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Mechanisms underlying chemotherapy-induced vascular proliferation in ovarian cancer

Zeynep Gülsüm Kahramanoğlu, *The University of Western Ontario*

Supervisor: Dr. Zia A. Khan, *The University of Western Ontario*
Joint Supervisor: Dr. Michele M. Weir, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology
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Abstract

Ovarian cancer is a leading cause of gynecological cancer-related death in Canadian women. Ovarian cancer is managed through surgical cytoreduction and carboplatin-based chemotherapy. Unfortunately, most patients often relapse or have reduced responses to initial chemotherapy. The mechanisms behind carboplatin resistance are poorly understood. In pilot studies, our group has observed vascular proliferation in patient samples following carboplatin treatment. The effectiveness of modulating neovascularization in combination with carboplatin has also been demonstrated in two large Phase 3 trials. In this study, I explore the underlying mechanisms of chemotherapy-induced vascular proliferation and potentially, tumour cell survival. I hypothesize that carboplatin induces angiogenic factors in ovarian cancer cells leading to microvascular endothelial cell survival.

To test my hypothesis, I screened for a variety of angiogenic factors in ovarian cancer cells and vascular endothelial cells following exposure to carboplatin. My results show that a number of angiogenic genes are upregulated in response to carboplatin exposure, including placental growth factor (PGF). Preclinical studies have shown that inhibition of PGF prevents tumour growth and metastasis. Therefore, I tested the effect of PGF and condition media prepared from ovarian cancer cells following carboplatin challenge on endothelial cell survival. My results show that PGF and ovarian cancer condition media facilitates endothelial cell survival. I also found that carboplatin may induce *PGF* expression in ovarian cancer cells through β-catenin activation.

Findings from this study may help better understand the effects of carboplatin exposure on ovarian cancer. Furthermore, the results may provide additional targets to increase carboplatin sensitivity in ovarian cancer patients.

Keywords

Serous ovarian cancer, angiogenesis, chemotherapy, carboplatin, vascular proliferation

Co-Authorship Statement

The work presented in this thesis is based on pilot studies conducted by Dr. Iram Siddiqui and Sandra Mekhaiel (2014) in the laboratory of Drs. Zia A. Khan and Michele M. Weir. The data showed increased vascular proliferation in ovarian cancer patient samples. A part of this pilot data is included in **Figure 2.1.1**. All other work shown was performed by Zeynep G. Kahramanoğlu.

Drs. Zia A. Khan and Michele M. Weir contributed to the experimental design and data interpretation.

Dedication

Koca kafalı kardeşim Selin’e.

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First and foremost, I would like to express my deepest gratitude to my supervisor Dr. Zia A. Khan, for his teachings, both in life and in research. Thank you for your continued support, guidance and encouragement. Your passion and curiosity for my project fueled my own. It has been a privilege to be a part of the Khan lab.

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To the Khan Laboratory members Jina, Niamh and Natalie (© Khan Sisters): these two years would not have been nearly as much fun without friends like you.

Thank you to my loving family and friends for always being there for me.

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List of Abbreviations

ACTB	Beta actin
ADGRB1	Adhesion G protein-coupled receptor B1
AKT	Protein kinase B
AKT1	V-akt murine thymoma viral oncogene homolog 1
ANG	Angiopoietin
ANGPT1	Angiopoietin 1
ANGPT2	Angiopoietin 2
ANGPTL4	Angiopoietin-like 4
ANOVA	Analysis of Variance
ANPEP	Alanyl (membrane) aminopeptidase
AUC	Area under concentration
B2M	Beta-2-microglobulin
BLT2	Leukotriene B4 receptor 2
BRCA	Breast cancer susceptibility gene
CA4P	Combretastatin A-4 phosphate (Fosbretabulin)
CCL11	Chemokine (C-C motif) ligand 11
CCL2	Chemokine (C-C motif) ligand 2
CDH5	Cadherin 5, type 2 (vascular endothelial-cadherin)
c-Myc	Myc proto-oncogene
CNA	Copy number alteration
COL18A1	Collagen, type XVIII, alpha 1
COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)
CTGF	Connective tissue growth factor (CCN family member 2)
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL5	Chemokine (C-X-C motif) ligand 5
CXCL6	Chemokine (C-X-C motif) ligand 6
CXCL8	Chemokine (C-X-C motif) ligand 8 (Interleukin 8)
CXCL9	Chemokine (C-X-C motif) ligand 9
DAPI	4’,6-Diamidine-2’-phenylindole
DMEM	Dulbecco’s modified eagle’s medium

ERCC1	Cross-complementation group 1
EDN1	Endothelin 1
EFNA1	Ephrin-A1
EFNB2	Ephrin-B2
EGF	Epidermal growth factor
EBM2	Endothelial basal medium 2
ELISA	Enzyme-linked immunosorbent assay
ENG	Endoglin
EPHB4	EPH receptor B4
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2
F3	Coagulation factor III (thromboplastin, tissue factor)
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF1	Fibroblast growth factor 1 (acidic)
FGF2	Fibroblast growth factor 2 (basic)
FGFR3	Fibroblast growth factor receptor 3
FIGF	C-fos induced growth factor (vascular endothelial growth factor D)
FLT1	Fms-related tyrosine kinase 1
FN1	Fibronectin 1
FRAT1	Frequently rearranged in advanced T-cell lymphomas
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFR	Glomerular filtration rate
HDMEC	Human dermal microvascular endothelial cells
HGDC	Human genomic DNA control
HGF	Hepatocyte growth factor
HGSOC	High grade serous ovarian carcinoma
HIF-1	Hypoxia inducible factor 1
HIF1A	Hypoxia inducible factor 1, alpha subunit
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HPSE	Heparanase
ID1	Inhibitor of DNA binding 1

IFNA1	Interferon, alpha 1
IFNG	Interferon, gamma
IGF1	Insulin-like growth factor 1 (somatomedin C)
IL1B	Interleukin 1 beta
IL6	Interleukin 6
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
JAG1	Jagged 1
KDR	Kinase insert domain receptor (vascular endothelial growth factor receptor 2)
LECT1	Leukocyte cell derived chemotaxin 1
LGSOC	Low grade serous ovarian carcinoma
LEP	Leptin
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MDK	Midkine (neurite growth-promoting factor 2)
MMP14	Matrix metalloprotease 14
MMP2	Matrix metalloprotease 2
MMP9	Matrix metalloprotease 9
MMR	DNA mismatch repair
mRNA	Messenger ribonucleic acid
mTOR	Mammalian/mechanistic target of rapamycin
NACT	Non-adjunct chemotherapy
NER	Nucleotide excision repair
NOS3	Nitric oxide synthase 3
NOTCH4	Notch 4
NRP1	Neuropilin 1
NRP2	Neuropilin 2
OS	Overall survival
OSE	Ovarian surface epithelium
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFA	Platelet derived growth factor alpha

PECAM1	Platelet/endothelial cell adhesion molecule (CD31)
PF4	Platelet factor 4
PFS	Progression free survival
PGF	Placental growth factor
PI3K	Phosphoinositide 3-kinase
PLAU	Plasminogen activator, urokinase
PLG	Plasminogen
PPC	Positive PCR control
PROK2	Prokineticin 2
PSF	penicillin, streptomycin, and amphotericin
PTGS1	Prostaglandin-endoperoxide synthase 1
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RPLP0	Ribosomal protein, large, P0
RRSO	Risk-reducing salpingo-oophorectomy
RTC	Reverse transcription control
S1PR1	Sphingosine-1-phosphate receptor 1
SERPINE1	Serpin peptidase inhibitor, clade E
SERPINF1	Serpin peptidase inhibitor, clade F
SPHK1	Sphingosine kinase 1
STIC	Serous tubal intraepithelial carcinoma
TEK	TEK tyrosine kinase
TGFA	Transforming growth factor alpha
TGFB1	Transforming growth factor beta 1
TGFB2	Transforming growth factor beta 2
TGFBR1	Transforming growth factor beta receptor 1
THBS1	Thrombospondin 1
THBS2	Thrombospondin 2
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TIMP1	TIMP metallopeptidase inhibitor 1
TIMP2	TIMP metallopeptidase inhibitor 2
TIMP3	TIMP metallopeptidase inhibitor 3

TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor alpha
TP53	Tumour protein p53
TPJ	Tubal-peritoneal junction
TSP	Thrombospondin
TYMP	Thymidine phosphorylase
VDA	Vascular disrupting agents
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VEGFB	Vascular endothelial growth factor B
VEGFC	Vascular endothelial growth factor C
WHO	World Health Organization
Wnt	Wingless-type MMTV integration site family member

Chapter 1

1 INTRODUCTION

1.1 Ovarian cancer: the “silent killer”

Ovarian cancer is the leading cause of death among gynecological malignancies in Canadian women[1-3]. The term “silent killer” is commonly used for ovarian cancer as it is often diagnosed at an advanced stage resulting in poor prognosis[4]. The high mortality rates associated with ovarian cancer is largely due to a general lack of understanding of the disease that leads to late-stage detection and diagnosis[4]. The etiology of ovarian cancer has been subject to ongoing debates and several mechanisms have been postulated. One proposed mechanism involved in the development of ovarian cancer is termed the “wear-and-tear” mechanism, where incessant ovulation causes the repeated destruction and repair of the ovarian surface epithelium (OSE)[3,5] and tubal epithelial cells[6]. This repeated trauma to the OSE and fallopian tube during ovulation increases the likelihood of DNA damage and acquisition of carcinogenic mutations[3]. The trauma to the OSE and tubal epithelial cells is also shown to elicit an inflammatory response[5,7]. Ovulation also impacts the fallopian tube similarly by increasing DNA damage as well as evoking inflammatory responses[6]. Inclusion cysts may form within the ovarian cortex post-ovulation, where wound repair may increase the risk of genetic abnormalities and result in dysplastic changes in the epithelial cells[8]. Along with chronic inflammation, the increased level of different growth factors may drive the transformation of these epithelial cells towards malignant growth[7]. Lastly, it is also speculated that hormones secreted during menopause, including gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) may contribute to the development of ovarian cancer[5,7,8]. Gonadotropins among other hormones initiate ovulation, thus ovulation is often termed a hormone-induced injury[9]. The late onset of ovarian cancer in 60-62 year olds[10] can perhaps be attributed to a lifetime of ovulatory cycles accompanied by the cyclic destruction of the OSE and fallopian tube epithelium, as well as changes in hormonal levels during these cycles and menopause.

1.2 Ovarian cancer: origin and clinical features

According to the World Health Organization (WHO) Histological Classification, ovarian neoplasms are categorized by the most probable cell of origin, and the site of origin (ovary vs. fallopian tube) is under intense debate[11]. Over 80% of ovarian cancers are surface epithelial tumours, but ovarian cancer is also comprised of germ cell, sex cord-stromal, and metastases categories[11,12]. Surface epithelial tumours are further classified by cell type: serous, mucinous, endometrioid, clear cell and undifferentiated. Due to a lack of early detection, most cases of ovarian cancer are diagnosed at a later, more aggressive stage known as high-grade serous ovarian carcinoma (HGSOC)[13]. HGSOCs are the most common histological subtype and are responsible for the most deaths from ovarian cancer[14]. Kurman and Shih (2010) have proposed a dualistic model which divides epithelial ovarian cancers into two broad groups (type I and II) based on genetic and morphological studies[15,16]. Molecularly, ovarian cancer is heterogeneous[17]. Low-grade surface epithelial tumours such as low grade serous carcinoma (LGSOC) are categorized as type I tumours, where morphology is distinct and tumours are genetically stable. Consistent mutations in type I tumours include Kirsten RAS oncogene homolog (*KRAS)*, *BRAF* and/or *ERBB2*[5,16,18]. These genes are all upstream regulators of mitogen-activated protein kinase (MAPK) which is believed to facilitate cell proliferation[18]. *TP53* mutations are uncommon in type I tumours like LGSOC[18]. LGSOCs are slow growing and involve a step-wise progression from a serous cystadenoma or adenofibroma to a serous borderline tumour eventually progressing to an invasive micropapillary serous carcinoma[18]. Meanwhile, type II tumours can have a range of morphological patterns: papillary, glandular, and solid[16]. Type II HGSOCs are high grade, genetically unstable and involve several *TP53* mutations, rapid growth and metastatic behaviour[5,15,16]. *KRAS*, *BRAF* or *ERBB2* mutations are rare in HGSOCs[18]. In addition, approximately 10% of ovarian carcinomas are hereditary, where most involve *BRCA* mutations[18]. Other major genomic features of HGSOCs include copy number alterations (CNA), where the genome is altered by up to 46%, whereas LGSOCs have close to normal CNAs[14].

The most recent accepted origin of HGSOC is the fallopian tube[16,19]. Dutch investigators proposed the fallopian tube as the primary site for most type II ovarian cancers[19]. Small

primary lesions on the fallopian tube, termed serous tubal intraepithelial carcinoma (STIC), were reported in women that exhibited genetic predisposition for ovarian cancer development[19]. STIC was postulated to share a common origin with HGSOC tumours because the genomic signatures often harboured identical *TP53* mutations[20]. In addition, over 70% of sporadic ovarian and peritoneal HGSOCs involved STIC[16,21]. In contrast, conclusive precursor lesions in ovaries have not been found[5,16]. Thus, the fallopian tube is believed to be the origin of ovarian cancer. It has been proposed that the invasive lesions found in the fallopian tube may shed malignant cells that implant on the ovary with subsequent tumour growth mimicking a primary ovarian tumour[5]. Some evidence supports that the tubal-peritoneal junction (TPJ), where the tubal lumen communicates with the peritoneal cavity, is the site of STIC origin[22]. This junction region is where the ciliated columnar epithelial lining of the tubal fimbriae meets the peritoneum, which is the serosal surface of the fallopian tube[22]. Risk-reducing salpingo-oophorectomy (RRSO) specimens from *BRCA* mutation carriers have also shown primary lesions of tubal origin[23,24]. In summary, due to the many different cell types involved in ovarian cancer, identifying the exact origin is difficult and possibly involves various sites.

1.3 Ovarian cancer: current treatments

The current treatment of ovarian cancer in most patients consists of debulking surgical resection of the primary tumour and platinum-based chemotherapy such as carboplatin, as well as a taxane drug, paclitaxel, commonly administered in a cyclic manner[10,25]. Some patients receive initial chemotherapy followed by debulking surgery and further chemotherapy[10]. Carboplatin is an alkylating agent, which consists of a platinum atom complexed with two ammonia groups and a cyclobutane-dicarboxyl residue[26]. Carboplatin dosage is measured by Calvert's formula, which accounts for the area under concentration versus the time curve (AUC) and creatinine clearance, or renal function via determination of the glomerular filtration rate (GFR)[26]. It is most often used to treat gynecological cancers, head and neck as well as lung cancer[27]. Carboplatin undergoes activation inside the cell and forms reactive platinum complexes, covalently binding to DNA that causes intra- and inter-strand cross-linkages[13,28]. Activation of carboplatin inside the cell is through hydrolysis, resulting in a positively charged molecule which is

able to interact with nucleophilic molecules[28]. Carboplatin-induced modification of DNA inhibits replication and transcription leading to cell death[13,28].

Most patients are diagnosed at a late disease stage where the cancer has metastasized to adjacent pelvic organs and the peritoneal cavity. When diagnosed early, the 5-year survival rate with current treatment regimens is over 90%[29], but the 5-year survival rate for late diagnosis (i.e. high grade stage) is approximately 15%[30]. However, despite positive response to initial chemotherapy, more than 80%[29] of patients relapse after a period of improvement leading to a significant clinical challenge[31]. Recurrent disease which is unresponsive to chemotherapy treatment is the primary cause of mortality in HGSOC patients[32]. Intratumoral heterogeneity and selection of chemo-resistant subclones may account for chemotherapy resistance[32]. Mechanisms attributing to acquired resistance from platinum-based chemotherapy have been recently proposed. These include reversion of *BRCA1* and *BRCA2* mutations, and upregulation of AKT signalling, which ultimately leads to tumour cell survival[32]. Understanding the mechanisms underlying carboplatin failure is under intensive investigation. Within the last ten years, targeting angiogenesis for therapy is on the rise in combination with chemotherapy treatments.

1.4 Physiological and pathological angiogenesis

Angiogenesis, the sprouting of new blood vessels from existing ones, is well established to occur during embryonic development, and during the female reproductive cycle and wound healing[33]. Conditions such as psoriasis and rheumatoid arthritis are also dependent on angiogenesis[34]. During embryonic development, endothelial cells are initially derived *de novo* from precursor cells in a process termed vasculogenesis[35]. Following vasculogenesis, the majority of the blood vessels are formed by angiogenesis[35]. Angiogenesis involves the following fundamental steps: the basement membrane of the existing blood vessel is broken down and the extracellular matrix is degraded[33,36]. Then, endothelial cells migrate into the interstitial space, towards the angiogenic stimuli, facilitated by proteases as well as cell adhesion molecules[36]. Finally, endothelial cells proliferate and form the new capillary lumen followed by functional maturation via

recruitment of other mural cells such as pericytes[33,36,37]. Blood vessels provide nutrients and oxygen to all tissues as well as facilitate the removal of waste products[33,38]. In addition, blood vessels deliver immune cells, macrophages and even humoral factors to tissues[38].

In the ovary, a cyclic reproductive process drives ovarian vascular development[36,37]. Physiological angiogenesis takes place during folliculogenesis, ovulation and luteal development[37]. The outer cortex of the ovary contains a number of ovarian follicles in different stages of growth before their maturity and release into the fallopian tube[39,40]. Ovarian follicles contain a single oocyte enclosed by a basal lamina, further surrounded by layers of theca cells, which are critical for ovulation and only appear in the early secondary stage of ovarian follicle development[41]. Angiogenesis occurs in the theca layer and is essential in the transition of the primary ovarian follicle into the vascular secondary follicle[36,37]. The capillary network does not traverse the basement membrane of the granulosa layer. However, follicular growth is dependent on gonadotrophins secreted by the theca layer of the ovarian follicle, which stimulates ovulation, causing the basement membrane to collapse and allow the blood vessels to penetrate the granulosa layer[36].

Angiogenesis also plays a critical role in cancer progression and is one of the vital alterations driving malignant tumour growth[42]. Tumour cells release numerous growth factors to mediate dysregulated angiogenesis[35]. This dysregulation involves both excessive vascular expansion and maintenance of a chaotic and immature vascular network. Tannock *et al.* (1968) found that the closer the capillary, the more likely the tumour cell will undergo mitosis[33,43]. Without angiogenesis, solid tumours are unable to grow beyond 2 mm^3 [44]. The induction of tumour angiogenesis that results in leaky and immature vasculature promotes alteration of perivascular cells, greater permeability and the inability to remodel the newly formed vessels[35,45]. Moreover, tumour cells utilize neo-vessels to metastasize to distant sites in the body[33].

It has been reported that after menopause or oophorectomy, the decrease in estrogen levels leads to elevated gonadotropin hormone levels, which promote tumour growth *in*

vivo by inducing angiogenesis[46]. Given that the ovarian medulla consists of loose connective tissue, vasculature and nerves[39], these blood vessels are readily accessible and in close proximity to the primary ovarian or fallopian tube and contribute to early tumorigenesis.

1.5 Angiogenesis signaling pathways and involvement in ovarian cancer

Once the tumour reaches a certain size, the tumour microenvironment becomes hypoxic and induces the angiogenic switch[35,47]. The angiogenic switch results from an imbalance between pro- and anti-angiogenic factors, facilitating vascular expansion[33,47]. Various growth factors are involved in tumour angiogenesis. Perhaps, the most studied is vascular endothelial growth factor (VEGF). VEGF was identified as an endothelial cell-specific mitogen that exhibited the capacity to induce physiological and pathological angiogenesis[48,49]. This VEGF is now known as VEGFA and is a member of a large family that includes VEGFB, VEGFC, VEGFD and placental growth factor (PGF). These VEGF family members differ in expression patterns, cell surface receptor specificity, and biological functions[50]. Increased microvessel density and VEGF expression has been shown to correlate with poor prognosis in ovarian cancer[34]. Signaling by VEGF in tumour cells is mediated by cell surface VEGF receptor tyrosine kinases and neuropilins (NRPs)[51]. The classical VEGF receptors include VEGFR1 (also known as FLT1), VEGFR2 (also known as FLK1 or KDR) and VEGFR3[52]. Although, most VEGFRs are expressed by endothelial cells and numerous tumour cell types[53], VEGFR2 is considered the predominant receptor that mediates VEGF signaling and drives VEGF-mediated angiogenesis[52]. Engagement of VEGFR2 by VEGF initiates a tyrosine kinase signaling cascade that causes endothelial cell proliferation, migration and tubule formation by production of other pro- and anti-angiogenic factors[33,37,54]. Intracellular mediators downstream of VEGF-VEGFR include the phosphoinositide 3-kinase (PI3K) pathway, which is required for vascular permeability[33]. Subsequently, protein kinase B (AKT) is activated; AKT is responsible for protein synthesis and cell growth via phosphorylation of mammalian target of rapamycin (mTOR) signaling pathway[33,55]. The PI3K/AKT pathway also modulates the expression of other angiogenic factors such as nitric oxide,

and angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2)[56]. Ang1 ensures stable and mature blood vessels by securing endothelial cell-cell junctions, decreasing endothelial sprouting and enhancing pericyte coverage[17]. Ang1 is also widely expressed in normal adult tissue[17]. Ang2, on the other hand, functions as an endothelial-derived negative regulator of Ang1 signaling during angiogenesis, thereby controlling the responsiveness of vascular endothelial cells to exogenous stimuli[57].

Unlike VEGF, family member PGF is dispensable for development but exhibits non-redundant roles in tissue ischemia, malignancy, and multiple other diseases. In addition, PGF binds primarily to VEGFR1, unlike VEGFA which binds to both VEGFR1 and VEGFR2[58]. The pro-angiogenic activity of PGF is mediated by direct effects on endothelial and perivascular cells, resulting in enhanced vascular cell proliferation, migration, and survival[59-62]. Since PGF primarily binds VEGFR1, it was initially thought that PGF limits and displaces VEGFA from VEGFR1, thereby liberating VEGFA to activate VEGFR2[63]. This suggests that endothelial cells or cancer cells can enhance or modulate responsiveness to VEGFA by releasing PGF[64]. PGF may also indirectly contribute to tumour angiogenesis through upregulation of factors such as VEGFA, fibroblast growth factor-2 (FGF2), platelet-derived growth factor-B (PDGFB), and matrix metalloproteinases (MMPs)[65,66].

Levels of angiogenic factors VEGF, FGF, PDGF, tumor necrosis factor-alpha (TNF-α), ephrins, angiopoietins and interleukins such as IL6 and IL8 have been shown to be increased in ovarian cancer specimens compared to normal ovarian tissue[34,45]. Furthermore, many of these angiogenic factors regulate the expression of other angiogenic factors. For example, TNF-α upregulates different growth factors, cytokines and chemokines, which ultimately contribute to a pro-angiogenic microenvironment[67]. Moreover in HGSOCs, VEGF increases vascular permeability of peritoneal blood vessels; the leaky vessels consequently lead to malignant ascites[45]. VEGF also increases expression of MMPs, enhancing tumour cell invasion[45].

Counteracting pro-angiogenic factors are the anti-angiogenic factors such as thrombospondins (TSPs), angiostatin, and endostatin[33]. TSP-1 for example, supresses

angiogenesis in two ways: 1) by suppressing migration and inducing apoptosis of endothelial cells or 2) by inhibiting co-receptors shared with pro-angiogenic factors on the endothelial cell surface[68]. However, the receptor engaged by TSP is of paramount importance in mediating any anti-angiogenic effects. For example, CD36 is expressed by endothelial cells as a receptor for TSP-1 and is necessary for the anti-angiogenic activity[69]. However, binding of TSP to alpha-3/beta-1, alpha-4/beta-1, and alpha-9/beta-1 integrin results in pro-angiogenic effects[70-72]. In addition, oncogenes such as *c-jun* and loss of tumour suppressor genes such as *TP53* have been shown to regulate TSP expression, thus disturbing the angiogenic balance in favour of angiogenesis[68]. Although several *TP53* mutations result in gain-of-function in HGSOC, whether TSP is altered and which receptors may be engaged is not fully known[20,73].

1.6 Anti-angiogenic therapies in ovarian cancer

The inhibition of angiogenic factors involved in ovarian cancer is emerging as a therapeutic strategy[74,75]. These therapies are being explored in conjunction with standard chemotherapy[56,76-78]. Monoclonal antibodies, endogenous peptide inhibitors, small molecule drugs and microRNAs have been developed over the past ten years to counteract angiogenesis[78]. Bevacizumab (Avastin), a monoclonal anti-VEGF antibody, was one of the first US Food and Drug Administration (FDA) approved anti-angiogenic drugs[17,77]. Another example is VEGF Trap, or Aflibercept, which is a fusion protein with affinity to VEGFA, VEGFB and PGF[17]. VEGF Trap prevents binding of VEGF ligands to cell-surface receptors[79]. In a phase 2 clinical trial, Avastin was administered to relapsed platinum-resistant ovarian cancer patients as a monotherapy, and showed improvement in median progression-free survival (PFS) of less than 5 months and overall survival by 17 months[17]. These monotherapies were also accompanied by adverse side effects such as hypertension and gastrointestinal perforations among others[17]. Combination of chemotherapy and anti-angiogenic therapies has also been tested in Phase 3 trials with varying treatment regimens. Although improving PFS by a few months, overall survival by the combination therapy did not change[17]. Other novel developments to combat pathological angiogenesis have included small molecule vascular disrupting agents (VDAs) to target existing blood vessels as opposed to targeting

factors that play a role in neovascularization[79,80]. VDAs target vasculature by binding to endothelial cell tubulin. Combretastatin A4-phosphate (CA4P), for example, targets endothelial cells without complete perivascular cell coverage. CA4P has shown improved PFS in combination with Avastin in ovarian cancer[79]. A phase 2/3 clinical trial is currently underway to assess the therapeutic effect of a combination of VDAs, chemotherapy, and anti-angiogenic agents[79]. Although these are promising developments, the efficiency and dose-limiting toxicities accompanying some of the treatment regimens are lingering concerns. In addition, resistance to anti-angiogenic agents involving VEGF are poorly understood[80]. Therefore, it is imperative to explore alterative pathways that may be involved in ovarian cancer angiogenesis to develop new targets and increase the efficiency of current ones.

Chapter 2

2 PURPOSE AND OBJECTIVES OF THESIS

2.1 Purpose of thesis

Resistance to platinum-based chemotherapy is a major clinical challenge in ovarian cancer treatment and the reasons behind chemotherapy failure is poorly understood[28,81]. Anti-angiogenic therapies targeting VEGFA and similar angiogenic genes have shown to disrupt tumour angiogenesis[81]. Pilot studies performed in our laboratory by previous members showed unexpected vascular proliferation in ovarian serous carcinoma following carboplatin treatment (unpublished data). Specifically, histological analyses of paraffin-embedded ovarian serous adenocarcinoma specimens from nine pre-chemotherapy biopsies and patient-matched post-chemotherapy resections showed microvascular proliferation in carboplatin-treated specimens. Moreover, early morphological observations have shown viable tumour cells near vascular proliferation following carboplatin treatment (unpublished data). In addition, a number of cytokines, matrix proteins and growth factor exhibited increased levels in post-chemotherapy specimens, such as pro-angiogenic Ang1 (unpublished data, **Figure 2.1.1 –** Dr. Iram Siddiqui and Sandra Mekhaiel, 2014). Little is known about the effect of chemotherapy on ovarian cancer tumour vasculature, and vascular proliferation following carboplatin therapy may point to an important mechanism by which ovarian cancer may escape the therapeutic effect of carboplatin. Interestingly, Wild *et al.* (2004) have shown that carboplatin significantly increased the expression of VEGFA in endothelial cells, and when VEGFA was inhibited, endothelial cell sensitivity to carboplatin significantly increased[81]. The investigators also showed that carboplatin treatment did not alter VEGFA expression in tumour cells. Therefore, it is imperative to understand the response of ovarian cancer and endothelial cells to carboplatin. These studies may lead to a better understanding of the tumour microenvironment post-carboplatin exposure, to the development of new therapeutic targets for ovarian cancer and/or rationalize combination treatment regimens.

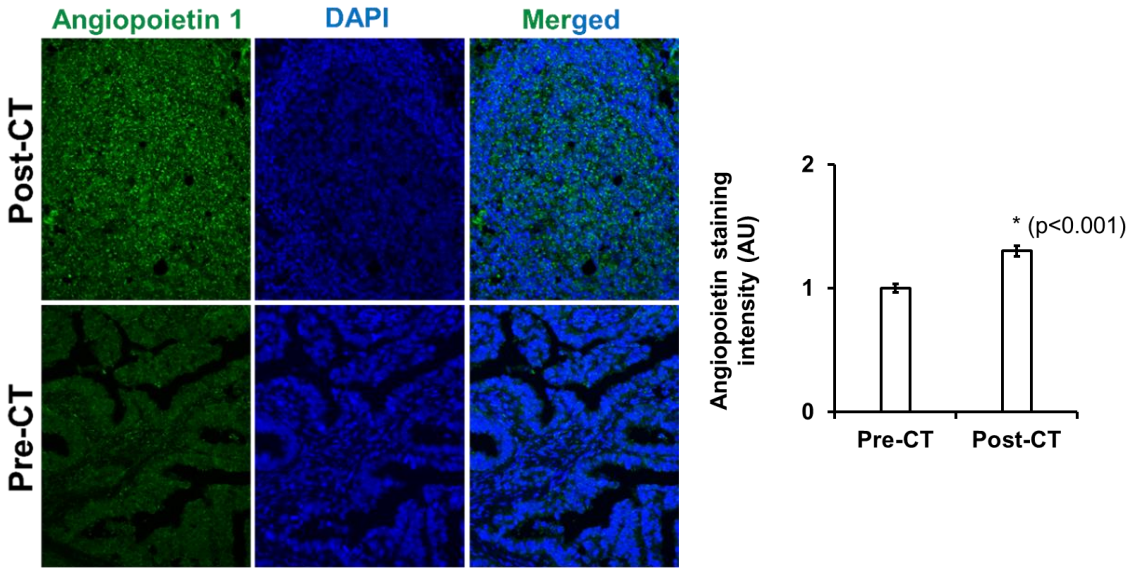


Figure 2.1.1 Angiopoietin 1 staining in patient pre-chemotherapy (Pre-CT) treatment biopsy versus patient-matched post-chemotherapy (Post-CT) treatment surgical resection. Representative immunofluorescence (IF) staining of angiopoietin 1 (green) in ovarian serous adenocarcinoma specimens [pre-CT = biopsy specimens prior to initiating chemotherapy, post-CT = specimens resected following chemotherapy; nuclei stained with DAPI (blue)]. Right panel shows quantification of fluorescence intensity by Image J (https://imagej.nih.gov/ij/). Staining intensity is presented in arbitrary units (AU) [Student's t-test was used for data analysis].

2.2 Hypothesis and objectives

My studies explore the mechanisms underlying the effects of carboplatin on ovarian cancer angiogenesis. I hypothesize that carboplatin induces angiogenic factors in ovarian cancer cells leading to microvascular endothelial cell survival.

My specific aims are as follows:

1: Identify alteration of angiogenic genes in ovarian cancer cells following carboplatin exposure.

2: Investigate the functional significance of carboplatin-induced angiogenic genes using vascular endothelial cells.

Chapter 3

3 MATERIALS AND METHODS

3.1 Ovarian cancer and vascular endothelial cells

HGSOCs make up approximately 80% of ovarian cancer diagnoses, are clinically aggressive and are responsible for the most gynecological cancer-related deaths in Canadian women[29]. Therefore, I elected to use two ovarian cancer cell lines of this histological subtype for my studies. Ovarian cancer cell line COV362 was obtained from Sigma-Aldrich (Oakville, ON, Canada). COV362 was originally identified as epithelial-endometrioid carcinoma[82], but is now considered a top-ranking HGSOC-like line[14]. This confusion is not surprising as some high-grade endometrioid adenocarcinomas are difficult to distinguish from HGSOC molecularly[83]. Some high grade endometrioid adenocarcinomas also have *TP53* mutations as well as a high correlation of CNA with HGSOCs[84]. In addition to COV362, I used an established ovarian serous carcinoma cell line, COV318 (Sigma-Aldrich). Human dermal microvascular endothelial cells (HDMECs; Lonza Inc.) were used for functional tests and to identify autocrine angiogenic factors.

3.2 Cell culture

COV362 and COV318 ovarian cancer cells were cultured on uncoated plastic plates in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS; Life Technologies) and 1x antibiotic antimycotic media (penicillin, streptomycin, and amphotericin, PSF; Mediatech Inc.). HDMECs were cultured on uncoated plastic plates in complete Endothelial Basal Media-2 (EBM-2; Lonza Inc.) supplemented with 20% FBS, 1x PSF, and EGM-2 SingleQuots (Lonza Inc.) containing VEGF, insulin-like growth factor-1, human epidermal growth factor, human FGF, ascorbic acid, heparin, hydrocortisone and gentamicin/amphotericin B. Besides different culture media, all other conditions were identical.

3.3 Cell viability assays

To establish the effect of carboplatin exposure, COV362, COV318 and HDMECs were seeded at 10,000 cells/cm^2 in respective growth media and allowed to attach overnight. The next day, media was changed to fresh growth media containing varying concentrations of carboplatin (Sigma; molecular weight 371.3 g/mol) including 0 (control), 1, 10, 25, and 50 µg/mL (3 µM, 79 µM, 67 µM, and 135 µM, respectively). Carboplatin challenge was carried out for 72 hours with three experimental replicates per condition. This concentration range was based on a study by Wild *et al.* (2004) where investigators exposed endothelial cells and human ovarian carcinoma MA148 cells to carboplatin[81]. In that study, endothelial cells showed 50% viability upon exposure to 50-70 µg/mL (135 µM – 190 µM) carboplatin as assessed by MTT assay. In my study, viable cell counts and live/dead percentages were measured using Scepter Handheld Automated Cell Counter (Millipore) and Trypan blue exclusion test by Countess II (Life Technologies), respectively. Scepter cell counter uses the Coulter principle of impedance-based particle detection to reliably and accurately count cells. The concentration which showed significant decrease in cell viability across all cell types relative to control was used for subsequent experiments.

3.4 RNA isolation and qRT-PCR

Identification of angiogenic factors was performed by profiling mRNA levels of known angiogenesis factors in cells. Cells were seeded at 10,000 cells/cm^2 and cultured overnight before exposing to 50 µg/mL (135 µM) carboplatin for 72 hours. RNA was isolated using RNeasy Micro Plus Kit (Qiagen). Amount of RNA yield was determined by Qubit RNA Broad Range Assay in a Qubit Fluorometer (Life Technologies). Then, cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Gene expression analysis was performed using RT^2 Human Angiogenesis PCR arrays (PAHS-072ZD; Qiagen) (**Plate 1**) in Bio-Rad CFX Connect. All reactions were performed using RT² SYBR® Green qPCR Mastermix (Qiagen) and nuclease-free H_2O. Target mRNA levels were analyzed by CFX Manager Software (Bio-Rad Laboratories), and by using the ΔΔCT method. Normalization to five housekeeping genes was carried out. These

housekeeping genes included β-actin (*ACTB*), β2-microglobulin (*B2M*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hypoxanthine-guanine phosphoribosyl transferase (*HPRT1*) and ribosomal protein lateral stalk subunit P0 (*RPLP0*). A full list of all angiogenic genes and appropriate PCR controls on **Plate 1** can be found in **Appendix 1**.

1	2	3	4	5	6	7	8	9	10	11	12
AKT1	ANG	ANGPT1	ANGPT2	ANGPTL4	ANPEP	ADGRB1	CCL11	CCL2	CDH5	COL18A1	COL4A3
CTGF	CXCL1	CXCL10	CXCL5	CXCL6	CXCL9	EDN1	EFNA1	EFNB2	EGF	ENG	EPHB4
ERBB2	F3	FGF1	FGF2	FGFR3	FIGF	FLT1	FN1	HGF	HIF1A	HPSE	ID1
IFNA1	IFNG	IGF1	IL1B	IL6	CXCL8	ITGAV	ITGB3	JAG1	KDR	LECT1	LEP
MDK	MMP14	MMP2	MMP9	NOS3	NOTCH4	NRP1	NRP2	PDGFA	PECAM1	PF4	PGF
PLAU	PLG	PROK2	PTGS1	S1PR1	SERPINE1	SERPINF1	SPHK1	TEK	TGFA	TGFB1	TGFB2
TGFBR1	THBS1	THBS2	TIE1	TIMP1	TIMP2	TIMP3	TNF	TYMP	VEGFA	VEGFB	VEGFC
ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Plate 1 RT^2 Human Angiogenesis PCR Array. The plate contained a selection of 84 different angiogenic genes, as well as housekeeping genes and PCR controls.

All reactions were performed for 50 cycles with the following temperature profiles: 95ºC for 10 minutes (initiation), and 50 cycles of 95ºC for 15 seconds (denaturation), 60ºC for 1 minute (annealing, extension, and measurement). All PCR studies were coupled to melting curve analysis with the following profile: 95ºC for 10 seconds, followed by 65ºC for 5 seconds, and 95ºC for 5 seconds.

3.5 Carboplatin-condition media

To prepare condition media, ovarian cancer cells were seeded at 10,000 cells/cm^2 overnight. Cells were then exposed to 0 μg/mL (control) and 50 μg/mL (135 μM) carboplatin for 72 hours. Following exposure, viable cell number was measured in replicates using Sceptor cell counter. This cell number was used to normalize condition media as described below. Subsequently, media was replaced with DMEM/0.5% FBS media and cells were incubated for 24 or 48 hours. DMEM/0.5% FBS was selected because it adequately sustains viable cells without complete starvation, this was determined empirically in our laboratory. After the appropriate time point, the media was collected and passed through 2 μm filters. Viable cell number was measured at time of

media collection. Condition media was normalized relative to the number of cells measured after 72 hours of initial exposure to carboplatin (**Figure 3.5.1**). In addition, carboplatin-containing media was added to culture dishes without any cells. This group was designed as negative control to test for any potential effects of residual carboplatin which may be transferred while preparing condition media.

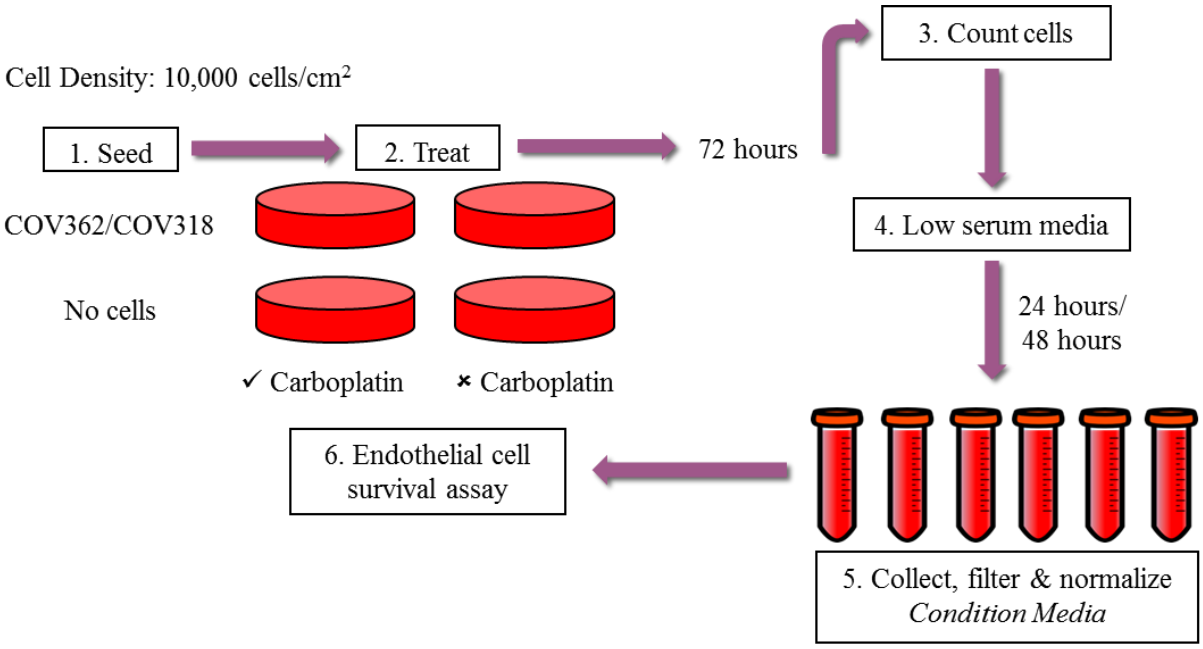


Figure 3.5.1 Preparation of condition media. Cells were plated at 10,000 cells/cm^2 and exposed to 50 µg/mL (135 µM) carboplatin for 72 hours. Media was replaced with a low serum-containing media (DMEM/0.5% FBS) for 24 or 48 hours. Viable cell count was measured at 72 hours following carboplatin exposure, and following low serum media collection. The condition media was filtered, collected and normalized to the number of cells from the 72-hour count.

3.6 Endothelial cell survival assay

To assess the effect of potential angiogenic factors released by ovarian cancer cells following exposure to carboplatin, I utilized an endothelial cell survival assay. HDMECs were seeded at 5,000 cell/cm^2 in EBM2 media supplemented with 20% FBS and 1x PSF. After overnight culture, cells were washed and exposed to condition media from COV318 and COV362 (**Methods and Materials 3.5**; **Figure 3.5.1**). HDMECs were also

cultured in fresh EBM2/20% FBS as positive control. Following 48 hours of incubation, viable cell number was measured and data was normalized to DMEM/0.5% FBS control.

Endothelial cell survival assay was also performed with recombinant proteins of interest. *PGF* and *IL1B* were significantly upregulated after carboplatin exposure in my qRT-PCR experiments across all three cell types used in this study: HDMEC, COV362 and COV318 (**Results 4.3**). To that end, I investigated the functional significance of PGF as well as its potential interaction with VEGFA, the main driver of angiogenesis, on endothelial cell survival by adding recombinant human proteins to HDMEC cultures. HDMECs were plated and allowed to attach. Cells were then exposed to DMEM/0.5% FBS media containing 10 ng/mL recombinant human VEGFA (rVEGFA, R & D Systems) and/or 10 ng/mL recombinant human PGF (rPGF, PeproTech Inc.). Cells were incubated for 48 hours and viable cell number was measured and normalized to DMEM/0.5% FBS control.

3.7 Immunocytochemistry

Ovarian cancer cells COV318 and COV362 were seeded at 10,000 cells/cm^2 on uncoated Nunc™ Lab-Tek™ II 8-chambered slides (Thermo Fisher). Cells were then exposed to 50 μg/mL (135 μM) carboplatin for 72 hours. Some cells were treated with 500 nM or 1 μM Wingless-type MMTV integration site family member (Wnt) agonist. Initially, cells were probed for PGF by labeling cells with primary PGF antibody (based on results, see section **4.3**). However, PFG staining was not robust and did not yield meaningful data. As a positive control for the staining studies, I used primary rabbit anti-β-catenin antibody (ab6302, Abcam). Briefly, cells were fixed with methanol and incubated with anti-β-catenin antibody for 1 hour at room temperature. PBS containing 1% bovine serum album was used as diluent. Subsequently, all slides were incubated with the appropriate fluorophore-conjugated secondary antibody for 1 hour at room temperature. Slides were counterstained using ProLong® Diamond Antifade Mountant containing 4, 6’ diamidino-2-phenylindole (DAPI) (Life Technologies). To test for non-specific secondary antibody staining, secondary antibody incubation without primary antibody incubation was used as negative control. Images were obtained using Olympus BX-51 fluorescence microscope

(Olympus Canada Inc.) equipped with SPOT digital camera. Images were processed by SPOT Imaging Software (SPOT Imaging Solutions).

3.8 Effect of Wnt pathway activation on *PGF* and *IL1B* expression

I then wanted to investigate whether the angiogenic factors of interest (*PGF* and *IL1B*) induced by carboplatin may potentially be induced by the Wnt/β-catenin pathway using Wnt agonist (**Material and Methods 3.7**). I cultured ovarian cancer cells and exposed COV362 and COV318 to 500 nM, 1 μM and 2 μM Wnt agonist (Millipore Calbiochem; CAS 853220-52-7) for 48 hours. RNA was isolated and cDNA was synthesized as previously described (**Material and Methods 3.4**). Gene expression was assessed by qRT-PCR, using *PGF* and *IL1B* primers (Qiagen; **Table 3.4.1**) and RT^2 SYBR Green Mastermix. Data was analyzed by CFX Manager Software by the ΔΔCT method and target gene mRNA data was normalized to *β-actin*.

Table 3.8.1 List of primers used.

Gene	*Source (Catalogue ID)*
B-actin	Qiagen (QT01680476)
PGF	Qiagen (QT00030688)
IL1B	Qiagen (QT00021385)

3.9 Statistical analysis

Statistical analysis was performed using student's t-test or ANOVA where applicable. Quantitative PCR data was analyzed by CFX Manager Software. Data are expressed as ± SD. P values less than 0.05 were considered statistically significant.

Chapter 4

4 RESULTS

4.1 Effect of carboplatin on ovarian cancer and endothelial cells

My first aim was to determine a carboplatin concentration that significantly decreases viability of ovarian cancer cells. This was essential to identify potential angiogenic factor production in response to carboplatin exposure. Based on a recent study[81], I anticipated that carboplatin may reduce viability at 50 µg/mL (135 µM), at least in endothelial cells. Wild *et al.* (2004) also showed that MA148 ovarian cancer cells exhibit significantly reduced viability at 0.7 µg/mL (2 µM) [81]. However, these MA148 cells are less characterized as compared to other available cell lines and are simply described in the literature as epithelial ovarian carcinoma[85]. Therefore, I exposed ovarian cancer cells and endothelial cells to carboplatin at concentrations of 0, 10, 25, and 50 µg/mL (3 µM, 79 µM, 67 µM, and 135 µM, respectively) for 72 hours. My results show that 50 µg/mL carboplatin significantly decreases viability of both ovarian cancer cell lines as well as that of vascular endothelial cells compared to respective control media (**Figures 4.1.1 A, B and C**). Contrary to the previous report[81], my results indicate that HDMECs are more sensitive to carboplatin than ovarian cancer cells. HDMECs exhibited significantly lower viability at 10 µg/mL (79 µM), whereas viability of ovarian cancer cells was not affected by carboplatin at 10 µg/mL. Approximately 50 percent reduction in viable cell counts were noted in ovarian cancer cells following carboplatin exposure at 25-50 µg/mL (67 µM - 135 µM). Based on these results, 50 µg/mL carboplatin was selected for subsequent studies as it produced significant reduction in viability in all three cell types.

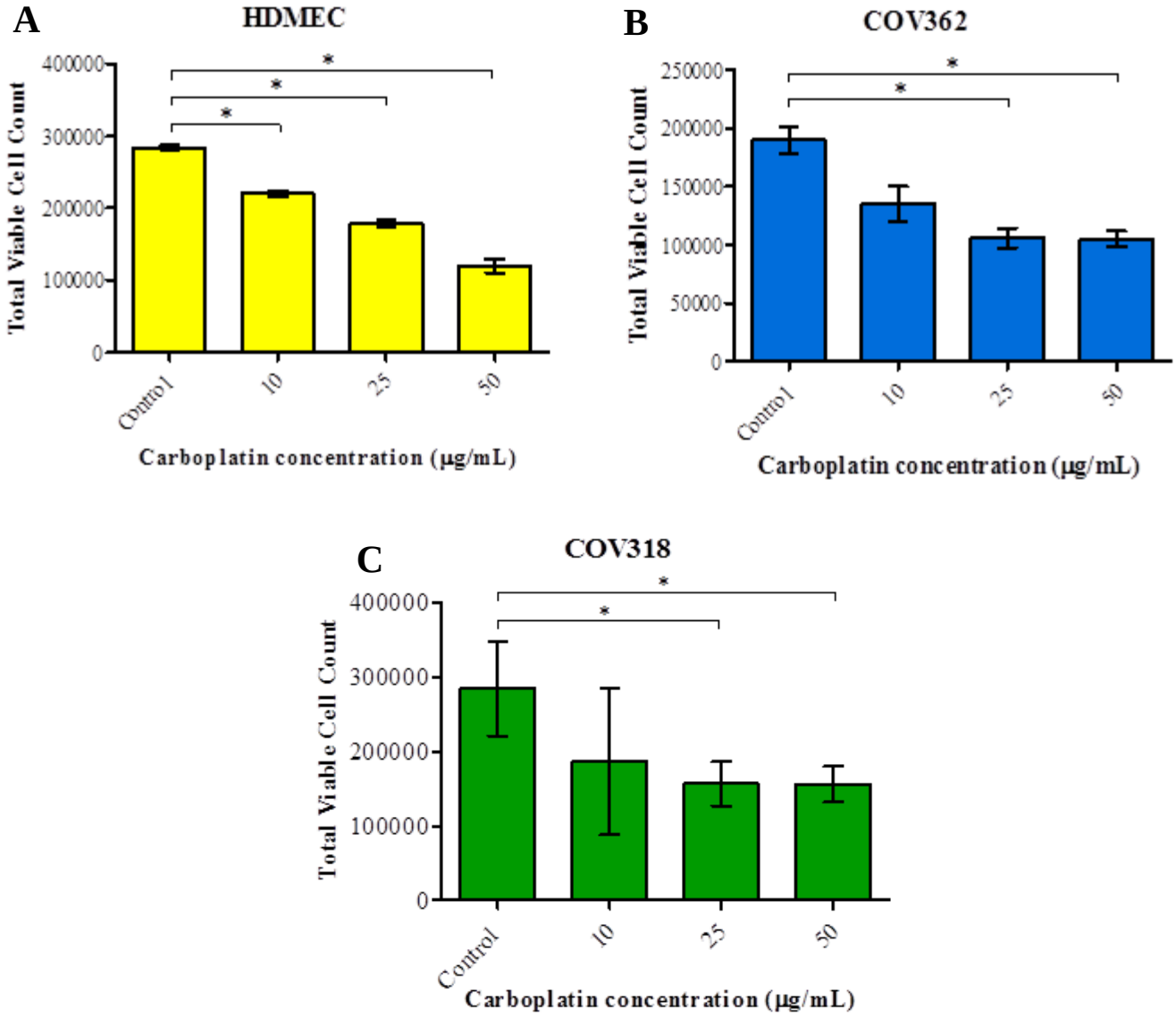


Figure 4.1.1 Effect of carboplatin on ovarian cancer cells and endothelial cells. Cells were exposed to 0 µg/mL, 10 µg/mL, 25 µg/mL or 50 µg/mL (3 µM, 79 µM, 67 µM, and 135 µM respectively) carboplatin for 72 hours. Total cell count was measured for HDMEC (endothelial cells- yellow **A**), COV362 (endometrioid ovarian cancer cells- blue **B**) and COV318 (serous ovarian cancer cells- green **C**). n=3. Data expressed as mean ± SD; *$p < 0.05$.

4.2 Carboplatin induced cell death

As viability is a sum of both positive and negative growth signals, I examined whether carboplatin was inducing cell death in this experimental platform. To measure cell death, I utilized the trypan blue exclusion test[86]. Since the focus of my studies was to identify angiogenic factors produced in response to carboplatin and not necessarily the mechanism of cell death, trypan blue dye test was sufficient for this purpose. HDMECs, COV362 and COV318 cells were exposed to 50 μg/mL carboplatin for 72 hours and then stained with trypan blue. Live and dead percentage was measured using Countess II automated cell counter. Results show approximately 70% cell death in HDMECs following carboplatin exposure (**Figure 4.2.1A**). A similar level was obtained in COV362 (**Figure 4.2.1B**) and COV318 cells (**Figure 4.2.1C**). Therefore, carboplatin reduces viability by inducing cell death.

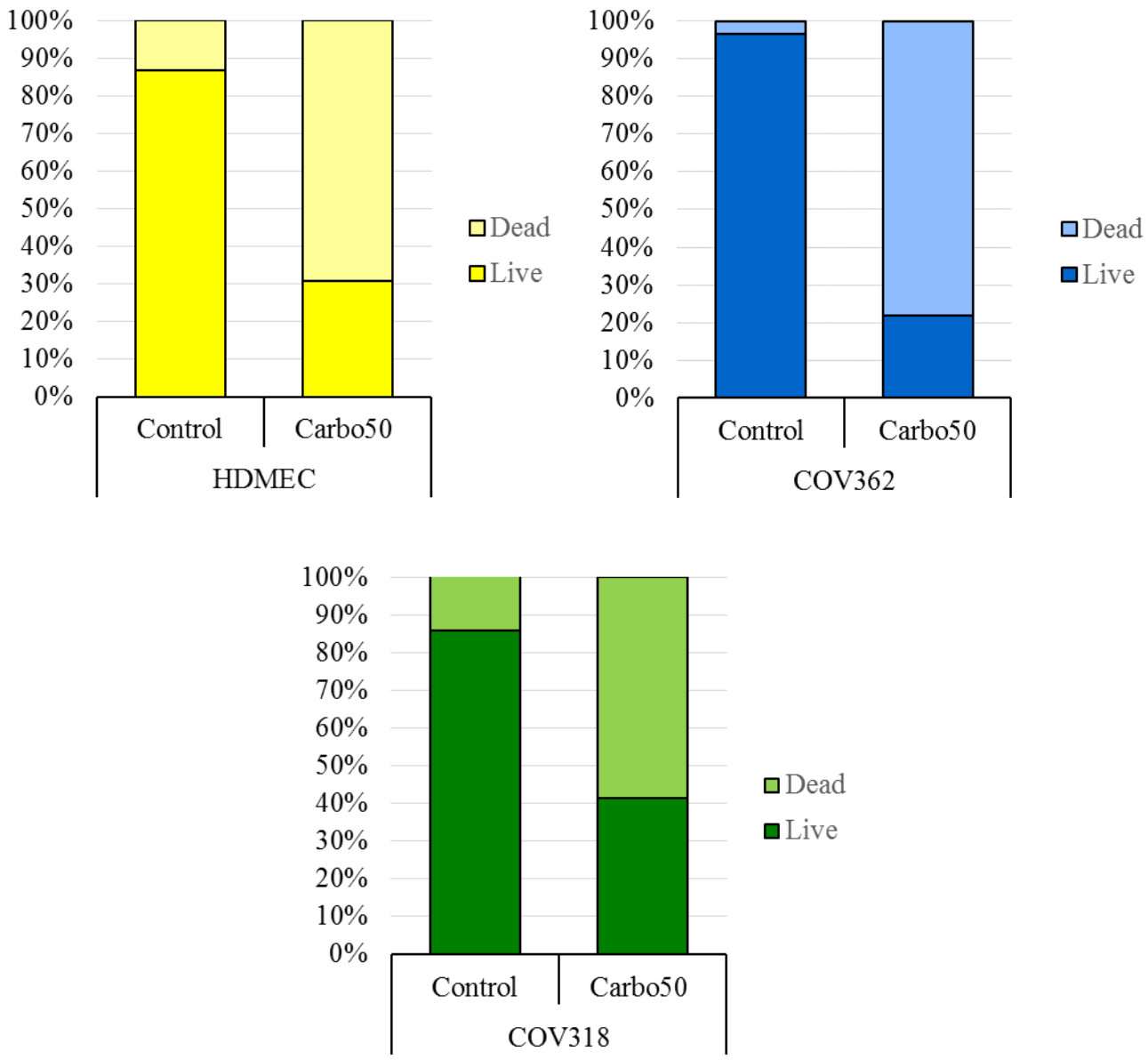


Figure 4.2.1 Total Live/Dead percentage of endothelial cells and ovarian cancer cells post-carboplatin exposure. Cells were exposed to 50 µg/mL (135 µM) carboplatin for 72 hours and live/dead percentage was measured by trypan blue exclusion test. Results shown represent average of at least 2 experimental replicates.

4.3 Effect of carboplatin on angiogenic gene expression profile

My next objective was to identify potential angiogenic factors which may be induced in ovarian cancer cells following carboplatin exposure. To achieve this, HDMECs, COV362 and COV318 cells were exposed to 50 μg/mL carboplatin for 72 hours. RNA was isolated and qRT-PCR was performed to profile angiogenic factors. RT^2 PCR array comprised of various growth factors, cytokines, extracellular matrix proteins, intracellular mediators and cell surface protein genes involved in angiogenesis (**Plate 1**, **Appendix 1**). There were several genes that showed greater than two-fold increase after carboplatin exposure in HDMECs (**Figure 4.3.1**), COV362 (**Figure 4.3.2**) and COV318 cells (**Figure 4.3.3**).

It is interesting to note that HDMECs also showed increased angiogenic factor expression possibly indicating autocrine regulation of neovascularization. Levels of angiopoietin-like 4 (*ANGPTL4*), interferon alpha 1 (*IFNA1*), interleukin 1 beta (*IL1B*), *PGF*, plasminogen activator, urokinase (*PLAU*), plasminogen activator inhibitor type-1 (*SERPINE1*), transforming growth factor beta receptor 1 (*TGFBR1*) and *VEGFA* were all increased in HDMECs (**Figure 4.3.1**). Of these numerous factors, only VEGFA has been shown to be induced by carboplatin previously[81].

In comparison to HDMECs, COV362 cells showed significant increases in *ANGPTL4*, Chemokine (C-X-C motif) ligand 5 (*CXCL5*), Ephrin-A1 (*EFNA1*), coagulation factor (*F3*), fibroblast growth factor 1 (*FGF1*), fibroblast growth factor receptor 3 (*FGFR3*), *IL1B*, interleukin-6 (*IL6*), leukocyte cell derived chemotaxin 1 (*LECT1*), matrix metallopeptidase 9 (*MMP9*), *NOTCH4*, *PGF*, sphingosine-1-phosphate (*S1PR1*), sphingosine kinase 1 (*SPHK1*) and transforming growth factor alpha (*TGFA*) (**Figure 4.3.2**).

COV318 were included to strengthen the results. My data showed significant increase in angiogenin (*ANG*), collagen, type IV, alpha 3 (*COL4A3*), *EFNA1*, *F3*, *FGF1*, *FGF2*, hepatocyte growth factor (*HGF*), *IL1B*, *IL6*, midkine (*MDK*), *NOTCH4*, *PDGFA*, *PGF*,

S1PR1, *SERPINE1*, *SPHK1*, transforming growth factor beta 1 (*TGFB1*) and thrombospondin (*THBS1*) (**Figure 4.3.3**).

Comparison of all three cell types showed that *PGF* and *IL1B* were induced by carboplatin in all cells used in my studies (**Table 4.2.1**).

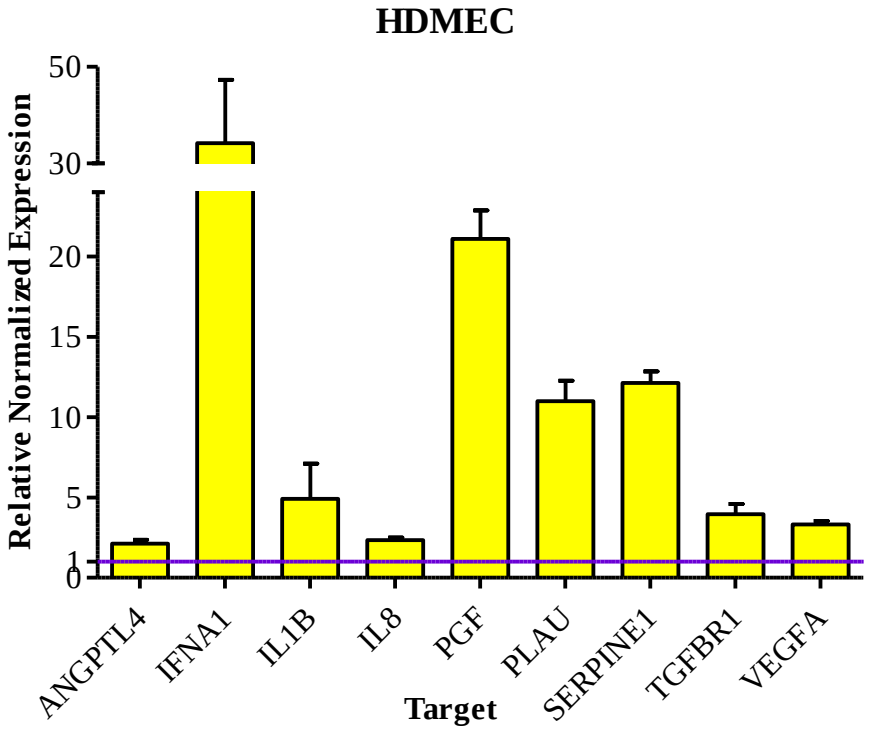


Figure 4.3.1 Angiogenic gene expression profile of HDMEC in response to carboplatin. Gene expression of various growth factors with significantly increased (greater than 2-fold, $p < 0.05$) levels post 72-hour 50 µg/mL (135 µM) carboplatin exposure relative to control (0 µg/mL). Control represented by value line 1 (purple). Relative expression normalized to *ACTB*, *B2M*, *GAPDH*, *HPRT1* and *RPL0P*. Data is derived from at least 3 experimental replicates and expressed as mean + SD.

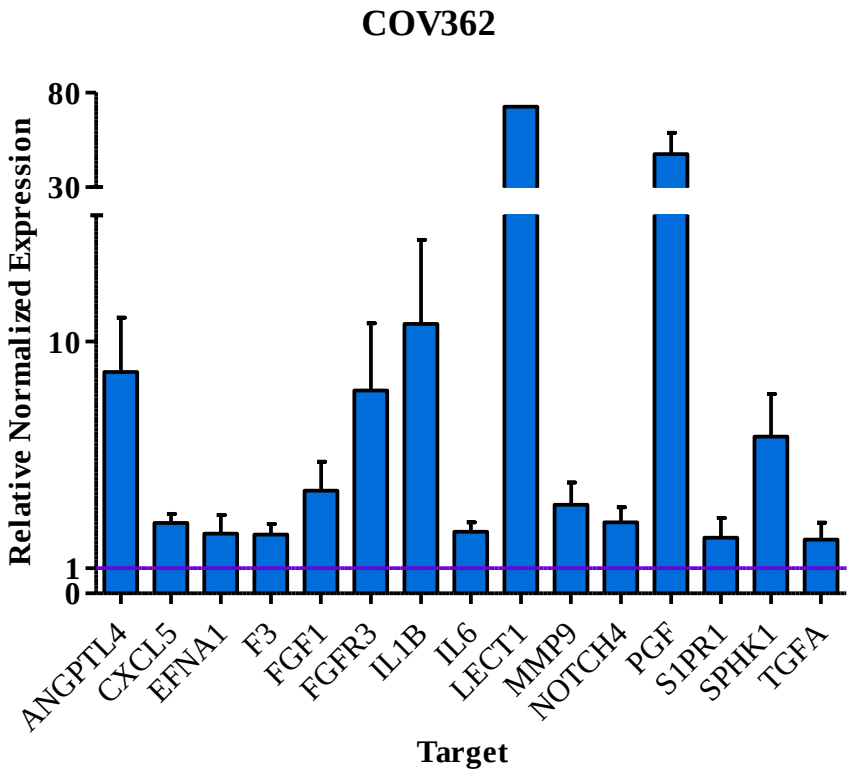


Figure 4.3.2 Angiogenic gene expression profile for COV362 in response to carboplatin. Gene expression of various growth factors with significantly increased (greater than 2-fold, $p < 0.05$) levels post 72-hour 50 µg/mL (135 µM) carboplatin exposure relative to control (0 µg/mL). Control represented by value line 1 (purple). Relative expression normalized to *ACTB*, *B2M*, *GAPDH*, *HPRT1* and *RPL0P*. Data is derived from at least 3 experimental replicates and expressed as mean + SD.

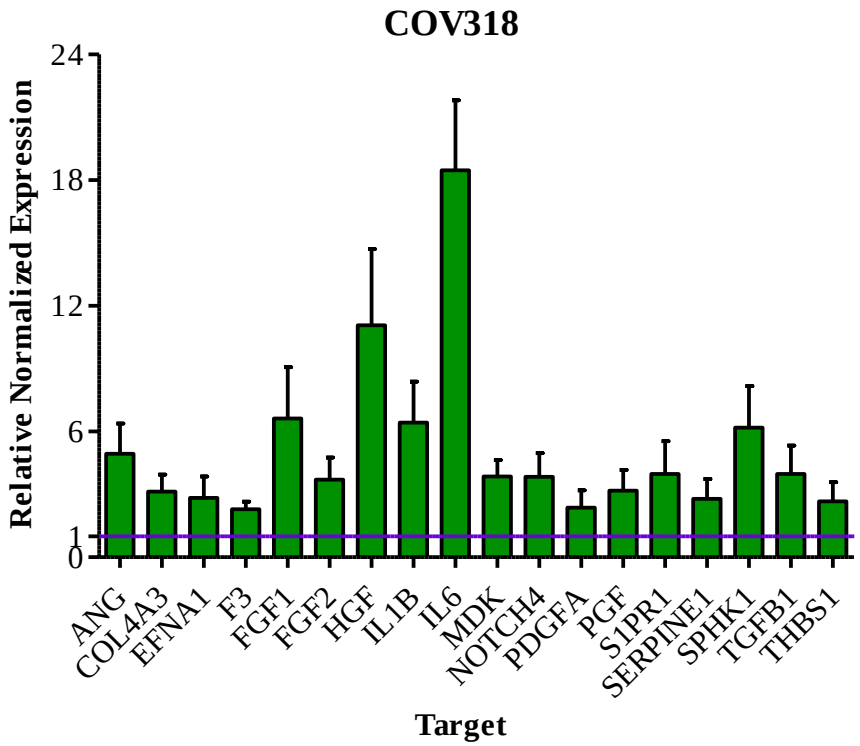


Figure 4.3.3 Angiogenic gene expression profile for COV318 in response to carboplatin. Gene expression of various growth factors with significantly increased (greater than 2-fold, $p < 0.05$) levels post 72-hour 50 μg/mL (135 μM) carboplatin exposure relative to control (0 μg/mL). Control represented by value line 1 (purple). Relative expression normalized to *ACTB*, *B2M*, *GAPDH*, *HPRT1* and *RPL0P*. Data is derived from at least 3 experimental replicates and expressed as mean + SD.

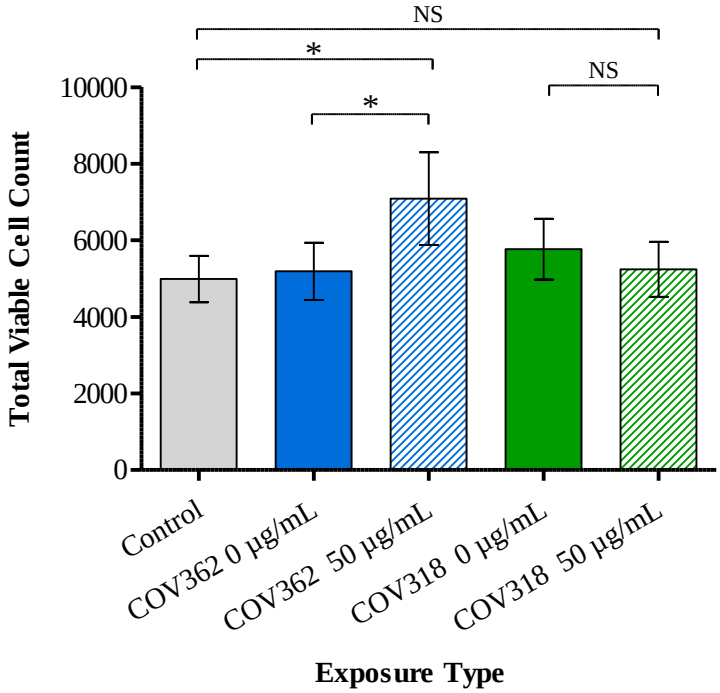
Table 4.3.1 Common target mRNA expression-fold in HDMEC, COV362 and COV318.

	Relative normalized expression		
Gene	**HDMEC**	**COV362**	**COV318**
IL1B	4.9	10.7	6.4
PGF	21.1	47.4	3.2

4.4 The effect of ovarian cancer cell condition media on endothelial cells

My results show that a host of angiogenic factors are induced by carboplatin in ovarian cancer cells and endothelial cells. My next objective was to determine the functional significance of this induction. I subjected endothelial cells to condition media prepared from ovarian cancer cells exposed to carboplatin and assessed viability. This is a modified cell survival assay and employs low serum levels to determine whether condition media is able to sustain or enhance cell viability. Endothelial cells were plated and allowed to attach. Cells were then exposed to ovarian cancer condition media.

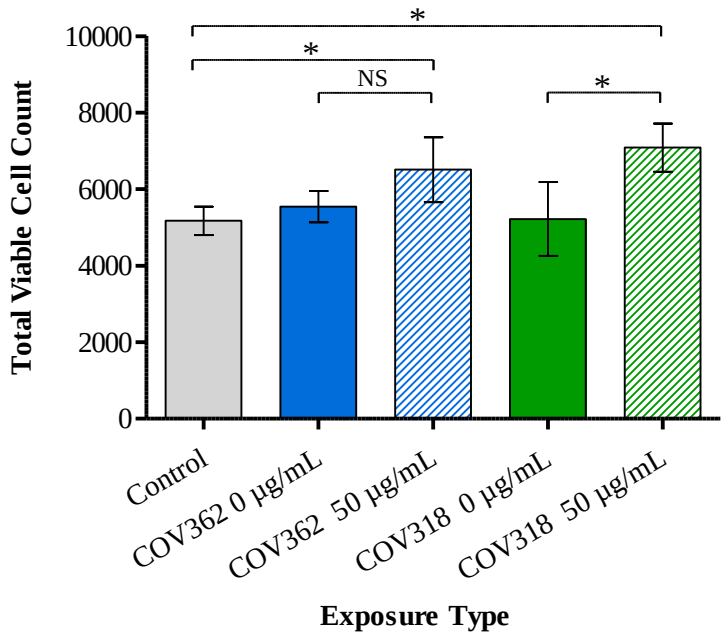
Negative control condition media was used to determine whether any residual carboplatin from media transfers and washes may negatively affect the experimental readout. My results show that negative control media does not significantly change viable cell counts (data not shown). HDMECs also showed increased viability counts in EBM2/20% FBS media as expected (data not shown). Incubation of endothelial cells with condition media produced interesting results. First, I tested condition media collected over a 24-hour period following carboplatin exposure. Condition media prepared from COV362 cells with 50 μg/mL carboplatin increased cell viability as compared to control media (no carboplatin) (**Figure 4.4.1**). This increase was also evident when comparing the condition media to fresh DMEM/0.5% FBS control. However, condition media prepared from COV318 cells did not show any significant alterations (**Figure 4.4.1**).



Collection: 24 hours
Cell density: 5000 cells/cm^2
Time: 48 hours

Figure 4.4.1 HDMEC response to 24-hour condition media. HDMECs were exposed to condition media prepared from COV362 or COV318 cells for 48 hours. N=2 (endothelial cell preparation); n=6 (experimental replicates). Data expressed as mean ± SD. NS = not significant, *p < 0.05.

It is possible that 24-hour collection period for COV318 cell condition media is not sufficient to produce any effects. In support of this notion, my studies show that COV318 increase *PGF* by approximately 3-fold as compared to 40-fold induction in COV362 cells (**Table 4.3.1**). The same pattern is seen with *IL1B*. Therefore, I increased the collection period to 48 hours. Endothelial cells exposed to COV362 condition media did not indicate significant increase in viable cell number after carboplatin exposure versus control (**Figure 4.4.2**). However, compared to DMEM/0.5% FBS control, viability was significantly increased (**Figure 4.4.2**). In addition, HDMEC viability was significantly increased when exposed to COV318 condition media with carboplatin (**Figure 4.4.2**).



Collection: 48 hours
Cell density: 5000 cells/cm^2
Time: 48 hours

Figure 4.4.2 HDMEC response to 48-hour condition media. HDMECs were exposed to condition media prepared from COV362 or COV318 cells for 48 hours. N=1 (endothelial cell preparation); n=6 (experimental replicates). Data expressed as mean ± SD. NS = not significant, *$p < 0.05$.

4.5 Recombinant PGF induces endothelial cell growth

As all three cell types showed increased expression of *PGF*, it represented an interesting target as it may directly affect endothelial cells, as well as indirectly by eliciting other angiogenic factors[76]. PGF is postulated to stimulate angiogenesis by binding to VEGFR1 and stimulating downstream signals[87]. Binding to VEGFR1 by PGF also displaces VEGFA which is then able to bind VEGFR2 to initiate angiogenic effects[87]. Moreover, Carmeliet *et al.* (2001) showed that the addition of PGF or VEGF alone resulted in minimal outgrowth in aortic ring capillary growth assay. However, PGF and VEGF together strongly stimulated capillary outgrowth[87].

To elucidate the roles of PGF and VEGF and investigate possible synergy, I exposed HDMECs to recombinant human PGF (rPGF) and recombinant human VEGFA (rVEGFA) for 48 hours. There was a significant increase in viable cell number when HDMECs were treated with 10 ng/mL rPGF, rVEGFA, or the combination of the two factors compared to the control DMEM/0.5% FBS (**Figure 4.5.1**). rVEGFA was more potent in increasing cell viability compared to rPGF (**Figure 4.5.1**). However, there was no significant difference between HDMECs exposed to rVEGFA versus combination exposure of rPGF and rVEGFA. This suggests two important biological phenomena. First, rPGF alone is able to increase HDMEC growth and second, that rPGF does not enhance the effect of rVEGFA.

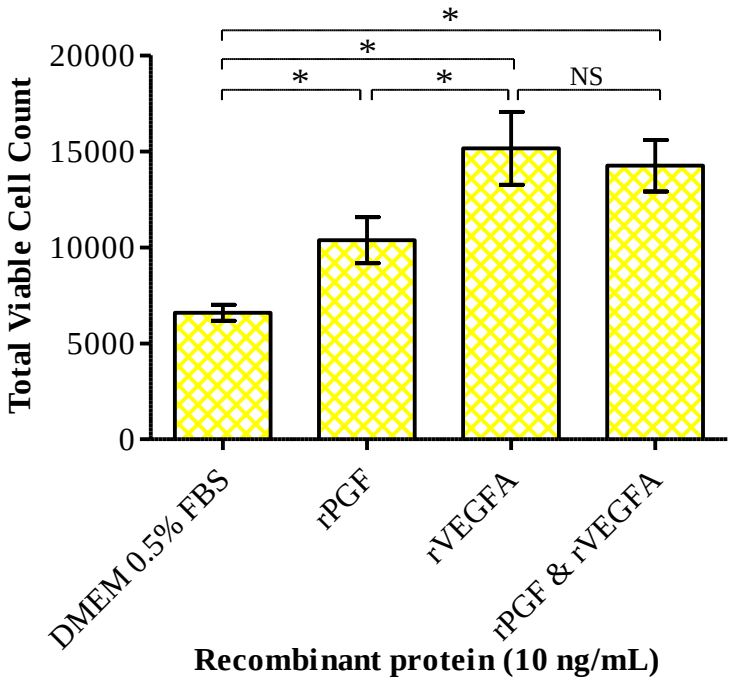


Figure 4.5.1 HDMEC viability in response to recombinant PGF and VEGFA. HDMECs were exposed to 10 ng/mL recombinant PGF and/or VEGFA for 48 hours. N=2 (endothelial cell preparations); n=6 (experimental replicates). Data expressed as mean + SD. NS = not significant, *$p < 0.05$.

4.6 Nuclear β-catenin translocation following carboplatin exposure

I performed immunofluorescence (IF) staining to confirm PGF induction in ovarian cancer cells after carboplatin exposure as seen in **Figure 4.3.2** (COV362) and **Figure 4.3.3** (COV318). β-catenin was initially included as a positive control. β-catenin is found in a complex with epithelial (E)-cadherin at cell-cell junctions in epithelial cells. IF staining was unable to provide a robust signal from PGF (data not shown). This is not fully unexpected as these are secreted growth factors. However, interesting and unexpected results were obtained when ovarian cancer cells were stained for β-catenin. Following carboplatin exposure, decreased membrane localization of β-catenin and increased nuclear β-catenin was noted in COV362 and COV318 cells (**Figure 4.6.1** and **Figure 4.6.2**). Nuclear β-catenin has been reported to be implicated in mediating the epithelial-mesenchymal transition (EMT) in a variety of human cancers. During this process, epithelial cells lose expression of proteins like E-cadherin, and β-catenin translocates to the nucleus to transcribe β-catenin-responsive genes. Interestingly, elevated β-catenin activity was recently shown to contribute to carboplatin resistance in A2780cp ovarian cancer cells[88].

To confirm whether nuclear β-catenin following carboplatin exposure is analogous to Wnt pathway activation, I treated ovarian cancer cells with a Wnt agonist and localized β-catenin. My results show that Wnt agonist reduces membrane-associated β-catenin after 48 hours relative to control (unexposed to Wnt agonist) in both COV362 cells and COV318 cells (**Figure 4.7.1**). These results may indicate canonical Wnt pathway activation[89].

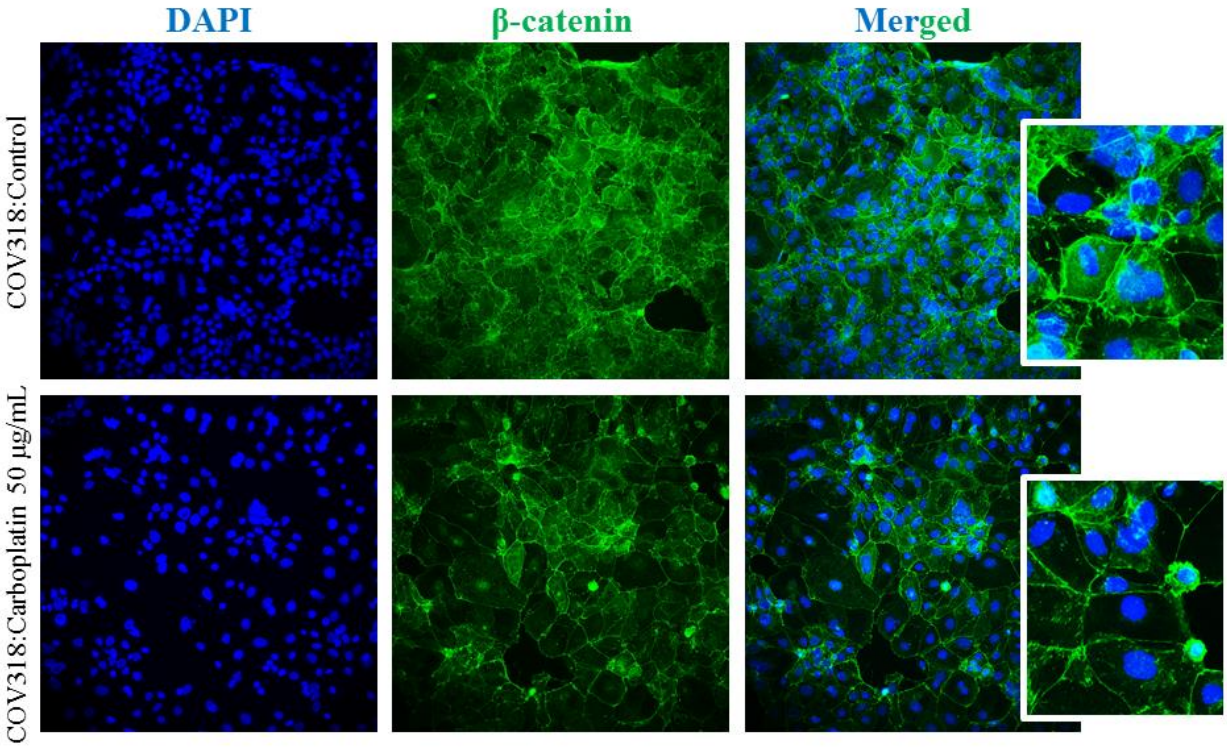


Figure 4.6.1 β-catenin localization in COV318 cells. Immunofluorescence **(**IF) staining of β-catenin (green) in control and carboplatin-challenged cells. Carboplatin exposure was carried out for 72 hours. Cells were counterstained with DAPI nuclear stain (blue).

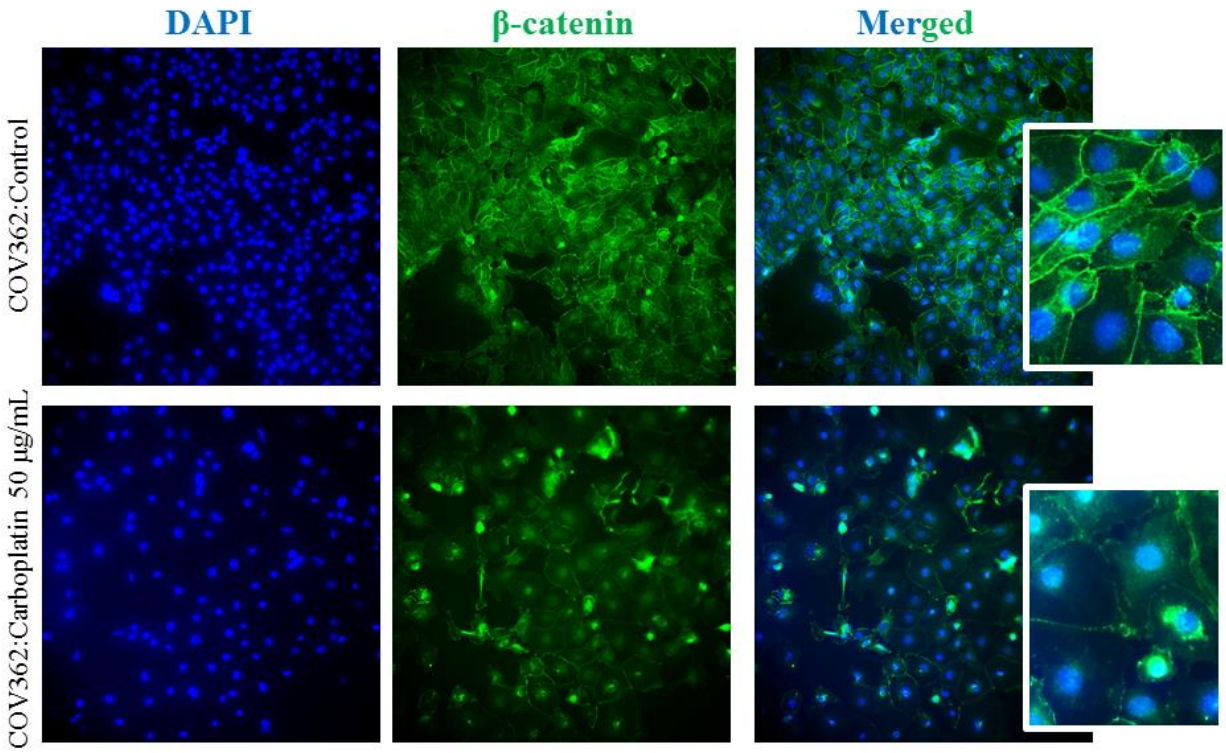


Figure 4.6.2 β-catenin localization in COV362 cells. Immunofluorescence **(**IF) staining of β-catenin (green) in control and carboplatin-challenged cells. Carboplatin exposure was carried out for 72 hours. Cells were counterstained with DAPI nuclear stain (blue).

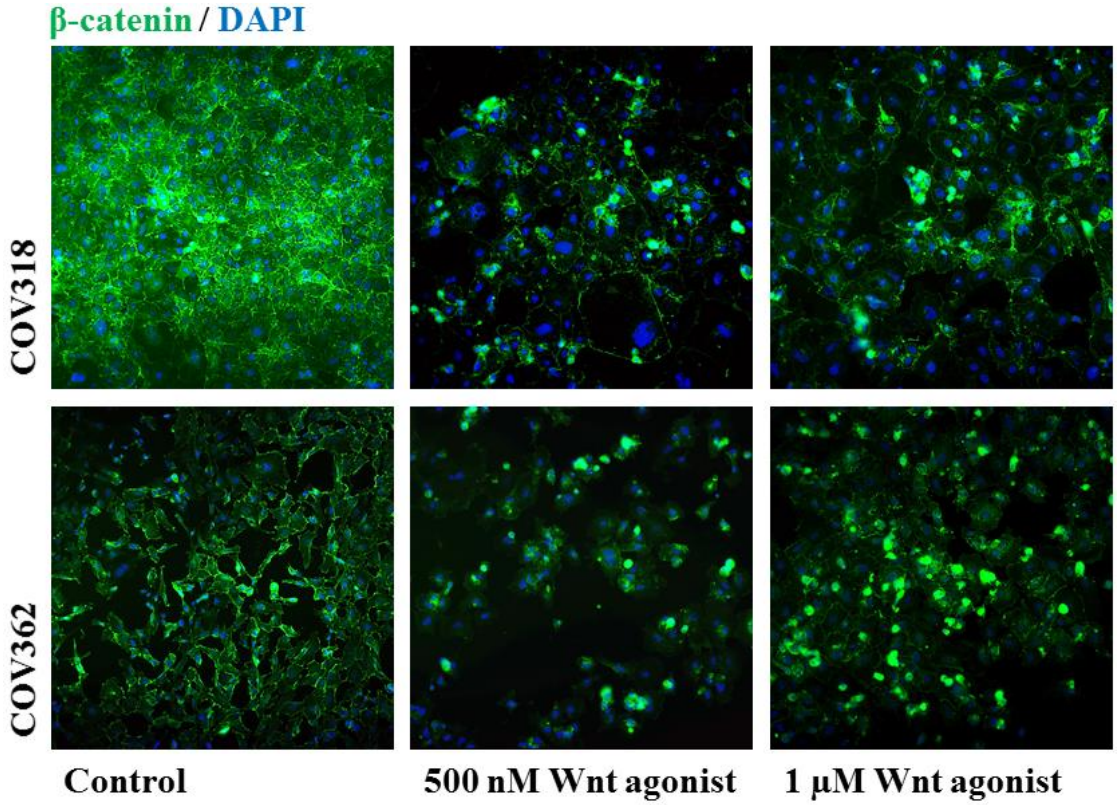


Figure 4.6.3 β-catenin localization after Wnt agonist treatment in COV318 and COV362 cells. Immunofluorescence **(**IF) staining of β-catenin (green) in control and Wnt agonist-treated cells. Cells were counterstained with DAPI nuclear stain (blue).

4.7 IL1B and PGF mRNA expression are upregulated after Wnt pathway activation

My results pointed to a novel mechanism involving activation of the Wnt pathway by which carboplatin may induce angiogenic factors. Therefore, I examined mRNA levels of *PGF* and *IL1B* following Wnt agonist exposure. I treated ovarian cancer cells to 500 nM, 1μM or 2 μM Wnt agonist for 48 hours. qPCR results show that *IL1B* is significantly upregulated following treatment of COV362 and COV318 with Wnt agonist (**Figure 4.7.1** and **Figure 4.7.2**). mRNA levels of *PGF* were also increased by Wnt agonist in COV362 cells (**Figure 4.7.1**). Interestingly, COV318 cells showed decreasing mRNA levels of *PGF* after treatment with Wnt agonist (**Figure 4.8.2**).

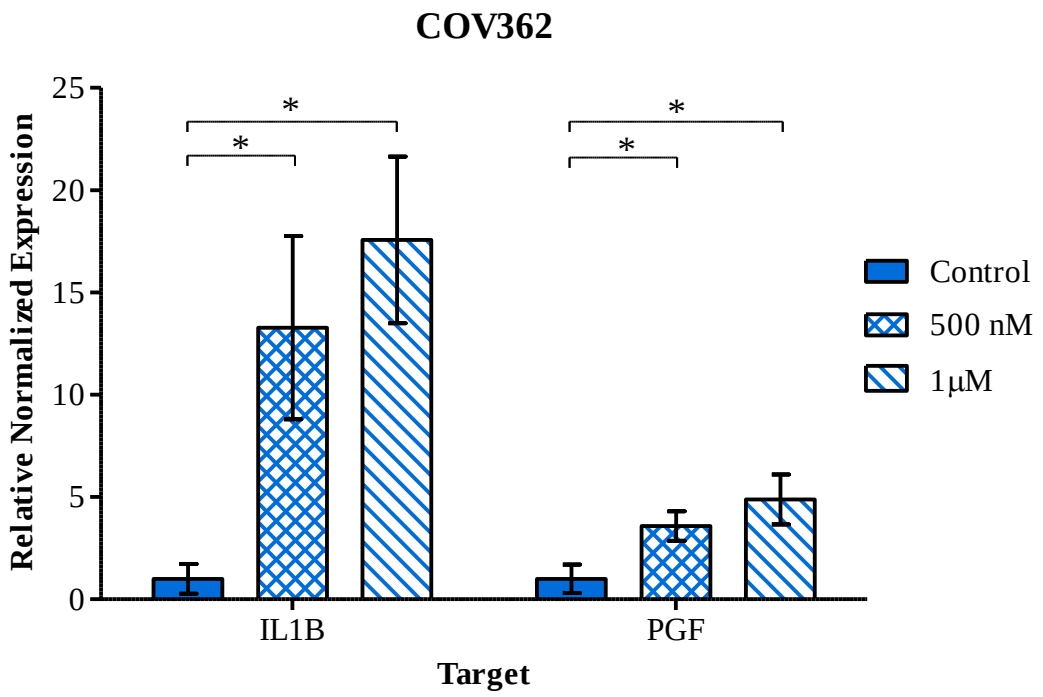


Figure 4.7.1 *IL1B* and *PGF* mRNA levels in COV362 cells treated with Wnt agonist. COV362 cells were exposed to 500 nM or 1 μM Wnt agonist for 48 hours. *PGF* and *IL1B* mRNA expression was normalized to *β-actin*. Results representative of three independent experiments. Data expressed as mean ± SD, *$p < 0.05$.

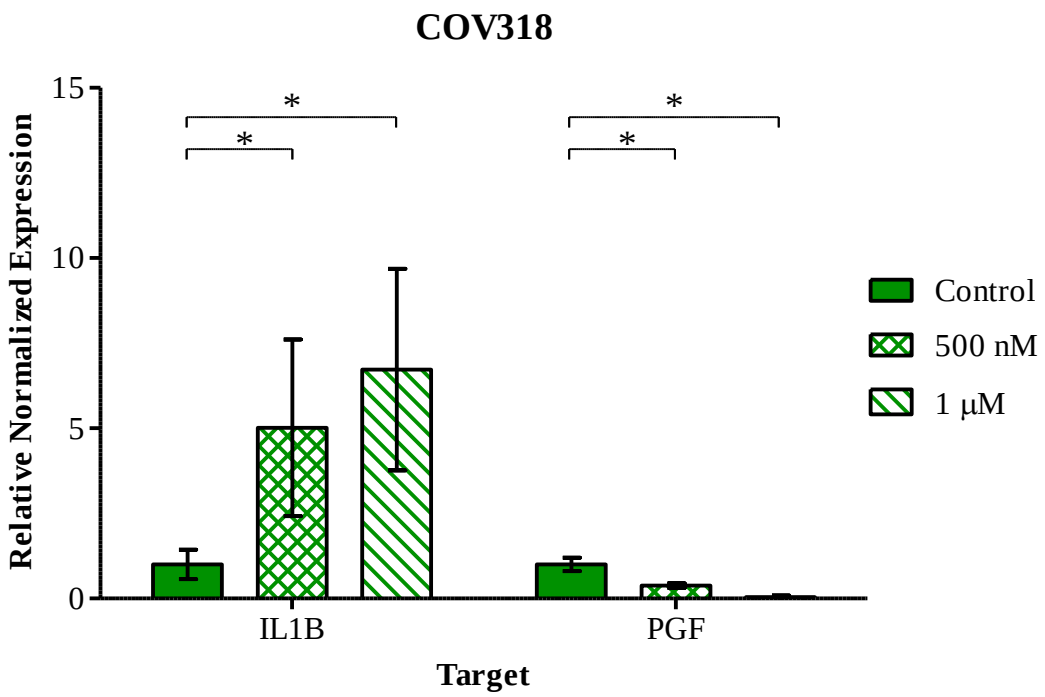


Figure 4.7.2 *IL1B* and *PGF* mRNA levels in COV318 cells treated with Wnt agonist. COV318 cells were exposed to 500 nM or 1 μM Wnt agonist for 48 hours. *PGF* and *IL1B* mRNA expression was normalized to *β-actin*. Results representative of three independent experiments. Data expressed as mean ± SD, *$p < 0.05$.

Chapter 5

5 DISCUSSION

The salient findings of my study show that carboplatin reduces viability of ovarian cancer cells and vascular endothelial cells. Interestingly, this reduced viability was associated with induction of a host of angiogenic genes. These factors included *PGF* and *IL1B* which were induced in the major cellular compartments of the ovarian tumour: tumour cells and vessel-lining endothelial cells. Ovarian cancer cell-induced factors increase viability of vascular endothelial cells as shown by my condition media studies. The results were mimicked by purified recombinant PGF. My studies also suggest that carboplatin may induce angiogenic factors including PGF and IL1B through the activation of Wnt/β-catenin pathway. I will dissect these key findings and discuss their implications below.

5.1 Ovarian cancer cells and endothelial cells in response to carboplatin

Ovarian cancer cells and endothelial cells showed significant cytotoxic response to carboplatin. After carboplatin exposure, percent of dead cells in cultures of HDMEC, COV362 and COV318 cells reached 69%, 78% and 59%, respectively (**Figure 4.2.1**). Variability between experimental and biological replicates (different cell preparations) may present some limitation in accuracy; but fundamentally, there was a cytotoxic effect to carboplatin exposure. Although statistical testing was not performed on data showing the percent of live and dead cells because of this inherent variability, the results showing dramatically reduced viability provide confidence. My results are contrary to ones obtained in a recent and only study of this type[81]. Wild and colleagues (2004) utilized human umbilical vein endothelial cells and MA148 ovarian carcinoma cells and showed that cancer cells are almost 100-fold more sensitive to carboplatin as compared to endothelial cells. There are potentially two reasons for the different results. First, human umbilical vein endothelial cells are typically used as models of large vessels which may be quite different in terms of proliferation and angiogenic responses[90]. Second, MA148

ovarian cancer cell line is not fully characterized[85]. In my study, I examined the effect of carboplatin in microvascular endothelial cells as well as two well characterized ovarian cancer cell lines. Even though the COV362 cell line was originally annotated as an endometrioid ovarian carcinoma, extensive molecular profiling by Domcke *et al.* (2013) has shown that it is one of the top-ranked HGSOC cancer line. Moreover, the COV362 line is known to be more resistant to platinum-based chemotherapy[91]; it is therefore conceivable that increased angiogenic factors released by these tumour cells upon carboplatin treatment may uncover drivers of tumour survival. To bolster my results, I also used the COV318 cell line which also matched its original annotation as HGSOC by molecular profiling[14]. Utilizing these two ovarian cancer cells and human microvascular endothelial cells, I show that carboplatin reduces the viability consistency to levels reaching 50% of controls when carboplatin is used at 25-50 µg/mL (67 µM - 135 µM). It is also interesting to note that viability did not differ in any of the cell types at 25 µg/mL carboplatin versus 50 µg/mL. Almost half of the cells in culture survived exposure to extremely high concentrations of carboplatin and it is possibly these surviving cells produce and release angiogenic factors.

As an alkylating agent, carboplatin causes DNA cross-linkages and disruptions which require complex mechanisms of repair[28]. The DNA mismatch repair (MMR) mechanism maintains genomic stability and recognizes DNA alteration caused by carboplatin and generates an injury signal that may initiate apoptosis of the cell[92]. It was reported that carboplatin resistance may occur due to the loss of MMR as well as the loss of its associated proteins[28]. Moreover, nucleotide excision repair (NER), which repairs changes in DNA helical structure recognizing intra-strand crosslinks, has been implicated in mediating platinum-based drug resistance by way of cross-complementation group 1 (ERCC1)[28]. It was reported that ERCC1 expression negatively correlated with patient survival and platinum therapy response, and knockout of ERCC1 increased sensitivity of platinum-resistant ovarian cancer cell lines[28]. It is possible that dysregulated repair mechanisms may explain acquired chemotherapy resistance by way of modification of downstream angiogenic genes. In support of this notion, gamma-histone H2A variant

H2AX (γ-H2AX) which is rapidly generated by DNA-damaging chemotherapeutic agents, is recently linked to angiogenesis and cancer progression[93].

5.2 Carboplatin induced upregulation of diverse angiogenic genes

One of my key objectives was to answer the question: what specific subset of angiogenic factors are released by ovarian cancer cells and endothelial cells in response to carboplatin exposure? Remarkably, after carboplatin exposure, ovarian cancer cells and endothelial cells exhibited upregulation of a large number of angiogenic genes (**Figure 4.3.1**, **Figure 4.3.2**, and **Figure 4.3.3**). There are a number of ways to digest this wealth of data. We could possibly examine genes which are induced in both ovarian cancer cell types or examine each induction individually. If examined individually, the variation may uncover tumour type-specific angiogenesis factors but would require the profiling of a large number of different ovarian cancer cell lines to provide meaningful results. We could also examine genes which are induced in both COV362 and COV318. In this case, the key factors include *EFNA*, *F3, FGF1, IL1B, Il6*, *NOTCH4*, *PGF, S1PR1* and *SPHK1*. Among these are factors which have been targeted in a number of human cancers. For example, IL6 has been extensively studied in ovarian cancer and is known to be secreted by ovarian cancer cells and through inflammation[94]. In effect, IL6 and its receptor have shown to decrease the effectiveness of taxol-based chemotherapeutic agents on endothelial cell apoptosis and migration[94,95]. Both COV362 and COV318 cells showed increased expression of IL6 post-carboplatin exposure, which may contribute to the survival of endothelial cells seen in the condition media assay (**Figure 4.4.1** and **Figure 4.4.2**). IL6 has also shown to act upstream of MMP9 secretion[96], and COV362 cells in my results reveal increased expression of both genes, which may contribute to overall tumorigenicity.

In my studies, I also profiled endothelial cells to identify autocrine factors which may be involved in neovascularization following carboplatin treatment. Similar to ovarian cancer cells, carboplatin induced the expression of a number of genes including *ANGPTL4*, *IFNA1*, *IL1B*, *IL8*, *PGF, PLAU, SERPINE1*, *TGFBR1*, and *VEGFA.* Some of these

factors have also been targeted in clinical and preclinical studies even if the reasoning was that these may potentially be released by cancer cells. For instance, VEGFA and its tyrosine kinase receptors are the main targets to counteract tumour angiogenesis. Clinical trials are investigating VEGFA inhibitors such as Avastin and Ranibizumab combined with chemotherapy and results have shown improved progression-free survival (PFS) but unchanged overall survival rates in comparison to chemotherapy alone[97]. In addition, Avastin has shown to improve progression-free survival (PFS) with regular maintenance therapy[77]. However, the upregulation of other pro-angiogenic factors shows that anti-VEGF therapies may not be enough to block angiogenesis[98]. Nevertheless, the reasons for varying responses to anti-VEGF therapy among some patients and tumour types remain unclear, and current research is turning to predictive biomarkers to distinguish patients that can benefit from anti-VEGFA therapies[17,97].

A limitation in my studies is the emphasis on upregulation of angiogenic genes and not their downregulation including that of anti-angiogenic factors. A number of genes were shown to be significantly decreased after carboplatin exposure in my studies (data not shown). As the balance between pro- and anti-angiogenic factors dictates the angiogenic switch, examining anti-angiogenic factors may further expose changes that take place post-carboplatin exposure in the tumour microenvironment.

5.3 PGF as a potential mediator of carboplatin-induced angiogenesis

PGF is reported to have controversial effects in cancer; highly expressed in some cancers such as gastric and breast carcinomas, but downregulated in others such as colon and lung carcinomas[99]. Also, the inhibition of PGF has shown to have antitumor and antiangiogenic effects in some cancers but again, not others[99]. Under physiological conditions, PGF is primarily known to be expressed in placenta; however, it is also expressed at low levels in other tissues such as the heart, lung, thyroid, skeletal muscle and adipose tissue[100]. PGF was shown to play a redundant role in vascular development and physiological vessel maintenance in healthy adults[101]. Yet, PGF is variably upregulated in tumours[87] and it is stipulated to contribute to pathological angiogenesis in

a number of ways[101]. One way that PGF stimulates angiogenesis is through modulating VEGF, a crucial factor involved in stimulating endothelial cells as well as increasing permeability of microvasculature[17,101]. VEGFA can bind to both VEGFR1 and VEGFR2; meanwhile PGF binds primarily to VEGFR1. Binding of PGF to VEGFR1 limits VEGFA binding and displaces bound VEGFA because of higher affinity[63]. This, in turn, amplifies endogenous VEGFA signaling through VEGFR2[87]. Signaling through VEGFR2 promotes tyrosine residue phosphorylation, activating downstream RAS/RAF/ERK/MAPK pathway and the PI3K/AKT pathway, ultimately stimulating endothelial cell proliferation and survival respectively[50]. Also, though with weaker effects, PGF can initiate a similar angiogenic cascade by binding to VEGFR1, inducing phosphorylation of tyrosine residue Y1309, which is involved in stimulating the AKT pathway, enhancing signaling leading to neovascularization[50]. By phosphorylation of tyrosine residue Y784 and Y1169, PGF also promotes endothelial cell proliferation[50]. Furthermore, although with controversial evidence, PGF and VEGF may form homodimers and heterodimers contributing to endothelial cell mitogenic and chemotactic activity[87,102]. **Figure 5.3.1** summarizes some of the important interactions regarding PGF and VEGF.

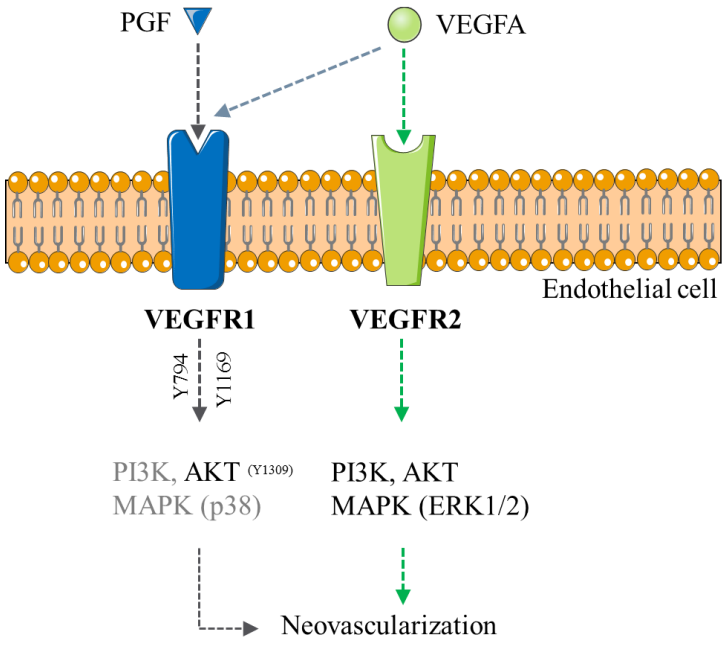


Figure 5.3.1 PGF and VEGFA interactions.

Increased PGF and VEGFA have been shown to recruit monocytes/macrophages to cancer tissues to induce angiogenesis[103]. In monocytes, PGF has shown to initiate VEGFR1 phosphorylation to lead to the PI3K/AKT and MAPK (p38) pathways[104]. Thus, angiogenesis can be induced by the constitutive activation of the MAPK pathway via either VEGF receptors[55]. Carmeliet *et al.* (2001) found that even at low doses of PGF, impaired VEGF response in *Pgf* $^{-/-}$ mouse endothelial cells was restored, concluding that PGF and VEGFA have a synergistic effect during pathological angiogenesis[87]. Additionally, when VEGFA binds to VEGFR2, in concurrence with PI3K/AKT pathway activation, it rescues endothelial cells from drug-induced cell death[55].

VEGF is known to have strong expression in malignant tumours such as serous adenocarcinomas[17]. In this study, VEGF expression levels with or without carboplatin exposure in COV362 and COV318 cells did not show a significant difference. A possible explanation may be that if there is indeed strong initial VEGF expression in COV362 and/or COV318 cells, it may contribute to tumour angiogenesis regardless of exposure to carboplatin. It is interesting to note that in this study, HDMECs have significantly elevated *VEGFA* mRNA levels post-carboplatin exposure. Taken together, carboplatin-induced *VEGFA* in HDMECs may add to the overall enhancement of angiogenic stimulus. Alternatively, varying levels of angiogenic factors in endothelial cells and ovarian cancer cells may contribute differently to overall angiogenic balance. Parallel to my results, Kuc *et al.* (2017) evaluated expression of angiogenic genes in ovarian cancer and healthy patient ovarian samples and found no difference in *VEGFA* expression. Moreover, the researchers did not find a difference in the expression of *PGF* in control versus ovarian cancer patient samples[54]. To that end, my results may confirm that *PGF* expression is induced by carboplatin. In contrast, examination of resected ovarian cancer specimens compared to paired non-tumour ovarian tissue, Song *et al.* (2015) have found increased protein levels of PGF in the cancer specimens. It is important to note that in both studies, it is unclear whether these ovarian cancer patient specimens had prior treatment[54,105]. In addition, PGF has been shown to be elevated when anti-VEGF drugs are used[106]. Horowitz *et al.* (2011) explored the efficacy of multiple anti-angiogenic drugs in recurrent platinum-sensitive ovarian carcinoma patients and found that Avastin

in combination with chemotherapy resulted in increased levels of circulating VEGF and PGF. The researchers claimed however, that PGF and VEGF were released by the host cells and not by cancer cells[107]. My results show that *PGF* was induced in endothelial cells as well as ovarian cancer cells after carboplatin-based therapy. Taken together, it is possible that carboplatin-based therapy and anti-VEGF therapy may have dual effects on PGF expression in the tumour microenvironment.

Interestingly, COV362 cell showed greater mRNA induction of *PGF* in comparison to COV318 cells after carboplatin exposure (**Figure 4.3.2**, **Figure 4.3.3** and **Table 4.3.1**). Using ovarian cancer cell condition media, I tested the effects of carboplatin-induced angiogenic factors such as *PGF* on microvascular endothelial cell survival. Perhaps due to this higher induction of *PGF*, endothelial cells exposed to COV362-derived condition media showed significantly greater endothelial cell survival (**Figure 4.4.1**). Whereas, this effect was only evident when I tested a longer-term collection from COV318 cells (**Figure 4.4.2**). One explanation of differential induction of angiogenic factors in COV362 and COV318 may be CNA, which is quite high[14]. However, a recent study has shown that there were no copy number changes in *PGF* and *IL1B1* in COV cells[*108*].

The main limitation of this study is the strict evaluation of gene expression profiles; further studies are required to confirm these genes by protein array assessment, possibly by enzyme-linked immunosorbent assay (ELISA). To that end, in this study, I have also explored the effects of human recombinant PGF and VEGFA (rPGF/rVEGFA). I found that there was an increase in endothelial cell viability with rVEGFA or rPGF, however rVEGFA had a greater effect on endothelial cell viability in comparison to exposure to rPGF alone (**Figure 4.5.1**). Also, there was no significant difference between HDMECs exposed to rVEGFA versus combination exposure of rPGF and rVEGFA. Taken together, rPGF and rVEGFA did not have a synergistic effect on endothelial cell survival and proliferation. One study found a synergistic effect, where upregulation of PGF and its receptor VEGFR1 made endothelial cells more responsive to VEGF[87]. Although synergism did not occur with rPGF and rVEGFA, it is important to note that endogenous PGF and VEGFA were not explored.

5.4 Inflammation, angiogenesis, and cancer: the role of IL1B

The tumour microenvironment is complex, and cancer-related inflammation also promotes angiogenesis and tumorigenesis. A number of pro-inflammatory genes were upregulated after carboplatin treatment such as *IL1B*. IL1β is a cytokine that affects many inflammatory processes; it is an actively secreted mature protein product by macrophages and blood monocytes[109]. Recombinant IL1β has been shown to increase tumour invasion and metastasis by stimulating endothelial cell proliferation as well as enhancing the expression of adhesion molecules on endothelial and malignant cancer cells[109]. Voronov *et al.* (2003) have shown lack of melanoma tumour growth in IL1β knockout mice but dramatic growth in WT mice[109]. Previous studies also provide evidence that PGF initiates inflammation by binding to its receptor VEGFR1, which in turn recruits monocytes[110]. Kim e*t al.* (2012) also show that fibroblasts produce PGF when exposed to pro-inflammatory factors such as IL1B[76]. Therefore, IL1B may potentially contribute to the propagation of PGF in the same manner in ovarian cancer.

In addition, Bohm *et al.* (2016) explored the effect of neoadjuvant chemotherapy (NACT) on immune activation in late stage HGSOC patient samples and found that chemotherapy may enhance the cytotoxicity of immune effector cells[111]. T-cell activation as well as low plasma levels of proinflammatory cytokines like TNF, IL8 and IL6 were found after NACT[111]. In contrast, my results have shown significant increase in *IL6* mRNA levels in ovarian cancer cells after chemotherapy exposure as well as elevated levels of *IL8* in HDMECs. Further studies are needed to explore plausible roles of increased inflammatory cytokine expression in ovarian cancer cells post-carboplatin exposure.

5.5 Wnt pathway activation via carboplatin may lead to downstream angiogenic effects

My results show increased nuclear β-catenin and decreased membrane β-catenin in COV318 and COV362 cells following exposure to carboplatin (**Figure 4.6.1** and **Figure 4.6.2**). β-catenin activation is known to activate specific genes involved in cell proliferation and survival[112]. Normally, β-catenin is associated with cell membrane

cadherins and cytoplasmic levels are kept in check by phosphorylation and ubiquitin-mediated proteosomal degradation[89]. Glycogen synthase kinase (GSK)-3 phosphorylates β-catenin, drawing in E3-ubiquitn ligase β-TrCP to initiate β-catenin degradation[89]. Upon activation of the Wnt pathway, cytoplasmic β-catenin levels increase. This may be due to reduced association with cadherins and/or reduced degradation[113]. Cytosolic β-catenin then translocates to the nucleus where it can initiate transcription of downstream genes[89]. β-catenin (*CTNNB1*) mutation frequencies are found to be between 16%[114] and 54% in endometrioid cancers, however *CTNNB1* mutations are rare in other ovarian cancer histological subtypes such as serous, clear cell and mucinous[115,116]. It is important to note that COV362 and COV318 ovarian cancer cell lines used in this study do not harbour a *β-catenin* mutation, an interesting study would be to evaluate carboplatin-induced β-catenin localization in a mutated ovarian cancer cell line, such as OVCAR8[14], in relation to pro-angiogenic activity.

Chemoresistance is associated with acquiring epithelial to mesenchymal transition (EMT) in ovarian cancer cells and a major signaling pathway involved is the Wnt/β-catenin pathway[116]. In ovarian cancer, emerging studies have proposed that the Wnt/β-catenin pathway is involved in cancer progression[116]. The key components of EMT entails the loss of polarity and cell-to-cell adhesion junctions of epithelial cells and gain of mesenchymal gene expression and motility[117]. When the canonical Wnt pathway is activated, cytosolic β-catenin is stabilized and translocates to the nucleus to transcribe downstream target genes such as *c-MYC*, *MMP*, and *VEGF*[116]. Interestingly, COV362 cells have amplified *c-MYC*, which may contribute to cell survival upon chemotherapy-induced Wnt pathway activation[14]. It is possible that *c-MYC* expression and potential subsequent expression of pro-angiogenic factors may influence the outcomes of endothelial cell survival in comparison to COV318 cells which do not have amplified *c-MYC*. Furthermore, Masckauchan *et al.* (2005) found that *IL8* is a transcriptional target of β-catenin which promotes pro-angiogenic activity[118]. This aligns well with my results, where *IL8* mRNA levels were elevated after carboplatin treatment (**Figure 4.3.1**). Another group showed that HGSOCs correlated with nuclear β-catenin expression[119]: Arend *et al.* (2014) reported that overexpression of oncogene frequently rearranged in

advanced T cell lymphomas-1 (*FRAT1*) in serous ovarian cancer inhibits the phosphorylation of β-catenin, eventually leading to its translocation to the nucleus.

Little is known of the relationship between PGF, β-catenin and Wnt pathway signaling; therefore, this possible axis could be further explored in future studies.

5.6 Concluding Remarks

Ovarian cancer is the most lethal gynecological malignancy in Canada and a combination of late diagnosis and ineffective therapies potentially contributes to relapse or resistance in patients. Angiogenesis plays a key role in solid tumour progression and metastasis. Despite the use of anti-VEGF therapies in combination with chemotherapy for ovarian cancer, overall survival is minimally impacted. Therefore, more studies are needed to catalogue angiogenic genes which are induced in ovarian cancer and factors which show alteration upon chemotherapy exposure. My studies showed that carboplatin induced a variety of angiogenic factors in ovarian cancer cells. Most interestingly, *PGF* and *IL1B* were induced in all types tested and may therefore play a pivotal role in promoting angiogenesis in ovarian cancer. Carboplatin also induced Wnt pathway activation shown by nuclear β-catenin localization. It is possible that PGF is a downstream target of activated Wnt/β-catenin pathway and promotes angiogenesis after carboplatin exposure (**Figure 5.6.1**).

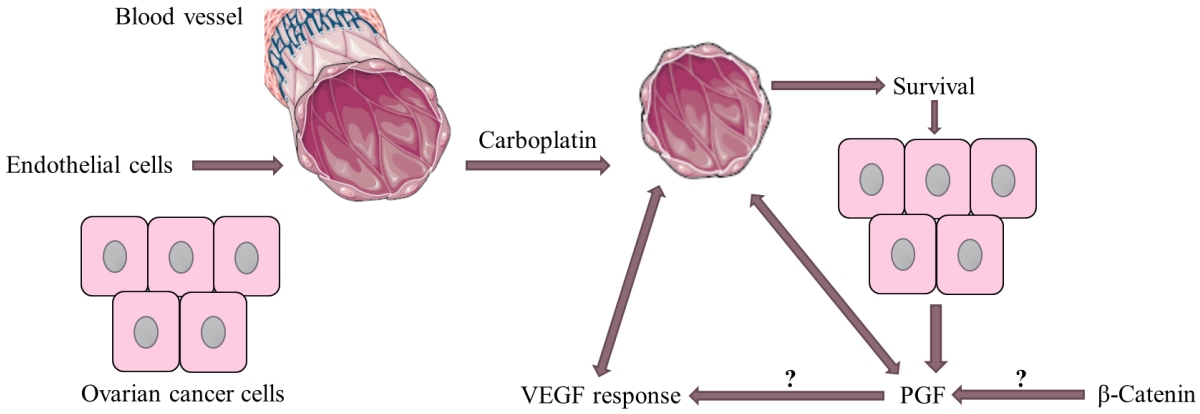


Figure 5.6.1 Possible interactions between PGF, VEGF and β-catenin after carboplatin exposure.

5.7 Future Directions

Further studies are needed to elucidate the possible interactions between the Wnt pathway/β-catenin activation with angiogenic factor PGF. Exploring PGF and β-catenin inhibition in carboplatin-resistant ovarian cancer cells may also provide insight into the utility of PGF and β-catenin as targets for patients encountering relapse. Another possible future direction may be to investigate the role of Wnt pathway and β-catenin transcriptional activity which may determine the differing responses of HGSOCs that resist chemotherapy or acquire chemotherapy resistance.

It is also essential to identify possible angiogenic targets so that effective combination therapies can be devised. A cohort evaluation of multiple ovarian cancer cell lines may provide insight into tumour type-specific carboplatin-induced angiogenic factors. The evaluation of angiogenic factors post-chemotherapy patient samples exposure by gene expression profiling or by proteomic evaluation using mass spectrometry can also be essential.

The angiogenic process primarily involves endothelial cell survival, proliferation, tubule formation and migration[120]. Further functional tests can be performed to elucidate the role of angiogenic factors post-carboplatin exposure and post-Wnt pathway activation. For instance, individual factors can be evaluated by functional assays to test tubule formation ability *in vitro* by three-dimensional tubule formation assay, or *ex vivo* by chick chorioallantoic membrane assay[121]. Scratch assays can be used to evaluate the migratory ability of endothelial cells in response to ovarian cancer cell carboplatin-induced angiogenic factors[122]. Knowing the mechanisms that govern tumour cell-to-host cell interactions in response to carboplatin may also provide additional targets. By co-culturing ovarian cancer cells and endothelial cells with a particular gene of interest knocked out, the effect of possible interactions in regard to survival, proliferation, migration and tubule formation can be explored.

Furthermore, many other cells that exist within the tumour environment also contribute to angiogenesis and consequently tumour survival[120]. Therefore, further studies elucidating *in vivo* pro-angiogenic response and cross-talk of angiogenic pathways induced by

carboplatin (in mouse tumour models[123] for example) must be further investigated to include these dynamic cell interactions which may ultimately provide a better understanding of the tumour microenvironment in response to carboplatin therapy.

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Appendix

Appendix 1 RT^2 Human Angiogenesis Profiler^{TM} PCR Array as prepared by Qiagen

Symbol	*Description*
AKT1	V-akt murine thymoma viral oncogene homolog 1
ANG	Angiogenin, ribonuclease, RNase A family, 5
ANGPT1	Angiopoietin 1
ANGPT2	Angiopoietin 2
ANGPTL4	Angiopoietin-like 4
ANPEP	Alanyl (membrane) aminopeptidase
ADGRB1	Brain-specific angiogenesis inhibitor 1
CCL11	Chemokine (C-C motif) ligand 11
CCL2	Chemokine (C-C motif) ligand 2
CDH5	Cadherin 5, type 2 (vascular endothelium)
COL18A1	Collagen, type XVIII, alpha 1
COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)
CTGF	Connective tissue growth factor
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL5	Chemokine (C-X-C motif) ligand 5
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
CXCL9	Chemokine (C-X-C motif) ligand 9
EDN1	Endothelin 1
EFNA1	Ephrin-A1
EFNB2	Ephrin-B2
EGF	Epidermal growth factor
ENG	Endoglin
EPHB4	EPH receptor B4
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
F3	Coagulation factor III (thromboplastin, tissue factor)
FGF1	Fibroblast growth factor 1 (acidic)
FGF2	Fibroblast growth factor 2 (basic)
FGFR3	Fibroblast growth factor receptor 3
FIGF	C-fos induced growth factor (vascular endothelial growth factor D)
FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
FN1	Fibronectin 1
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HPSE	Heparanase

ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
IFNA1	Interferon, alpha 1
IFNG	Interferon, gamma
IGF1	Insulin-like growth factor 1 (somatomedin C)
IL1B	Interleukin 1, beta
IL6	Interleukin 6 (interferon, beta 2)
CXCL8	Interleukin 8
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
JAG1	Jagged 1
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
LECT1	Leukocyte cell derived chemotaxin 1
LEP	Leptin
MDK	Midkine (neurite growth-promoting factor 2)
MMP14	Matrix metallopeptidase 14 (membrane-inserted)
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
NOS3	Nitric oxide synthase 3 (endothelial cell)
NOTCH4	Notch 4
NRP1	Neuropilin 1
NRP2	Neuropilin 2
PDGFA	Platelet-derived growth factor alpha polypeptide
PECAM1	Platelet/endothelial cell adhesion molecule
PF4	Platelet factor 4
PGF	Placental growth factor
PLAU	Plasminogen activator, urokinase
PLG	Plasminogen
PROK2	Prokineticin 2
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
S1PR1	Sphingosine-1-phosphate receptor 1
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1
SPHK1	Sphingosine kinase 1
TEK	TEK tyrosine kinase, endothelial
TGFA	Transforming growth factor, alpha
TGFB1	Transforming growth factor, beta 1
TGFB2	Transforming growth factor, beta 2
TGFBR1	Transforming growth factor, beta receptor 1
THBS1	Thrombospondin 1
THBS2	Thrombospondin 2

TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TIMP1	TIMP metallopeptidase inhibitor 1
TIMP2	TIMP metallopeptidase inhibitor 2
TIMP3	TIMP metallopeptidase inhibitor 3
TNF	Tumor necrosis factor
TYMP	Thymidine phosphorylase
VEGFA	Vascular endothelial growth factor A
VEGFB	Vascular endothelial growth factor B
VEGFC	Vascular endothelial growth factor C
ACTB	Actin, beta
B2M	Beta-2-microglobulin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPLP0	Ribosomal protein, large, P0
HGDC	Human Genomic DNA Contamination
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
PPC	Positive PCR Control
PPC	Positive PCR Control
PPC	Positive PCR Control

Curriculum Vitae

PERSONAL INFORMATION Zeynep G. Kahramanoğlu

EDUCATION

2015 – 2017	**M.Sc.**	**Western University**, London ON Master of Science, Research-based *Pathology and Laboratory Medicine* Thesis: *Mechanisms underlying chemotherapy-induced vascular proliferation in ovarian cancer* Supervisor: *Zia A. Khan, PhD*
2010 - 2014	**B.Sc.**	**University of Guelph**, Guelph, ON Bachelor of Science, Honours *Molecular Biology and Genetics (major)* *French Language (minor)* Research Project (major)*: A clonogenic survival assay for the study of* TGFβ*'s effect on chemotherapy response of canine osteosarcoma* Supervisor: *Alicia Viloria-Petit, PhD* Research Project (minor): *Charles Perrault: Des contes à l'écran* Supervisor: *Joubert Satyre, PhD*

PRESENTED RESEARCH

06/2017 **Oncology and Education Research Day**, London, ON.

- Abstract: *Carboplatin paradoxically increases angiogenic factors in ovarian cancer*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

05/2017 **Annual Developmental Biology Research Day**, London, ON.

- Abstract: *Transforming growth factor-β1 pathway regulates differentiation of bone marrow-derived progenitor cells*
- Authors: JJY Kum**, ZG Kahramanoglu**, CJ Howlett, ZA Khan

05/2017 **Annual Developmental Biology Research Day**, London, ON.
- Abstract: *Carboplatin paradoxically increases angiogenic factors in ovarian cancer*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

03/2017 **London Health Research Day**, London, ON.
- Abstract: *Carboplatin paradoxically increases angiogenic factors in ovarian cancer*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

03/2017 **Pathology and Laboratory Medicine Research Day**, London, ON.
- Abstract: *Carboplatin paradoxically increases angiogenic factors in ovarian cancer*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

10/2016 **Till and McCulloch Meetings**, Whistler, BC.
- Abstract: *Mechanisms underlying chemotherapy-induced hematopoietic stem cell deficits and myelosuppression*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

06/2016 **Oncology Research and Education Day**, London, ON.
- Abstract: *Mechanisms underlying chemotherapy-induced vascular proliferation in ovarian cancer*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

05/2016 **Annual Developmental Biology Research Day**, London, ON.
- Abstract: *Mechanisms underlying chemotherapy-induced vascular proliferation in ovarian cancer*
- Authors: **ZG Kahramanoglu**, JJY Kum, NC Richmond, MM Weir, ZA Khan

03/2016 **Pathology and Laboratory Medicine Research Day**, London, ON.
- Abstract: *Mechanisms underlying chemotherapy-induced vascular proliferation in ovarian cancer*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

03/2016 **London Health Research Day**, London, ON.
- Abstract: *Mechanisms underlying chemotherapy-induced vascular proliferation in ovarian cancer*
- Authors: **ZG Kahramanoglu**, MM Weir, ZA Khan

04/2014 **College of Biological Science Research Day**, Guelph, ON.
- Abstract: *Optimization of the clonogenic cell survival* in vitro *assay for the study of chemotherapy response of osteosarcoma cells*
- Authors: **ZG Kahramanoglu**, A Viloria-Petit

07/2013 **Institute for Comparative Cancer Investigation Research Symposium**, Guelph, ON.
- Abstract: *The role of transcriptional co-activator PDZ-binding motif (TAZ) in osteosarcoma metastasis*
- Authors: **ZG Kahramanoglu**, P Neogi, B Deheshi, A Viloria-Petit

07/2013 **Student Leadership and Research Program Symposium**, Guelph, ON.
- Abstract: *The role of stem cell transcription factors in osteosarcoma metastasis*
- Authors: **ZG Kahramanoglu**, P Neogi, B Deheshi, A Viloria-Petit

07/2012 **Research Training Center Summer Program Research Day**, Toronto, ON.
- Abstract: *Molecular validation of copy number variation in osteosarcoma*
- Authors: **ZG Kahramanoglu**, N Gokgoz, I Andrulis.

RELATED WORK EXPERIENCE

09/2016 - 12/2016 **Graduate Teaching Assistant**, Department of Basic Medical Sciences Undergraduate Education, Western University, London, ON.
- Course code: MEDSCI 4930

01/2015 - 08/2015 **Research Technical Assistant**, University Health Network, Toronto, ON.

05/2013 - 08/2013 **Undergraduate Research Assistant**, Department of Biomedical Sciences, University of Guelph, Guelph, ON.

05/2012 - 08/2012 **Research Training Center Summer Student**, Lunenfeld-Tanenbaum Research Institute, Toronto, ON.

AWARDS

04/2017	**Dr. Frederick Winnett Luney Graduate Research Award,** UWO
2015 - 2017	**Western Graduate Research Scholarship**, UWO
11/2016	**Ontario Institute for Regenerative Medicine Travel Award**, OIRM

DEPARTMENAL & COMMUNITY ACTIVITIES

09/2015 - present **Research Information Outreach Team (R.I.O.T)**, London

- Notably: Host/Guide for *Virtual Lab Tours* YouTube series, *Young Researchers* series (Author, *The Londoner* (06/2017 Article) and Interviewee for Rogers Television)
- "Let's Talk Cancer" event volunteer, program designer and organizing committee member (05/2016 and 05/2017)

10/2016 - present **Strong Bones, Strong Minds, Strong Muscles**, Western University

- Scientific education presenter in retirement homes
- Fundraising volunteer

11/2015 - present **Let's Talk Science**, Western University

- Presented scientific concepts to individuals ranging from grade 1- 12 in fun science-related activities; volunteer with the "Teacher Partnership Program"
- Mentor for Grade 10 Interdisciplinary Research Project

11/2015 - present **CHRW Radio**, Western University

- Morning show host for *Wake Up Western*
- Writer, editor and on-air presenter for *Morning News*

09/2015 - present **Canadian Cancer Society**, Elgin Middlesex Community Office, London

- Notably: Scientific Research Presenter for 2016 Volunteer Appreciation events for London, and Chatham-Kent, ON.

12/2016 - 04/2017 **Stem Cell Talks**, Western University

- Youth education event volunteer

03/2016 & 03/2015 **SciNapse**, Western University

- Poster judge; mentored and encouraged students to apply critical thinking