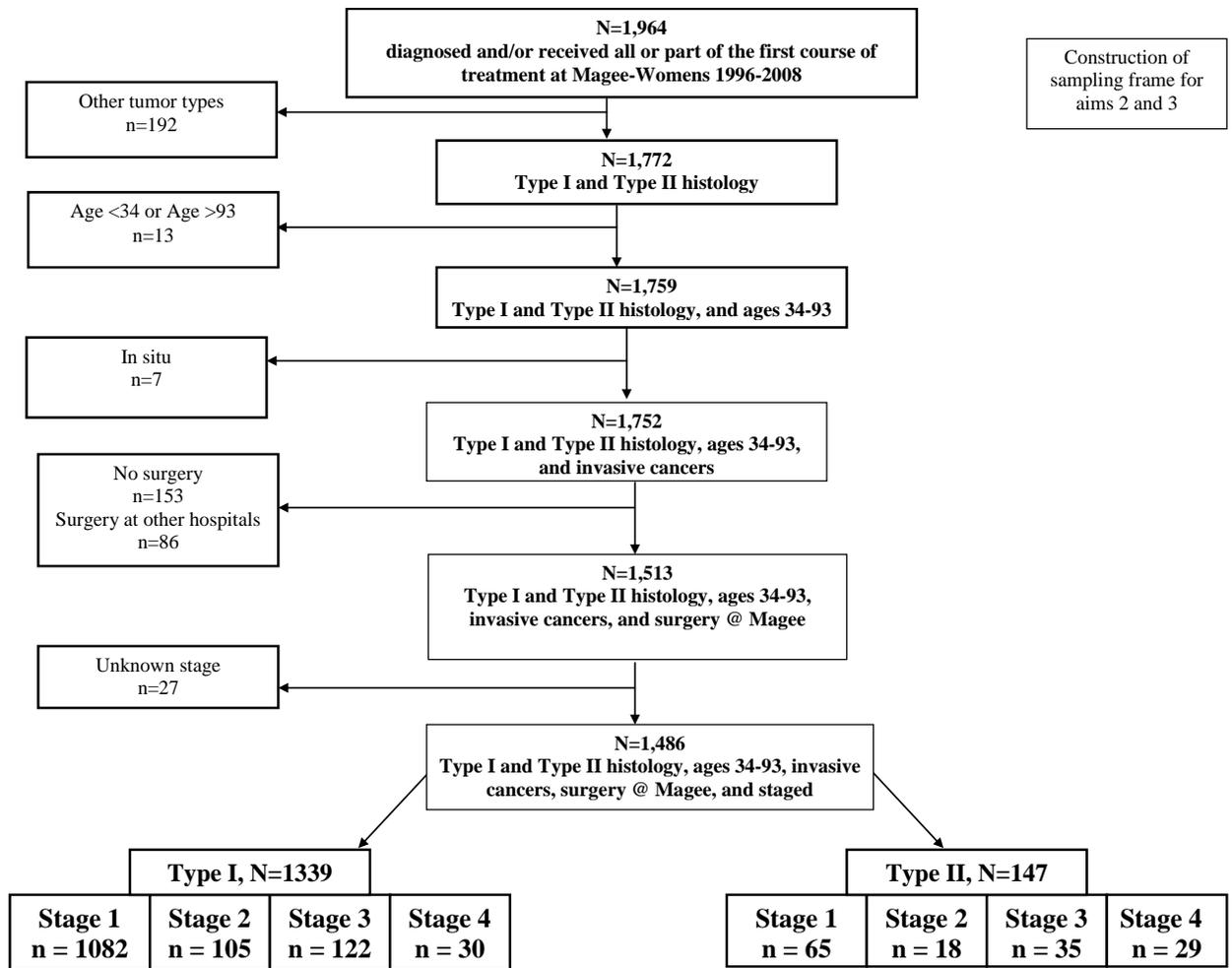


Figure 33 Construction of sampling frame, N=1,486

Table 28 Demographic and clinical characteristics of the sampling frame

Characteristics	N=1,486
Mean age (sd)	61 (12)
Race	N (%)
White	1421 (96)
Non-White	65 (4)
Body Mass Index	
Normal (BMI< 25 kg/m ²)	259 (17)
Overweight (BMI 25-30 kg/m ²)	324 (22)
Obese (BMI> 30 kg/m ²)	826 (55)
Unknown	77 (5)
Additional cancer primaries	
No	1247 (83)
Yes	222 (15)
Unknown	17 (1)
Histology type	
Low-grade Type I	1133 (76)
High-grade Type I	206 (14)
Type II	147 (10)
FIGO stage	
Early (I & II)	1270 (85)
Late (III & IV)	216 (15)
Lymph node involvement	
No nodes examined	462 (31)
Negative	857 (58)
Positive	109 (7)
Unknown	58 (4)
Tumor size	
< 2 cm	179 (12)
> 2 cm	458 (31)
Unknown	849 (57)
Therapy	
Surgery only	706 (47)
CT only	107 (7)
RT only	599 (40)
CT and RT	74 (5)

A.2 DIFFERENCES BETWEEN PATIENTS WITH AND WITHOUT TISSUE AVAILABLE FOR INCLUSION IN THE MOLECULAR STUDIES

Among the cases included in the sampling frame (N=1,486), 67% of cases (n=1,003) were indicated as having tissue blocks available for use in the molecular studies (Figure 34). The numbers in red indicate the number of cases included from each stratum. Table 29 shows the characteristics of patients with presumed tumor block unavailability (N=483) and patients with presumed tumor block availability (N=1,003). Patients with potential available tissue blocks were slightly older (61 vs. 60 years), more likely to have stage III and IV tumors (17% vs. 10%), more likely to have Type II tumors (14% vs. 1%), and be diagnosed at a hospital other than Magee-Womens Hospital (50% vs. 42%) compared to patients without tumor blocks available. The unavailability of tissue might reflect depletion of specimens. As these cases patients differ with respect to several major characteristics, selection bias is a potential threat to the internal and external validity of the study.

Table 30 shows the selection of the study sample (N=216) from the larger sampling frame (N=1,486) by histology subtype and stage categories, the variables used to construct the discrete sampling strata. A random number procedure in SAS was used to select a random sample of 252 cases. Although our goal was to include 200 cases in the molecular studies, we provided the tissue bank with 52 additional case identifiers to substitute for potential “blanks.” Blanks are cases thought to have available tumor blocks, however upon request of the tissue, no specimens were located. Of the 252 cases in our scientific random sample, 204 were located by the tissue bank. An additional 12 cases not included in the original random sample were provided by the Pathology department. Although these cases were not part of the sampling design, we assumed

that inclusion of these additional cases would not severely bias the internal validity of this study, resulting in a sample of 216 cases.

Once slide sectioning of the 216 cases was completed, additional cases were excluded from the molecular analyses of aims 2 and 3. In Aims 2 and 3, respectively, 17 and 5 cases did not have evaluable tumor present on the slides, resulting in sample sizes of 199 and 211.

The subject-specific predicted probability of having tissue available for this study was estimated after running the logistic regression model in Table 31 in order to adjust for characteristics associated with selection bias. Figure 35 shows the distribution of the predicted probabilities according to the eight sampling strata. The narrow intervals of the predicted probabilities show that within the strata, cases have a similar probability of having tissue available for the study. Unmeasured factors do not appear to be contributing to the propensity that tumor blocks were available.

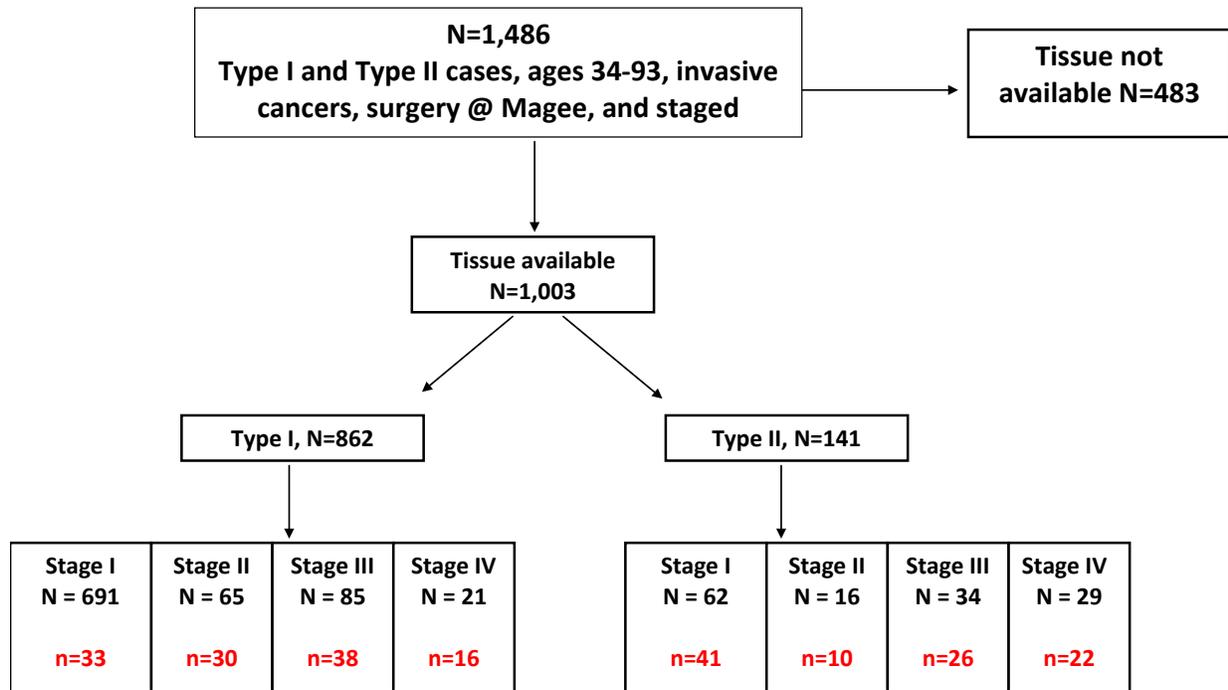


Figure 34 Reduced sampling frame based on availability of tissue, N=1,003

Table 29 Characteristics of cases in the sampling frame with tissue available compared to those without available tissue, N=1,486

	Presumed unavailable (N=483)	Presumed available (N=1003)	<i>p</i>
Age, median (range)	60 (34-91)	61 (34-93)	0.05*
BMI			
Underweight	26 (5)	67 (7)	0.31†
Normal weight	74 (15)	172 (17)	
Overweight	99 (21)	225 (22)	
Obese	284 (59)	539 (54)	
Stage			
I	394 (82)	753 (75)	0.002‡
II	42 (9)	81 (8)	
III	38 (8)	119 (12)	
IV	9 (2)	50 (5)	
Histology type			
Type I	477 (99)	862 (86)	<0.001†
Type II	6 (1)	141 (14)	
Type of surgery			
Local tumor destruction	0 (0)	1 (0.1)	0.04‡
Local tumor excision	0 (0)	2 (0.2)	
Subtotal hysterectomy	0 (0)	5 (0.5)	
Total hysterectomy w/o removal of ovaries	13 (3)	17 (2)	
Total hysterectomy with removal of ovaries	395 (82)	855 (85)	
Modified radical hysterectomy	56 (11)	94 (9)	
Hysterectomy, NOS	19 (4)	21 (2)	
Pelvic exenteration	0 (0)	1 (0.1)	
Surgery, NOS	0 (0)	7 (0.7)	
Race			
White	464 (96)	957 (95)	0.62†
Non-white	19 (4)	45 (5)	
Class of case			
Diagnosed and treated at Magee-Womens Hospital	280 (58)	497 (50)	0.002†
Diagnosed elsewhere and treated at Magee-Womens Hospital	203 (42)	506 (50)	
* Wilcoxon test <i>p</i> -value			
† Wald test <i>p</i> -value			
‡ Fishers exact test <i>p</i> -value			

Table 30 Selection of the study sample (N=216) from the larger sampling frame (N=1,486) by histology subtype and stage

	Stratum								ALL
	Type I				Type II				
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 1	Stage 2	Stage 3	Stage 4	
Eligible	1082	105	122	30	65	18	35	29	1,486
Eligible, tumor blocks assumed to be available	691	65	85	21	62	16	34	29	1,003
Scientific random sample	33	36	33	21	50	16	34	29	252
Blocks received from scientific sample	31	30	28	16	41	10	26	22	204
Blanks in sample frame, N	2	6	5	5	9	6	8	7	48
Blanks in sample frame, P	0.06	0.20	0.18	0.31	0.22	0.60	0.31	0.32	0.24
Blocks received not in random sample	2	0	10	0	0	0	0	0	12
Total blocks received	33	30	38	16	41	10	26	22	216
Aim 2: not missing IHC score	30	28	36	16	36	9	23	21	199
Aim 3: not missing IHC score	33	29	37	15	40	9	26	22	211

Table 31 Multivariable logistic regression model to predict available tumor block (N=1,003) vs. no available tumor block (N=483)

Variable	OR (95% CI)
Histology type	
Type I	1.00
Type II	11.25 (3.50 36.17)
Stage	
Stage I	1.00
Stage II	0.88 (0.58, 1.33)
Stage III	1.30 (0.86, 1.95)
Stage IV	1.33 (0.60, 2.94)
Type * Stage interactions	
Type II, Stage II	0.39 (0.06, 2.68)
Type II, Stage III	1.28 (0.12, 13.32)
Type II, Stage IV	>999.99 (<0.001, >999.99)
Place of diagnosis	
Diagnosed at other facility	1.00
Diagnosed at Magee	0.71 (0.57, 0.89)
Age at diagnosis	1.00 (1.00, 1.01)

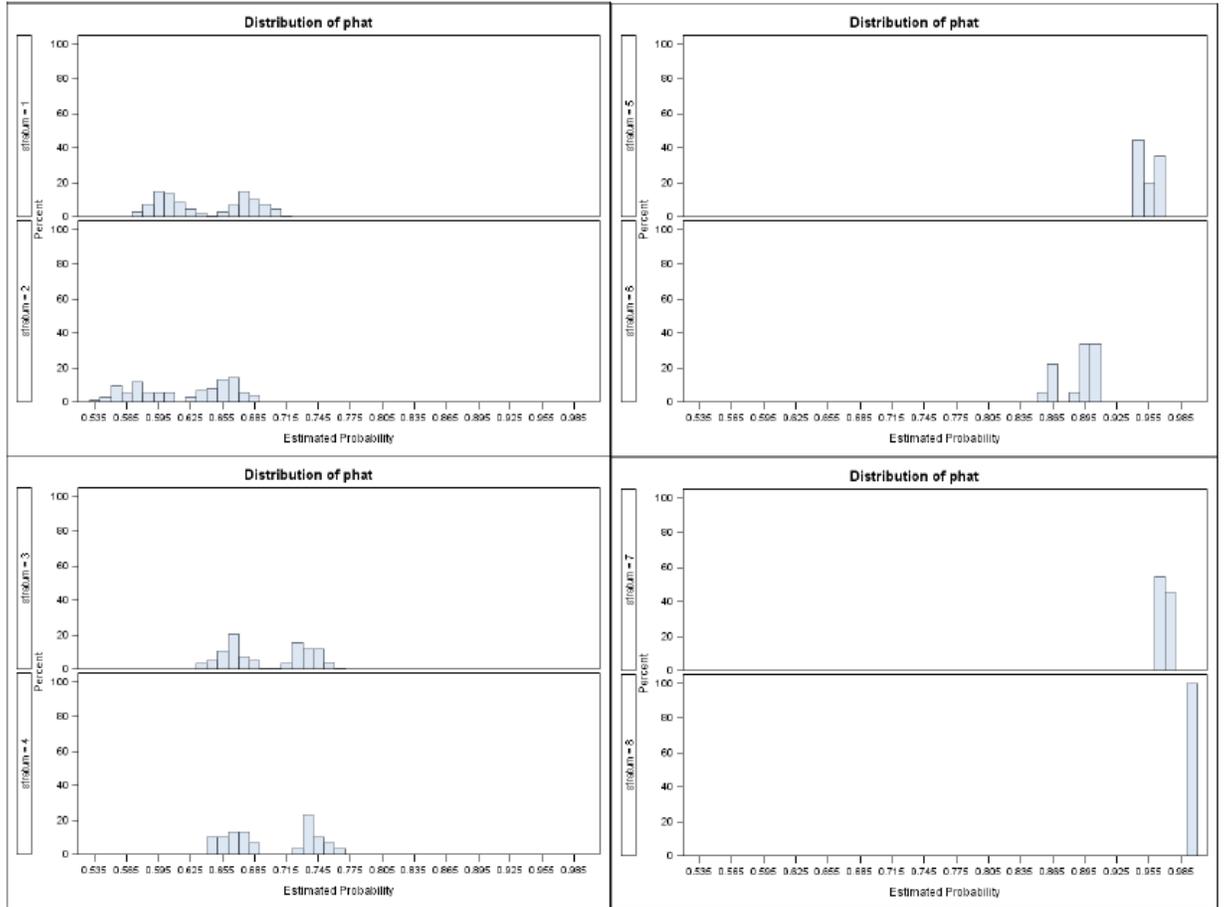


Figure 35 Distribution of predicted probability of tissue availability by sampling stratum

APPENDIX B: ASSOCIATION BETWEEN BIOMARKER EXPRESSION AND YEAR OF DIAGNOSIS

OF DIAGNOSIS

As this was a longitudinal study, we examined the association between biomarker expression and year of diagnosis. Figures 36 and 37 show the proportion of positive expression in each year included in the study for the two biomarker aims. We did not observe any statistically significant trends between year of diagnosis and CXCR4 (Adjusted Wald $p=0.19$), CXCL12 (Adjusted Wald $p=0.88$), HGF (Adjusted Wald $p=0.41$), c-Met (Adjusted Wald $p=0.06$), tumor bFGF expression (Adjusted Wald $p=0.27$), or stromal bFGF expression (Adjusted Wald $p=0.80$).

As mentioned in the methods section of Aim 2 and Aim 3, Cox regression models stratified by year of diagnosis were employed to control for this variable without estimating coefficients.

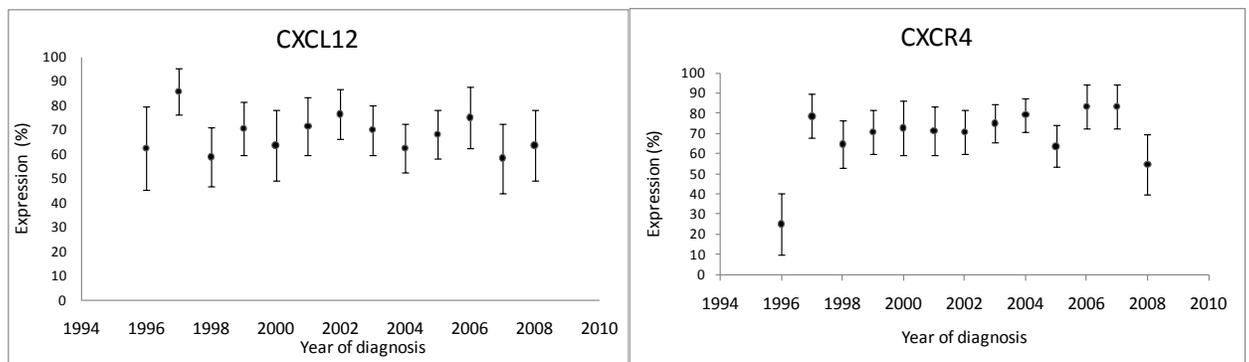


Figure 36 Expression of CXCL12 and CXCR4 by year of diagnosis

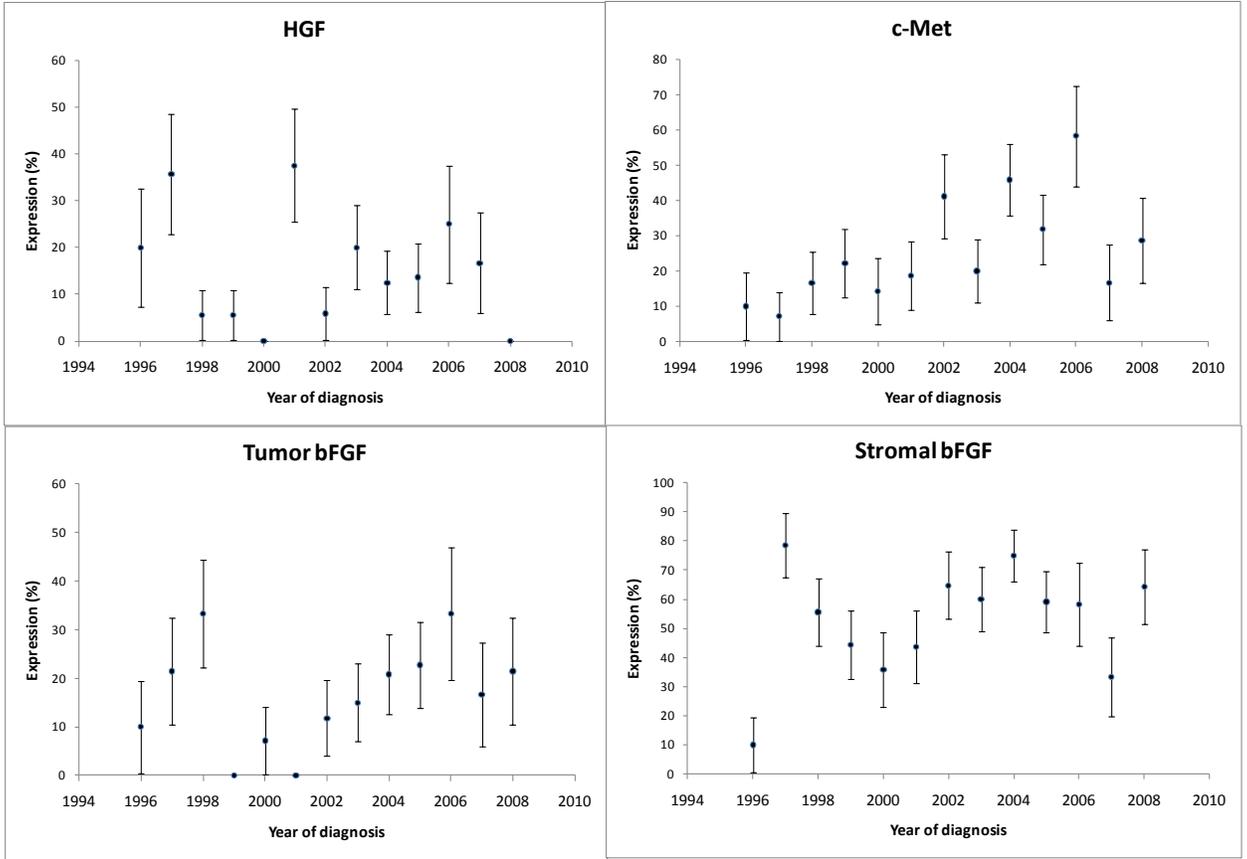


Figure 37 Expression of HGF, c-Met, tumor bFGF, and stromal bFGF expression and year of diagnosis

APPENDIX C: SUPPLEMENTARY DETAILS FOR ARTICLE 3

C.1 DIAGNOSTICS FOR THE COX PROPORTIONAL HAZARDS MODEL

The proportional hazards assumption was examined using the postestimation *stphplot* command in STATA. The resultant log-log survival graph plots an estimate of the log-log survival vs. $\ln(t)$ for each level of the covariate [209]. If the proportional hazards assumption holds, the lines for each level of a covariate should be roughly parallel. There is some evidence that the proportional hazards assumption for CXCL12 in the OS model does not hold, however the model tested by these curves does not account for the interaction between CXCL12 and histology subtype (Figure 38). Similarly, in the RFS model there is evidence that the curves for negative and positive CXCL12 expression overlap (Figure 39).

A limitation of using survey procedures (i.e. *svy*) in the setting of Cox regression modeling is the inability to obtain the Schoenfeld residuals which prevents the use of the analytic Grambsch and Therneau test. This test is useful because it investigates whether that a plot of the scaled Schoenfeld residuals vs. time has a slope of zero and reports an objective p -value based on this test.

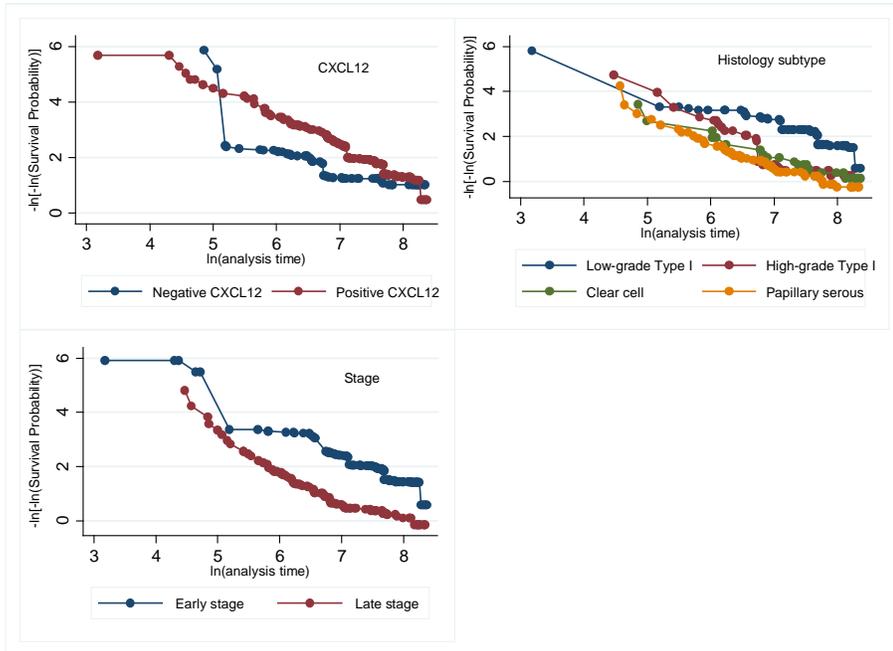


Figure 38 Tests of the proportional-hazards assumption for variables in Table 13

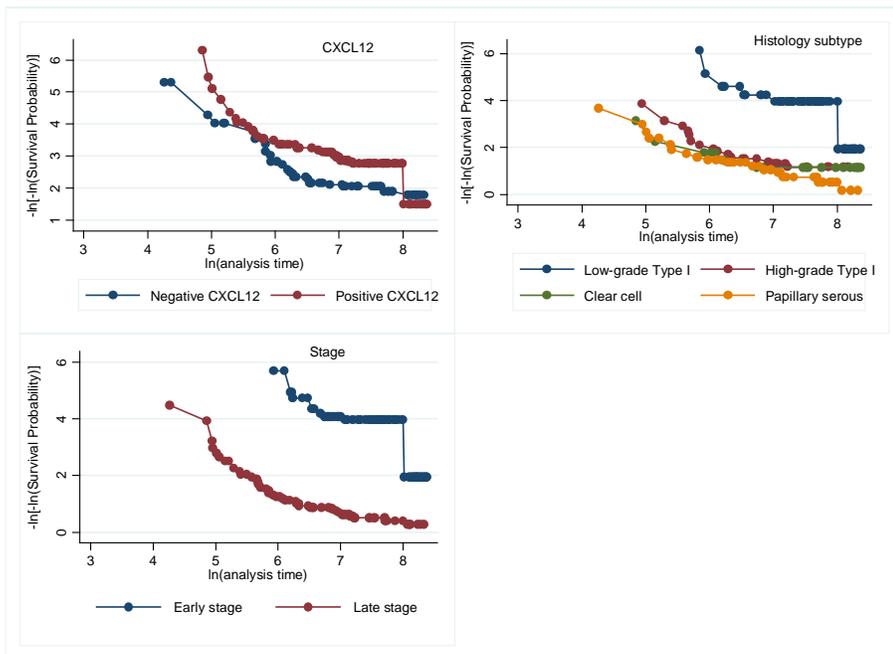


Figure 39 Tests of the proportional-hazards assumption for variables in Table 16

C.2 POWER CALCULATIONS

The statistical power to detect an association between CXCL12 expression and the survival outcomes (OS and RFS) was calculated using the *stpower* command in STATA. In this sample, the probability of death was 0.43 (86/199) and the probability of recurrence was 0.26 (42/163). Using an alpha of 0.05 and the log hazard ratio from the main effects model, the power to detect an association in the OS model was 30% and in the RFS model the statistical power was 52%.

C.3 PROGNOSTIC ROLE OF CXCL12/CXCR4 IN SUBGROUPS OF EC CASES

Kaplan-Meier curves and log-rank tests comparing negative vs. positive CXCL12 expression in subgroups of cases is shown in Figures 40-41 (OS) and Figures 42-43 (RFS). Positive CXCL12 expression was associated with better OS in high-grade Type I cases ($p=0.01$) and better RFS in late stage cases ($p=0.04$). Similarly, we compared moderate and strong CXCR4 expression in subgroups of EC cases (OS: Figures 44-45, RFS: Figures 46-47). No significant associations between CXCR4 and survival outcomes were noted in these subgroups.

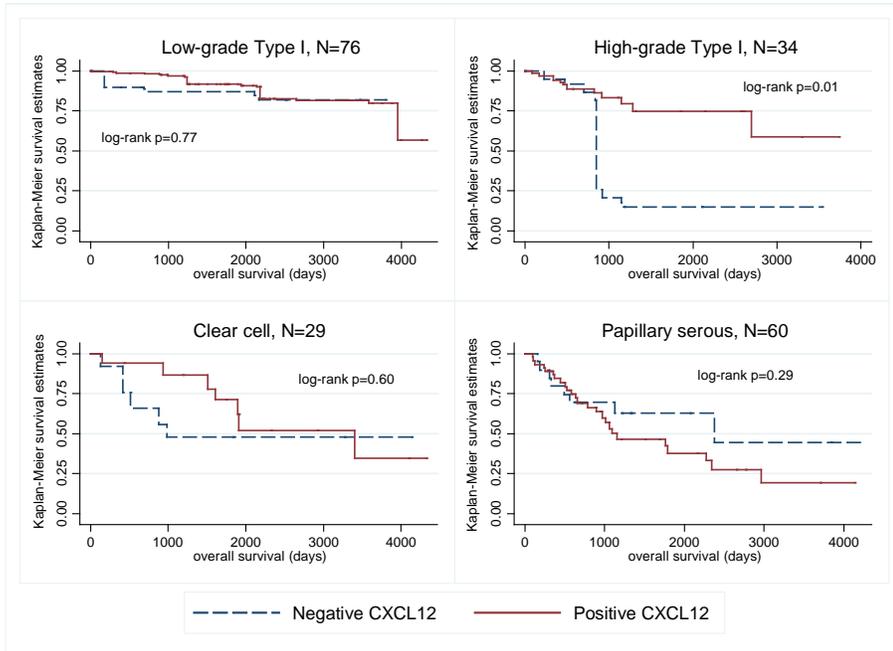


Figure 40 Association between CXCL12 and OS by histology subtype

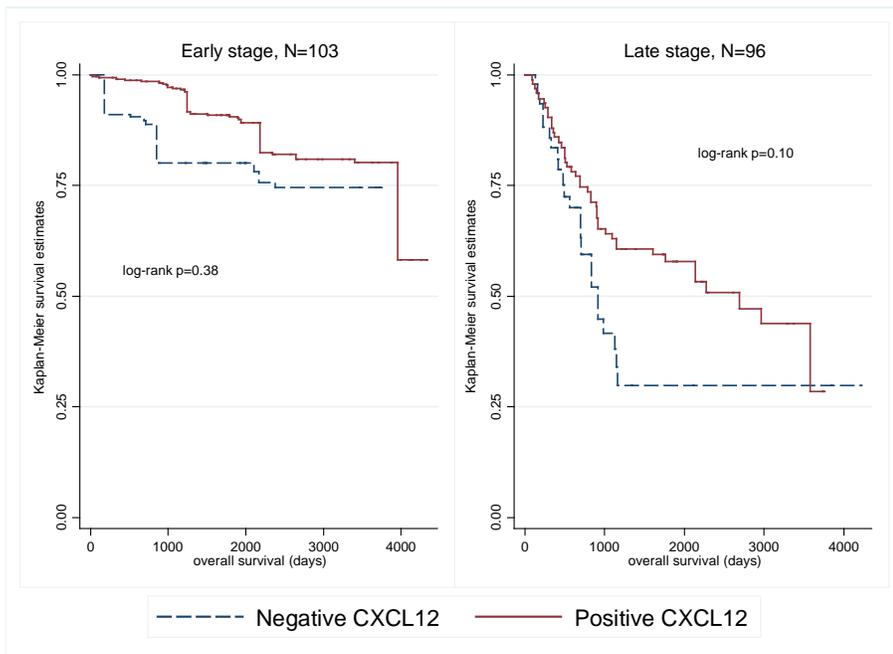


Figure 41 Association between CXCL12 and OS by stage

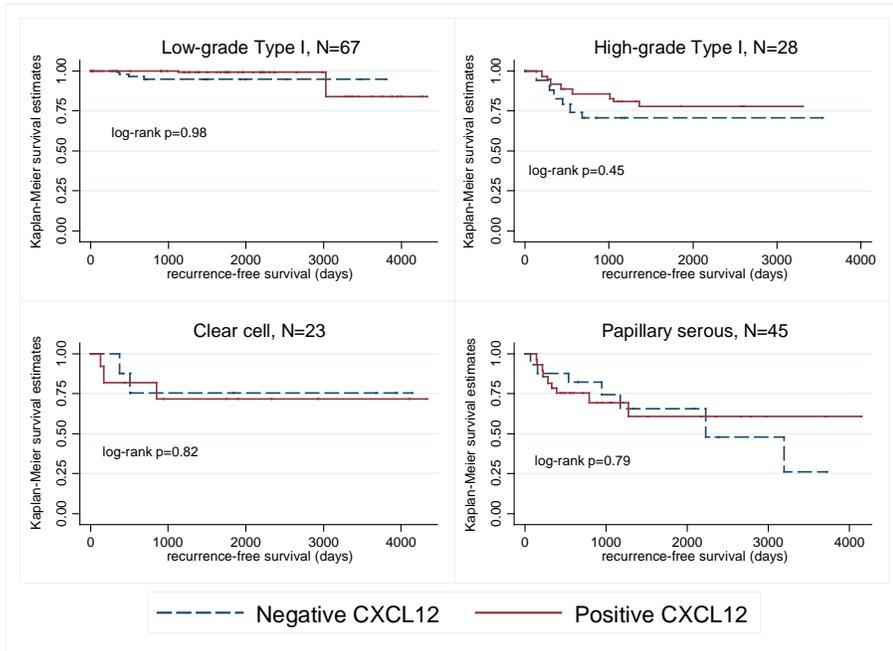


Figure 42 Association between CXCL12 and RFS by histology subtype

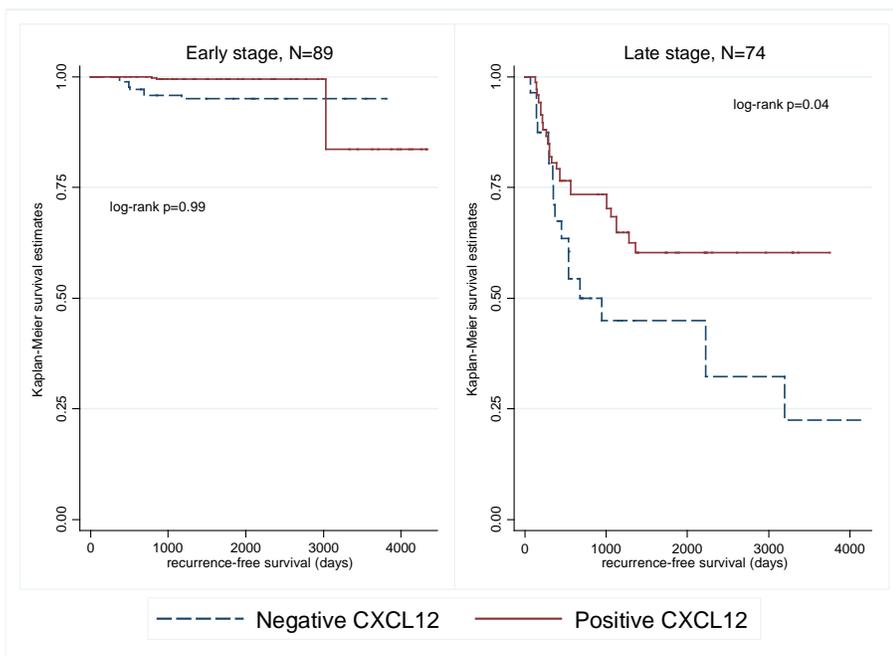


Figure 43 Association between CXCL12 and RFS by stage

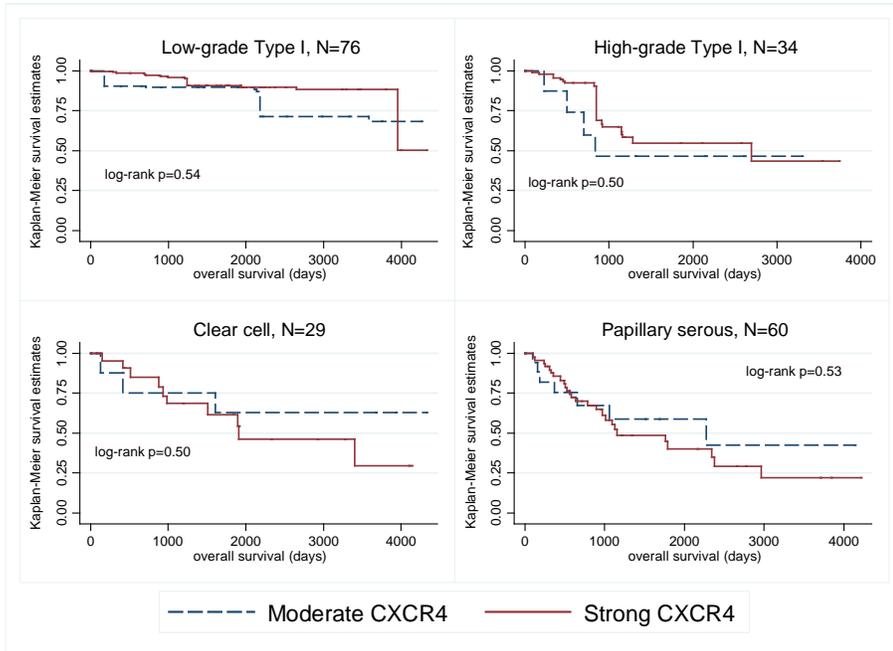


Figure 44 Association between CXCR4 and OS by histology subtype

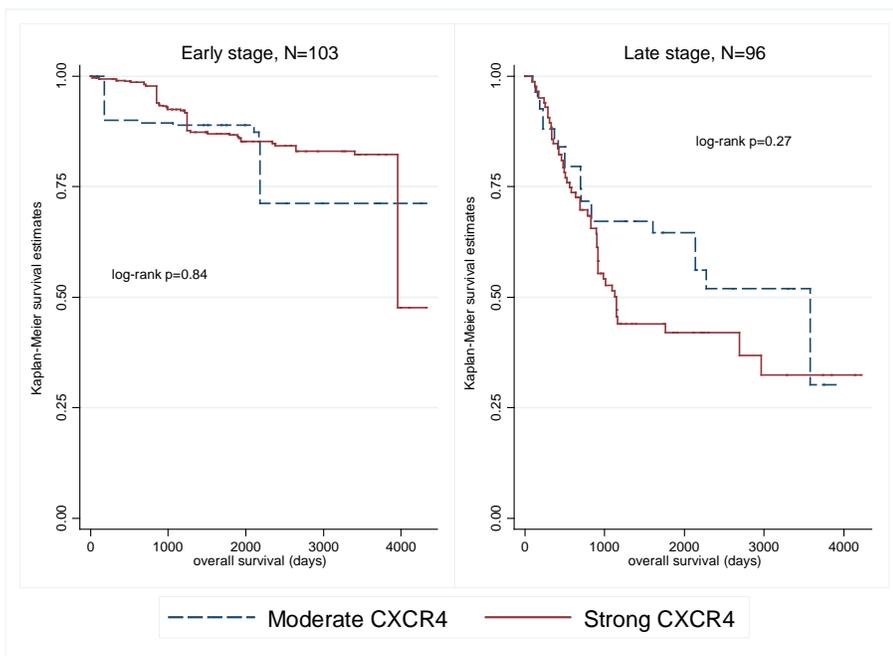


Figure 45 Association between CXCR4 and OS by stage

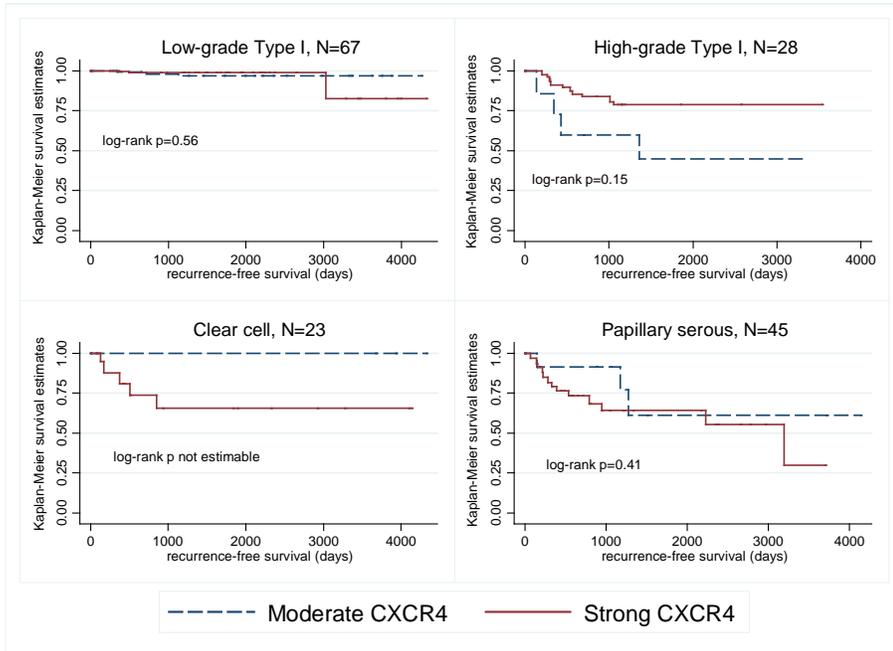


Figure 46 Association between CXCR4 and RFS by histology subtype

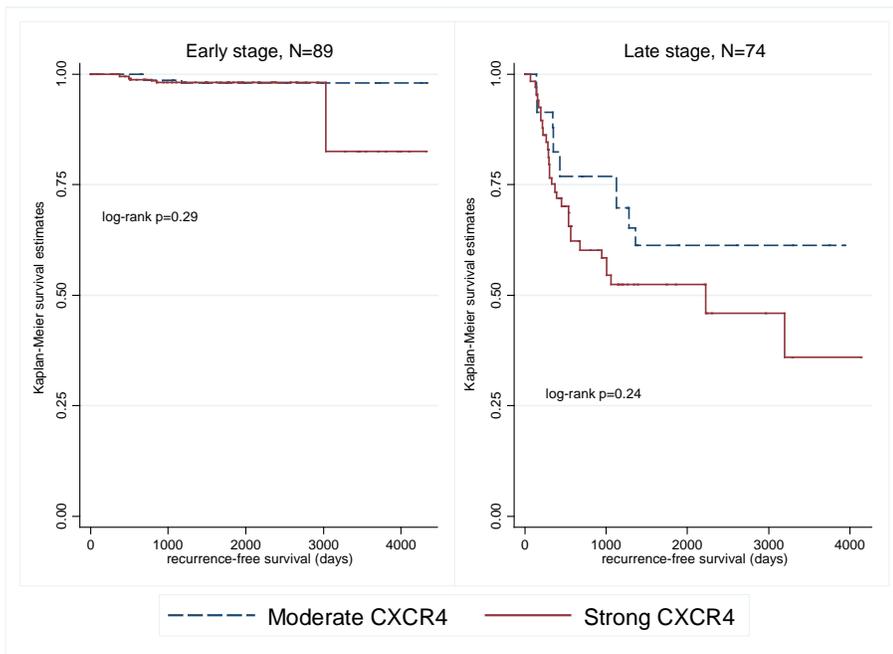


Figure 47 Association between CXCR4 and RFS by stage

C.4 ASSOCIATION BETWEEN CXCL12/CXCR4 EXPRESSION AND METASTASIS

As the CXCR4/CXCL12 pathway is thought to influence patient survival through directional movement of cancer cells, the association between CXCL12 and CXCR4 expression at the primary tumor site and distant metastasis was examined. Stage IV EC cases were classified as distant metastatic cases while stage I-III cases were classified as non-distant metastatic cases. Neither CXCL12 ($p=0.17$) nor CXCR4 expression ($p=0.97$) was significantly associated with distant metastasis (Table 32). Furthermore, a logistic regression model with distant metastasis as the outcome revealed that neither CXCL12 nor CXCR4 significantly predicted the odds of metastasis adjusted for histology type and age (Table 33).

Table 32 Association between distant metastasis, CXCL12, and CXCR4 expression

N=199	No metastasis (N=162)		Metastasis (N=37)		p^{\ddagger}
	n (%) [*]	% [†]	n (%) [*]	% [†]	
CXCR4					
Moderate	48 (30)	31	11 (30)	31	0.97
Strong	114 (70)	69	26 (70)	69	
CXCL12					
Negative	46 (28)	32	17 (46)	47	0.17
Positive	116 (72)	68	20 (54)	53	
[*] proportion in the study sample [†] weighted proportion [‡] Adjusted Wald p -value					

Table 33 Multivariable logistic regression model to predict metastasis (N=37) vs. no metastasis (N=162)

Variable	Adjusted OR (95% CI)	Wald <i>p</i>-value
CXCL12		
Negative	1.00	0.20
Positive	0.52 (0.29, 0.91)	
CXCR4		
Moderate	1.00	0.32
Strong	0.63 (0.25, 1.57)	
Histology type		
Low-grade Type I	1.00	<0.001
High-grade Type I	20.73 (5.06, 884.92)	
Clear cell	39.19 (11.34, 135.51)	
Papillary serous	55.82 (20.59, 151.38)	
Age at diagnosis	0.96 (0.93, 1.00)	0.06

C.5 AT-RISK POPULATION FOR RECURRENCE

Only cases known to be disease-free following the primary surgery were included in analyses of RFS. The following tables provide information on the type of first recurrence in the sample as a whole and by stage. In the total sample, 61% of patients were disease-free following primary surgery and did not have a recurrence over the follow-up period, while 22% of cases that entered a state of remission following surgery recurred over the follow-up (Table 34). The remaining 17% of cases were either never disease-free or it was unknown if they entered a state of remission after the primary surgery and were therefore excluded from RFS analyses. Furthermore, two early stage cases reportedly had a local recurrence of an invasive tumor (Table 35) despite having a total hysterectomy with removal of both ovaries; these two cases were excluded as it was deemed implausible that patients without an intact uterus would have a local recurrence to the site. Therefore, the final sample for RFS analyses was 163.

Table 34 Type of first recurrence in the CXCR4/CXCL12 study, N=199

Recurrence type	N	%
Patient became disease-free after treatment and has not had a recurrence	121	61
Local recurrence of an invasive tumor	2	1
Regional recurrence	3	2
Recurrence of an invasive tumor in adjacent tissue or organ	14	7
Recurrence of an invasive tumor in regional lymph nodes only	5	3
Recurrence of an invasive tumor in adjacent tissue or organ and in regional lymph node at the same time	3	2
Distant recurrence	1	1
Distant recurrence of an invasive tumor in the lung only	3	2
Distant recurrence of an invasive tumor in the liver only	2	1
Distant recurrence of an invasive tumor in bone only	2	1
Distant recurrence of an invasive tumor in the lymph node only	2	1
Distant recurrence of an invasive tumor in a single distant site and local, trocar, and or regional recurrence	1	1
Distance recurrence of an invasive tumor in multiple sites	4	2
Patient has never been disease-free	17	9
Disease has recurred but the type of recurrence is unknown	2	1
Unknown if disease recurred or if the patient was ever disease-free.	17	9

Table 35 Type of first recurrence stratified by stage

Early stage, N=103	N	%
Patient became disease-free after treatment and has not had a recurrence	81	79
Local recurrence of an invasive tumor	2	2
Recurrence of an invasive tumor in adjacent tissue or organ	5	5
Recurrence of an invasive tumor in regional lymph nodes only	1	1
Distant recurrence of an invasive tumor in bone only	2	2
Patient has never been disease-free	2	2
Unknown if disease recurred or if the patient was ever disease-free	10	10
Late stage, N=96		
Patient became disease-free after treatment and has not had a recurrence	40	42
Regional recurrence	3	3
Recurrence of an invasive tumor in adjacent tissue or organ	9	9
Recurrence of an invasive tumor in regional lymph nodes only	4	4
Recurrence of an invasive tumor in adjacent tissue or organ and in regional lymph node at the same time	3	3
Distant recurrence	1	1
Distant recurrence of an invasive tumor in the lung only	3	3
Distant recurrence of an invasive tumor in the liver only	2	2
Distant recurrence of an invasive tumor in the lymph node only	2	2
Distant recurrence of an invasive tumor in a single distant site and local, trocar, and or regional recurrence	1	1
Distance recurrence of an invasive tumor in multiple sites	4	4
Patient has never been disease-free	15	16
Disease has recurred but the type of recurrence is unknown	2	2
Unknown if disease recurred or if the patient was ever disease-free	7	7

APPENDIX D: SUPPLEMENTARY DETAILS FOR ARTICLE 4

D.1 DIAGNOSTICS FOR THE COX PROPORTIONAL HAZARDS MODEL

The proportional hazards assumption was examined using the post-estimation *stphplot* command in STATA. In the OS model there is some evidence that the proportional hazards assumption for HGF and stromal bFGF do not hold, however the model tested by these curves does not account for the interaction between HGF and stromal bFGF (Figure 48). In the RFS main effects model, no violations of the proportional hazards assumption were observed (Figure 49).

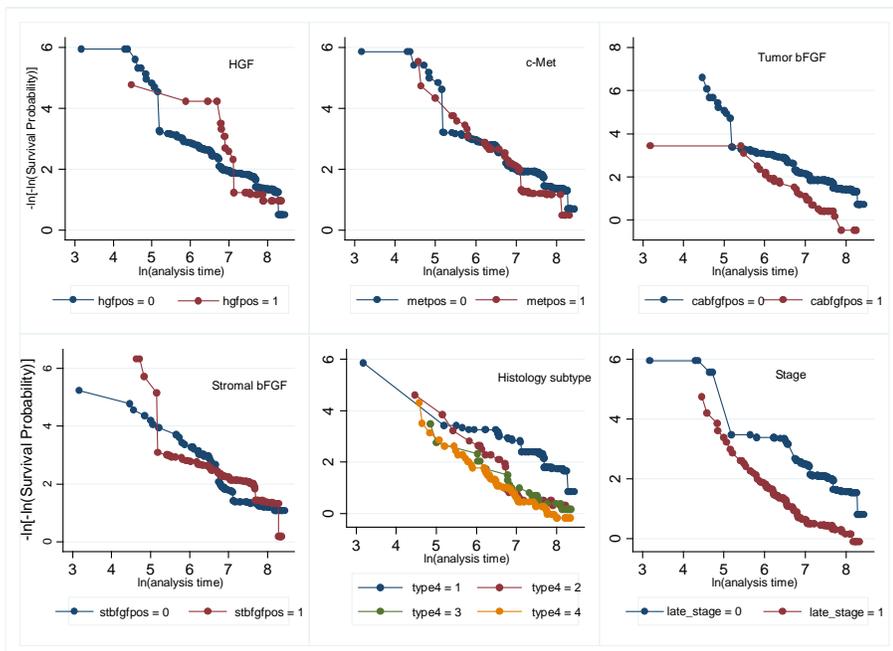


Figure 48 Tests of the proportional-hazards assumption for variables in Table 23

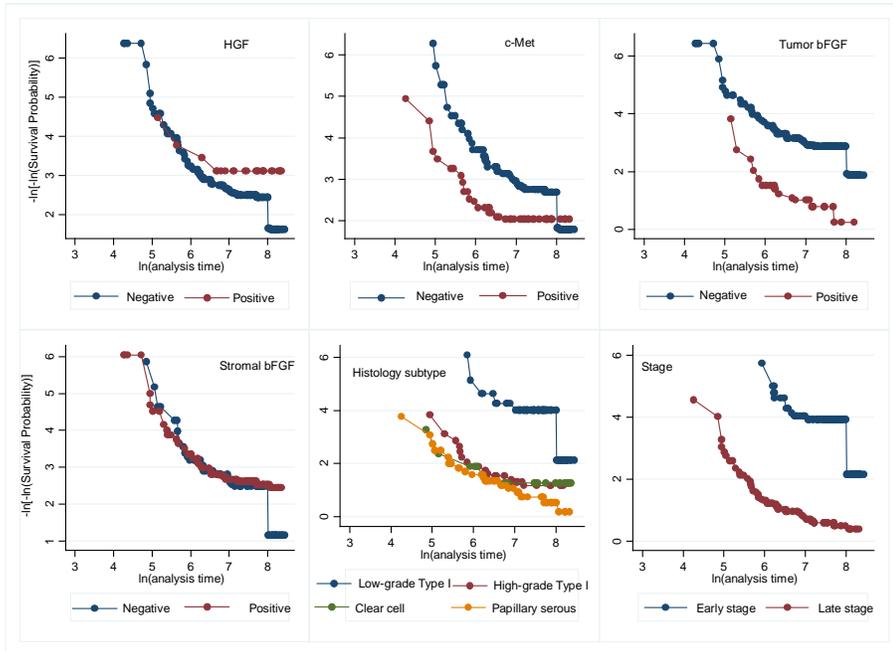


Figure 49 Tests of the proportional-hazards assumption for variables in Table 26

D.2 POWER CALCULATIONS

The statistical power to detect an association between the four markers (tumor HGF, tumor c-Met, tumor bFGF, and stromal bFGF) and survival outcomes (OS and RFS) was calculated using the *stpower* command in STATA (Table 36). The probability of death was 0.41 (87/211) and the probability of recurrence was 0.24 (42/174). The alpha was set at 0.05 and the log hazard ratios from the main effects model were specified as the effect sizes for the power calculations.

Table 36 Power calculations for the association between biomarker expression and EC survival outcomes

Marker	Power
Overall survival, N=211	
HGF	3%
c-Met	27%
Tumor bFGF	35%
Stromal bFGF	32%
Recurrence-free survival, N=174	
HGF	45%
c-Met	7%
Tumor bFGF	58%
Stromal bFGF	20%

D.3 PROGNOSTIC ROLE OF HGF/C-MET/BFGF IN SUBGROUPS OF EC CASES

OS and RFS Kaplan-Meier curves and log-rank tests comparing levels of HGF, c-Met, tumor bFGF, and stromal bFGF expression in subgroups of EC cases is shown in Figures 50-52. For space considerations, we only include graphs for associations significant at $p < 0.05$. Positive HGF expression was associated with better OS in late stage cases and better RFS in low-grade Type I and high-grade Type I cases (Figure 50). Positive c-Met expression was significantly associated with worse RFS in low-grade Type I and better RFS in late stage cases (Figure 51). Tumor bFGF expression was significantly associated with worse OS and RFS in low-grade Type I cases. Additionally, tumor bFGF expression was significantly associated with worse RFS in early stage and late stage cases (Figure 52). Stromal bFGF expression was not significantly associated with either OS or RFS in any of the subgroups.

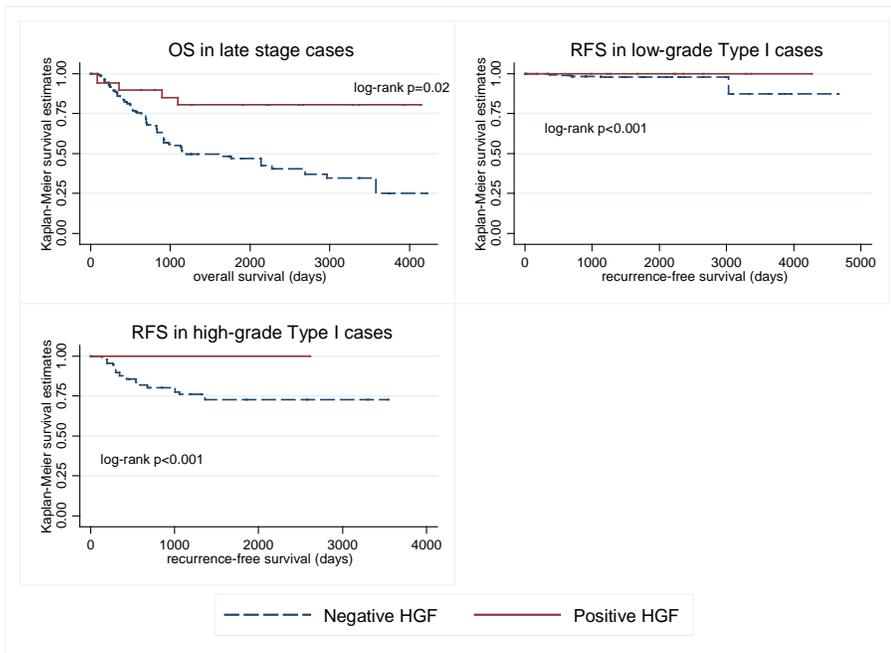


Figure 50 HGF in subgroups of EC cases

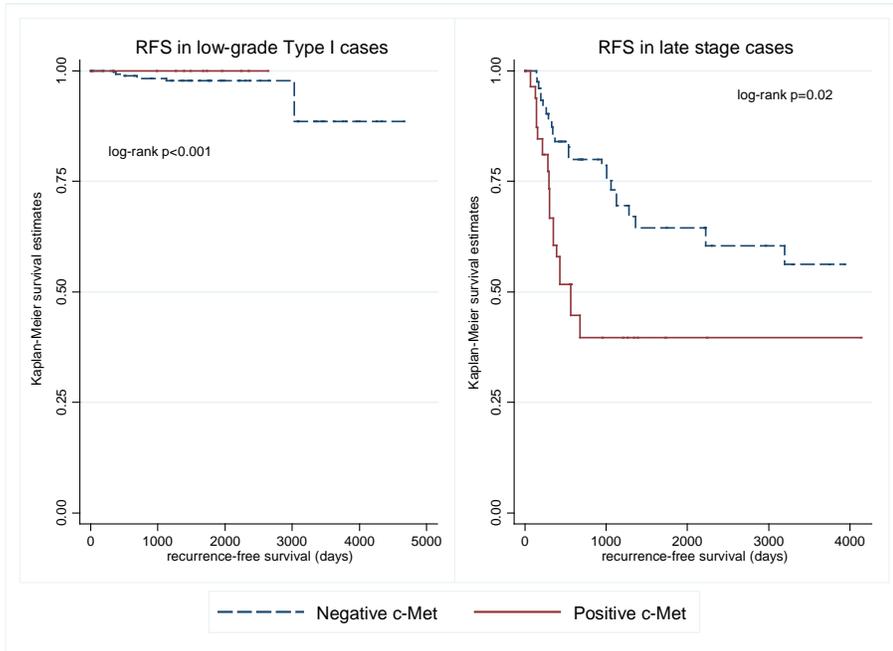


Figure 51 c-Met in subgroups of EC cases

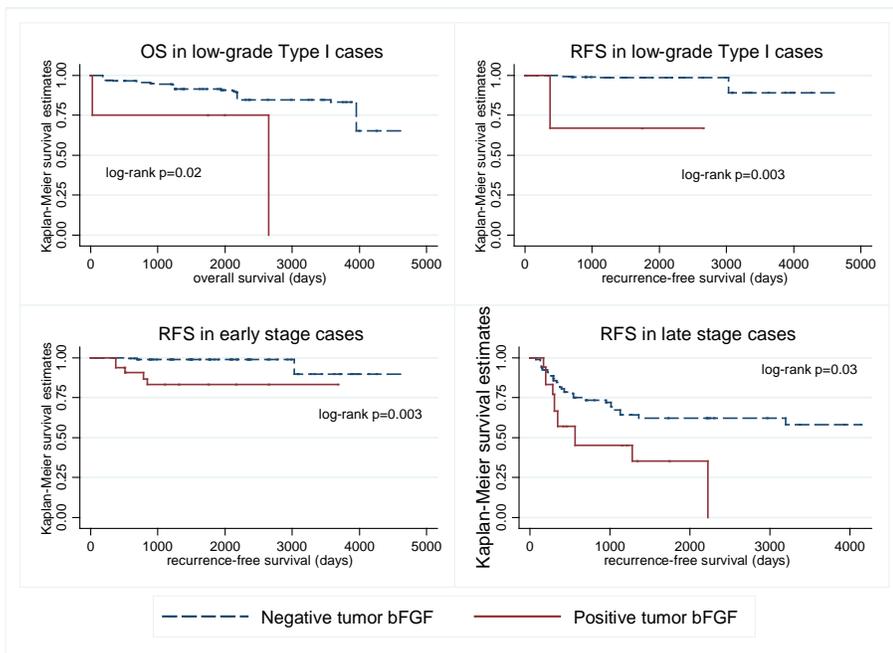


Figure 52 Tumor bFGF in subgroups of EC cases

D.4 ASSOCIATION BETWEEN HGF/C-MET/BFGF EXPRESSION AND METASTASIS

HGF, c-Met, and bFGF are thought to influence cancer prognosis through metastasis and activation of angiogenesis. We examined the relationship between metastasis and expression of these biomarkers independent of their effect on prognosis. Stage IV EC cases were classified as distant metastatic cases while stage I-III cases were classified as non-distant metastatic cases. Only tumor bFGF expression was significantly associated with distant metastasis ($p=0.01$, Table 37). Furthermore, a logistic regression model with distant metastasis as the outcome revealed that positive HGF was a significant predictor of the odds of metastasis (Table 38). Compared to negative HGF expression at the primary tumor site, positive HGF expression was associated with a higher odds of distant metastasis (OR: 3.29, 95% CI 1.06, 10.18) independent of c-Met, tumor bFGF, stromal bFGF, histology type, and age.

Table 37 Association between metastasis, HGF, c-Met, tumor bFGF, and stromal bFGF expression

N=211	No metastasis (N=174)		Metastasis (N=37)		p^{\ddagger}
	n (%) [*]	% [†]	n (%) [*]	% [†]	
HGF					0.40
Negative	151 (87)	86	29 (78)	78	
Positive	23 (13)	14	8 (22)	22	
c-Met					0.50
Negative	129 (74)	78	26 (70)	72	
Positive	45 (26)	22	11 (30)	28	
Tumor bFGF					0.01
Negative	149 (86)	94	27 (73)	76	
Positive	25 (14)	6	10 (27)	24	
Stromal bFGF					0.31
Negative	76 (44)	40	19 (51)	52	
Positive	98 (56)	60	18 (49)	48	
* proportion in the study sample					
† weighted proportion					
‡ Adjusted Wald p -value					

Table 38 Multivariable logistic regression model to predict metastasis (N=37) vs. no metastasis (N=174)

Variable	Adjusted OR (95% CI)	Wald <i>p</i>-value*
HGF		
Negative	1.00	0.04
Positive	3.29 (1.06, 10.18)	
c-Met		
Negative	1.00	0.72
Positive	0.84 (0.32, 2.20)	
Tumor bFGF		
Negative	1.00	0.48
Positive	1.47 (0.50, 4.34)	
Stromal bFGF		
Negative	1.00	0.47
Positive	0.71 (0.28, 1.80)	
Histology type		
Low-grade Type I	1.00	<0.001
High-grade Type I	20.96 (4.49, 97.88)	
Clear cell	30.10 (8.37, 108.28)	
Papillary serous	43.79 (14.83, 129.35)	
Age at diagnosis	0.97 (0.93, 1.00)	0.09

D.5 AT-RISK POPULATION FOR RECURRENCE

Cases known to be disease-free following the primary surgery were included in analyses of RFS. Tables 39 and 40 show the type of recurrence in the total cohort and stratified by stage. In the total sample 63% of patients were disease-free following primary surgery and did not have a recurrence over the follow-up period, while 20% of cases that entered a state of remission following surgery recurred over the follow-up. The remaining 17% of cases were either never disease-free or it was unknown if they entered a state of remission after the primary surgery and were therefore excluded from RFS analyses. Furthermore, two early stage cases reportedly had a local recurrence of an invasive tumor (Table 40) despite having a total hysterectomy with removal of both ovaries; these two cases were excluded as it was deemed implausible that patients without an intact uterus would have a local recurrence to the site. Therefore, the final sample for RFS analyses was 174.

Table 39 Type of first recurrence in the HGF/c-Met/bFGF study, N=211

Recurrence type	N	%
Patient became disease-free after treatment and has not had a recurrence	132	63
Local recurrence of an invasive tumor	2	1
Regional recurrence	2	1
Recurrence of an invasive tumor in adjacent tissue or organ	14	7
Recurrence of an invasive tumor in regional lymph nodes only	5	2
Recurrence of an invasive tumor in adjacent tissue or organ and in regional lymph node at the same time	3	1
Distant recurrence	1	0.5
Distant recurrence of an invasive tumor in the lung only	3	1
Distant recurrence of an invasive tumor in the liver only	2	1
Distant recurrence of an invasive tumor in bone only	2	1
Distant recurrence of an invasive tumor in the lymph node only	2	1
Distant recurrence of an invasive tumor in a single distant site and local, trocar, and or regional recurrence	1	0.5
Distance recurrence of an invasive tumor in multiple sites	5	2
Patient has never been disease-free	17	8
Disease has recurred but the type of recurrence is unknown	2	1
Unknown if disease recurred or if the patient was ever disease -free.	18	9

Table 40 Type of first recurrence stratified by stage

Early stage, N=111	N	%
Patient became disease-free after treatment and has not had a recurrence	87	78
Local recurrence of an invasive tumor	2	2
Recurrence of an invasive tumor in adjacent tissue or organ	5	5
Recurrence of an invasive tumor in regional lymph nodes only	1	1
Distant recurrence of an invasive tumor in bone only	2	2
Distance recurrence of an invasive tumor in multiple sites	1	1
Patient has never been disease-free	2	2
Unknown if disease recurred or if the patient was ever disease -free	11	10
Late stage, N=100		
Patient became disease-free after treatment and has not had a recurrence	45	45
Regional recurrence	2	2
Recurrence of an invasive tumor in adjacent tissue or organ	9	9
Recurrence of an invasive tumor in regional lymph nodes only	4	4
Recurrence of an invasive tumor in adjacent tissue or organ and in regional lymph node at the same time	3	3
Distant recurrence	1	1
Distant recurrence of an invasive tumor in the lung only	3	3
Distant recurrence of an invasive tumor in the liver only	2	2
Distant recurrence of an invasive tumor in the lymph node only	2	2
Distant recurrence of an invasive tumor in a single distant site and local, trocar, and or regional recurrence	1	1
Distance recurrence of an invasive tumor in multiple sites	4	4
Patient has never been disease-free	15	15
Disease has recurred but the type of recurrence is unknown	2	2
Unknown if disease recurred or if the patient was ever disease -free.	7	7

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