Skin capillary density in ovarian cancer

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Abstract

Introduction: Ovarian cancer is a diverse disease with a poor survival rate and complex treatments that have potential significant morbidity. The role of angiogenesis in ovarian cancer progression is well recognised and research into angiogenic inhibitors is novel and exciting. Skin capillary density (SCD) is a dynamic marker that may provide a surrogate indicator of angiogenic activity and alter in response to treatment in cancer patients.

Methods: I conducted a longitudinal prospective cohort study to investigate skin capillary density in ovarian cancer. I recruited 50 women with high grade serous carcinoma and measured SCD and angiogenic markers at five time points during treatment. Longitudinal and survival analysis was conducted to ascertain changes in the variables during treatment and association with cancer outcomes including surgical resection, overall survival (OS) and progression free survival (PFS).

Results: Capillary rarefaction occurred in all patients during cytotoxic treatment. Rarefaction also occurred in the subgroup who received anti angiogenic inhibitors and was correlated with a rise in blood pressure. Baseline SCD was strongly associated with the outcome of debulking surgery.

Conclusion: In this thesis I have demonstrated a dynamic change in SCD during cytotoxic and anti angiogenic treatment in women with ovarian cancer. Although this data requires validation in larger studies, it can be postulated that SCD could be useful as a biomarker of response to treatment and cancer outcomes and act as a surrogate marker of angiogenesis in cancer. It is a reproducible, cheap and non-invasive investigation that is acceptable to patients and shows promise in helping to guide treatment and prognostic information in the era of personalised medicine.

Dedication and Acknowledgements

This thesis is dedicated to the women who gave their time and support to the project when their lives had been turned upside down with cancer. Some of these women lost their fight and I shall never forget their bravery and kindness and thank them and their families from the bottom of my heart. I would like to thank my supervisors Professor Harry Mellor and Miss Claire Newton who have provided their precious time and encouragement. In addition I would like to thank Vivek Nama, without whom the work would not have been conceived. I am forever grateful to Professor Lopez Bernal for his kindness, wisdom and teaching. Thank you to Sarah Chir for your help with the slides and Joya Pawade who is the most amazing Pathologist, who despite having mountains of work gave her time. I am grateful to Ms Sofia Kanavou for her time and expertise to help me understand statistics much more than I did before. I'm so grateful to Carl May and Gavin Walsh for their patience and expertise and especially Rosie Lewes for her help with ELISAs. I had the honour of supervising her BSc during my research time and she is an outstanding student who will go on to change the world and who I hope to have convinced to pursue a career in Obs and Gynae! I want to personally thank Dr Jo Bailey, Mr Patel and Dr Platt who have mentored me in my clinical training and are an inspiration to me every day. I want to thank my friends and colleagues Sarah Newell, Kate Birchenall and Colin Down for their unwavering support and laughter on this journey. Last but not least to the people who have supported and loved me. To all the wonderful women I know who are juggling work, life and a million other things but yet still find time for a motivational chat and cake; Philippa Hefferan, Katie Ellison and my dear sister Joanna Rose, you inspire me. To my amazing dedicated parents who I can never thank enough for all that they do and my delightful boys Harry and George who can't wait to read mummy's book! To Tom, I could not live without you, thank you for your never ending love, kindness and generosity, endless cups of coffee and keeping our dear family going.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .....G Cass..... DATE:.....16/11/2020.....

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Abbreviations

Ang.	Angiopoietin
ANOVA.	Analysis of variance
BRCA.	Breast cancer gene
Ca125.	Cancer antigen 125
CCC.	Clear cell carcinoma
CEC:	Circulating endothelial cells
CEP:	Circulating endothelial progenitor cells
CI.	Confidence interval
CR.	Complete response
CRS.	Chemotherapy response score
CT.	Computed Tomography
DNA.	Deoxyribonucleic acid
EC.	Endometroid carcinoma
ELISA.	Enzyme linked immunosorbent assay
EMA.	European Medicines Agency
End.	Endothelin
ERK.	Extracellular signal related kinase
FDA.	Food and Drug Administration
FFPE:	Fixed formaldehyde and paraffin embedded
FGF.	Fibroblast growth factor
FIGO.	International Federation of Gynaecology and Obstetrics
GMC.	Good Medical Practice
GOG	Gynaecologic Oncology Group
H&E.	Haematoxylin and eosin **stain**
HER:	Human epidermal growth factor
HGF.	Hepatocyte growth factor
HGSC.	High grade serous carcinoma
HIF.	Hypoxia inducible factor
HR.	Hazard ratio
IHC:	Immunohistochemistry
IL.	Interleukin
LGSC.	Low grade serous carcinoma
MC.	Mucinous carcinoma
MDT.	Multi disciplinary team
MVD.	Microvessel density
NHS.	National Health Service.
NICE.	National Institute of Clinical Excellence
OR.	Odds ratio
OS.	Overall survival
PARP.	Poly (ADP) ribose polymerase
PDGF.	Platelet derived growth factor
PDGFR.	Platelet derived growth factor receptor
PFS.	Progression free survival
PIGF.	Placental growth factor
PR.	Partial response

RECIST:	Response evaluation criteria in solid tumours
ROC.	Receiver operating curve
SCD.	Skin capillary density
SD.	Stable disease
SMA.	Smooth muscle actin
SPRK.	Serine-arginine protein kinase
STAT:	Signal Transduction and Activator of Transcription
sVEGFR.	Soluble vascular endothelial factor receptor
TGF:	Transforming growth factor
Tie:	Tyrosine kinase
TPS:	Thrombospondin
UK.	United Kingdom
VEGF.	Vascular endothelial factor
VEGFR.	Vascular endothelial factor receptor
WHO.	World Health Organisation

Chapter 1. Introduction

1.1. Ovarian cancer

1.1.1. Epidemiology

Ovarian cancer is the most common gynaecological malignancy in developed countries. There were over 7,000 new cases of ovarian cancer in the UK in 2014 and the incidence is projected to rise by 15% between 2014-2035.[1] Ovarian cancer is associated with increasing age peaking in those aged 75-79 and over 53% of cases are diagnosed in women over 65 years.[1]

Most ovarian cancers are diagnosed at a late stage with 55-58% diagnosed at stage III-IV.[2, 3] A strong prognostic factor for survival is the stage of disease at presentation. Advanced stage ovarian cancer has a 5-year survival rate of 30-40%, while early stages have a 5-year survival of over 80%.[4]

1.1.2. Histopathology

Ovarian cancer is a diverse disease and epidemiology, risk factors, pattern of spread, response to treatment and prognosis vary significantly according histological subtype.[5] Consequently, as well as stage of disease histological subtype is fundamental to diagnosis and treatment when staging the disease. The primary site of disease is also given at histological diagnosis as recommended by the International Federation of Gynaecology and Obstetrics (FIGO).[6]

Ovarian cancers can be divided into five categories: epithelial, germ cell, sex cord stromal, metastatic and miscellaneous depending on their origin. The most common type of ovarian malignancies are epithelial in origin (carcinomas) accounting for 80-90%.[7] Five main

histological subtypes of epithelial ovarian carcinoma include high-grade serous carcinoma (HGSC, 70%); endometrioid carcinoma (EC,10%); clear-cell carcinoma (CCC,10%); mucinous carcinoma (MC, 3%); and low-grade serous carcinoma (LGSC, <5%).[6, 8] HGSC is the most common type.[6]

The different histological subtypes of ovarian cancer are associated with distinctive molecular features and HGSC cancers often have inactivation of p53 (a tumour suppressor gene) and are associated with *BRCA* (Breast cancer Gene) mutations[9]. *BRCA* genes are tumour suppressor genes that are important in cell DNA repair.

The most common presentation for HGSCs is stage III where disease has spread along the peritoneal surfaces to involve both pelvic and abdominal peritoneum and omentum.[6, 10] Table 1 demonstrates staging of ovarian cancer according to FIGO.

Table 1.1 FIGO staging ovarian cancer 2014 [11]

STAGE I: Tumour confined to ovaries	
IA	Tumour limited to 1 ovary, capsule intact, no tumour on surface, negative washings
IB	Tumour involves both ovaries otherwise like IA
IC: Tumour limited to 1 or both ovaries	
IC1	Surgical spill
IC2	Capsule rupture before surgery or tumour on ovarian surface
IC3	Malignant cells in the ascites or peritoneal washings

STAGE II: Tumour involves 1 or both ovaries with pelvic extension (below the pelvic brim) or primary peritoneal cancer	
IIA	Extension and/or implant on uterus and/or fallopian tubes
IIB	Extension to other pelvic intraperitoneal tissues

STAGE III: Tumour involves 1 or both ovaries with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes		
IIIA: Positive retroperitoneal lymph nodes and/or microscopic metastasis beyond the pelvis)		
IIIA1	Positive retroperitoneal lymph nodes only	
	IIIA1(i)	Metastasis ≤ 10 mm
	IIIA1 (ii)	Metastasis > 10 mm
IIIA2	Microscopic, extrapelvic (above the brim) peritoneal involvement ± positive retroperitoneal lymph nodes	
IIIB	Macroscopic, extrapelvic, peritoneal metastasis ≤ 2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen	
IIIC	Macroscopic, extrapelvic, peritoneal metastasis > 2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen	

STAGE IV: Distant metastasis excluding peritoneal metastasis	
IVA	Pleural effusion with positive cytology
IVB	Hepatic and/or splenic parenchymal metastasis, metastasis to extraabdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)

1.1.3. Risk factors

1.1.3.1. Genetic factors

Up to a fifth of ovarian malignancies are associated with inherited conditions, the majority being linked with *BRCA* 1 and 2 mutations.[12, 13] Both *BRCA* genes encode tumour suppressor proteins which are involved in DNA repair. *BRCA 1* and *2* mutations carry a significant increased risk of developing ovarian, breast, colon, pancreatic and prostate cancer. In fact patients who have germline mutations in *BRCA 1* and *2* have a higher risk of ovarian cancer compared to the general population with a cumulative risk by age 70 of 40-60%[14] and 10-30% % respectively.[15, 16] Women with Lynch syndrome have a 7% risk of developing ovarian cancer by 70 years[17] and 21% of women with Peutz-Jeghers syndrome are at risk of developing the disease by 65.[18] As well as mutations in *BRCA 1* and *BRCA 2*, derangements in other genes BRIP1, PALB2, RAD51C, RAD51D AND BARD1 were found to make up 20% of mutations in a group of nearly 2000 patients with ovarian cancer.[19]

Prophylactic risk reducing surgery is beneficial in prevention of ovarian cancer in women with germline *BRCA* mutation and bilateral salpingo-oophorectomy confers a 96% reduction in *BRCA* related ovarian cancer.[19] Data in unselected populations suggests that salpingectomy reduces the risk of ovarian cancer by 35-50% and supports the concept that many high grade serous cancers begin in the fallopian tube.[20] A study looking at PReventing Ovarian cancer Through early ExCision of Tubes and late Ovarian Removal (PROTECTOR) is ongoing to ascertain whether risk reducing salpingectomy only in pre menopausal women is effective in cancer prevention whilst avoiding morbidity from early surgical menopause.[21]

Nonetheless patients with germline *BRCA* mutation have a survival benefit compared to those without. This is likely to be multi factorial but increased sensitivity of platinum chemotherapy[22] and the advent of new drug therapies such as PARP inhibitors are likely to be contributory to improved outcomes.

1.1.3.2.Lifestyle factors

Protective factors for ovarian cancer include reduction in the number of ovulations, thus increased parity, breastfeeding and use of oral contraceptive pills.[23-25] Additionally these prove to be effective in reducing risk in those with inherited genetic mutations.[26] Smoking and obesity particularly in premenopausal women increases the risk of ovarian cancer.[27, 28]

1.1.3.3.Other factors

It is now recognised that the distal fallopian tube is the likely origin of high grade serous disease and Yoon *et al* demonstrated a significant decrease in the risk of ovarian cancer in patients who underwent bilateral salpingectomy compared to controls (OR 0.51, 95% CI 0.35-0.75).[29]

1.1.4.Presentation and screening

Symptoms of ovarian cancer are often vague and can be attributed to many other more common conditions. Goff *et al* demonstrated that symptoms that were significantly associated with ovarian cancer were pelvic/abdominal pain, urinary urgency/frequency, increased abdominal size/bloating, and difficulty eating/feeling full when they were present for <1 year and occurred >12 days per month.[30]

The National Institute of Clinical Excellence (NICE) recommends that women over the age of 50 should be offered a cancer antigen 125 (CA125) test if any of these symptoms are present persistently (>1 month) and frequently (>12 times a month).[31] CA125 is a glycoprotein that is expressed on the epithelial surfaces of the female genital tract and is a recognised biomarker of ovarian cancer. However, it should be recognised that only 50% of stage 1A cancers have a raised CA125 so the test does not achieve high specificity.[32]

The UKCTOCS trial demonstrated a reduction in mortality over 7 years in those women who had serum Ca125 measurements or ultrasound to detect early cancer although this was not significant.[33] With the lack of a reliable screening tool and the biological indolent nature of ovarian cancer most cases present with advanced disease.

1.1.5. Treatment

Primary debulking surgery followed by 6 cycles of platinum and taxane chemotherapy is the treatment of choice for ovarian cancer.[34] An anti angiogenic therapy Bevacizumab has been demonstrated to have survival benefit when given to those patients with advanced disease or suboptimal surgical outcomes and is available via the cancer drug fund and is administered alongside chemotherapy after surgery.[35] More recently poly ADP ribose polymerase (PARP) inhibitors have been licenced as a maintenance treatment after completion of surgery and chemotherapy.[36, 37]

Optimal surgery aims to achieve complete resection of disease, thus removing all visible disease or only leaving macroscopic disease <1cm in size.[38] In the primary instance this may involve hysterectomy, bilateral salpingo-oophorectomy, appendicectomy, omentectomy and in many cases bowel resection, diaphragmatic stripping, splenectomy and peritoneal resection. Primary surgery has a reported 30 day morbidity ranging from 11-67%.[39] In those patients undergoing upper abdominal surgery to resect disease morbidity is likely to be higher due to the length and complexity of surgery. Overall post-operative mortality has been reported as high as 2.8% although in more elderly patients this ranges between 5.4-11.7%.[39] Gerestien *et al* have developed a nomogram for predictive 30- day morbidity which includes age, WHO performance status (classification of physical activity), operative time and extent of surgery.[40]

In many women the morbidity of surgery due to the advanced nature of the disease may mean that neoadjuvant chemotherapy is offered to reduce tumour bulk. In this instance surgery is done as an interval procedure and followed by completion chemotherapy. There has been much debate as to the benefits of primary cytoreduction with likely increased surgical morbidity vs interval debulking surgery. However residual tumour after surgery and chemosensitivity are independent prognostic factors of recurrence and survival.[41, 42] Results of the EORTC trial revealed no significant overall survival advantage for those women with stage IIIc and IV disease undergoing primary surgery vs neoadjuvant chemotherapy. In fact post-operative morbidity and mortality was less after interval debulking surgery.[42]

Nonetheless it appears that complete cytoreduction at surgery is the most important prognostic factor for survival.[34, 43-47] The Gynaecological Oncologic Group (GOG) trials demonstrated that those patients with no residual disease had improved overall survival (OS)

compared to those with <1cm residual disease (64 months vs 29 months).[44] This trial did note however that advanced disease at presentation remained a significant prognostic indicator even after complete cytoreduction was achieved surgically.[44]

A Cochrane review which analysed 11 studies concluded that all attempts should be made by the surgeon to achieve complete cytoreduction at surgery.[48] A meta analysis of six studies (3447 participants) found that women who were optimally debulked had more than twice the risk of death compared to those who had no macroscopic disease remaining (HR 2.20 95% CI 1.90-2.54). Furthermore, a meta-analysis of 2 studies of 464 participants demonstrated increased risk of death in those who were sub-optimally debulked (disease >1cm remaining) compared to those optimally debulked (<1cm remaining) (HR 1.36 CI 1.10-1.68).[48] It is thus widely accepted that where complete cytoreduction is not achievable, any disease remaining should be small. If suboptimal surgery is likely, women may not undergo attempt of cytoreduction at all due to the associated morbidity with no improvement in survival. The data on surgical outcome is all retrospective and therefore likely to be associated with bias. The authors comment on the fact that surgical efforts may vary with age, cancer centre and those women who are more unwell are more likely to have less aggressive surgery and thus larger residual disease.

Due to the heterogeneity of disease burden at presentation alongside patient factors, it becomes a clinical judgement to determine the most appropriate treatment strategy. This is often a multidisciplinary decision considering radiographic opinion of staging CT images, diagnostic laparoscopic findings, co morbidities, patient's wishes and surgical expertise of the

particular cancer centre. Currently, there are no clinically useful biomarkers that predict morbidity and mortality from surgery of neoadjuvant chemotherapy.

1.1.6.Recurrent disease

Overall, 85% of women with ovarian cancer experience recurrence of their disease[45]. After completion of primary treatment for ovarian cancer women are followed up in an outpatient setting by clinicians over 5 years. Consultation involves eliciting symptoms of concern, physical examination and CA125 measurement in an aim to detect recurrence early.

In women with an asymptomatic recurrence detected by a raise in CA125 the OV05/EORTC trial demonstrated no improvement in survival when chemotherapy was administered compared to waiting to treat in the presence of symptomatic disease.[49]

1.1.6.1. Surgery for recurrent ovarian cancer

Those studies investigating secondary cytoreductive surgery demonstrated a significant morbidity with limited long term survival benefit.[50, 51] Much like primary surgery, complete resection of the disease was the strongest prognostic factor for survival.[50] The DESKTOP group of studies have created a score incorporating primary complete cytoreduction, good performance status and absence of ascites to predict likelihood of complete secondary resection.[52, 53] DESKTOP III randomised women to surgery or chemotherapy for recurrence. It demonstrated progression free survival (PFS) was 19.6 months for women randomized to undergo surgery followed by chemotherapy, compared with 14 months in women who were randomized to receive only second-line chemotherapy (HR, 0.66).[54] Overall, decisions

regarding management of recurrent disease is based on lengthy MDT discussions with a holistic approach to care.

1.1.6.2. Platinum resistant disease

Occurrence of tumour progression during or within six months of primary treatment with platinum chemotherapy or those who lack response to first line treatment is known as platinum resistant disease[55, 56]. Treatment options include additional surgery, non-platinum based chemotherapy, molecular targeted therapies such as PARP inhibitors for patients with BRACA mutations and anti-angiogenic inhibitors such as Bevacizumab.[57]

1.1.7 Poly ADP ribose polymerase (PARP) inhibition

Breaks in DNA can result in mutations and unregulated cell division. Homologous repair comprises of a series of pathways that function to repair DNA double stranded breaks.[58] Homologous repair deficiency (HRD) can occur due to germline or somatic mutations of genes involved in the homologous repair pathway and is associated with cancer and cell death.[58] PARP (poly ADP ribose polymerase) proteins also play a role in DNA repair by binding to single strand breaks to initiate repair and therapies that inhibit PARP are amongst the most novel treatments for women with ovarian cancer.[22] Tumours that have underlying homologous recombination repair deficiency (HRD) are more susceptible to PARP inhibition.[59]

PARP inhibitors bind and trap to PARP 1 and PARP 2 on DNA at sites of single strand breaks which causes double strand breaks. In cancer cells which are also HRD, these breaks are repaired by error prone pathways causing cell death.[59, 60] This process of depletion of two molecules in a DNA repair pathway causing cell death is known as 'synthetic lethality'.[19, 22]

BRCA 1 and *2* genes play multiple roles in homologous repair[19] and those tumours with *BRCA* mutation are consistent with HRD. Although germline (15%) and somatic (8-9%) *BRCA* mutations are common in high grade serous cancer[22], HRD occurs from mechanisms other than *BRCA* mutation and up to 50% of ovarian cancers have a mutation in a gene related to homologous recombination function.[22, 61] Although *BRCA 1* and *2* mutations are the most clinically relevant mediators targeted by treatments such as PARP inhibitors, the clinical application of these therapies is now much wider and PARP inhibitors therefore have a role in women without *BRCA* mutation.

The Solo 1 trial has led to unprecedented changes to primary treatment for women with ovarian cancer. Those women with advanced disease and *BRCA* mutation derived significant PFS benefit from maintenance olaparib vs placebo (median 41 month vs 13.8 months HR 0.30 p=<0.001).[36] Recent 5 year follow up data from this randomised trial has revealed that this benefit lasts beyond the 2 years of treatment with those women receiving olaparib (PFS 56 vs 14 months).[37]

Additionally another PARP inhibitor, niraparib, demonstrated an improvement in PFS and OS in both patients with and without *BRCA* mutation when given in first line treatment (PRIMA trial)[62] and both olaparib and niraparib are licensed by the FDA (Food and Drug Administration) and EMA (European Medicines Agency) for first line monotherapy.

In the recurrent disease setting, PARP inhibitors also improve outcomes for women. Both olaparib (Solo 2 trial)[63] and niraparib (NOVA trial)[64] demonstrate improved PFS in women with recurrent disease. Niraparib improved PFS from 5.5 to 21 months in those with *BRCA*

mutation although improvement was also seen in those with non *BRCA* cohort albeit more modest (3.9 vs 9.3 months).[64] Furthermore Rucaparib (ARIEL 3 trial) significantly improved progression free survival in patients with platinum sensitive cancer in all groups of women irrespective of *BRCA* mutation status.[65]

Olaparib received NICE (National Institute of Clinical Excellence) approval in 2019 for newly diagnosed ovarian cancer and *BRCA* mutation in the UK and Niraparib is available in England through cancer drugs fund.

These advances in treatment for ovarian cancer from PARP inhibitors make it imperative that patient have counselling and access to *BRCA* testing at the point of diagnosis to allow for treatments to be started in the first line setting.

1.1.8. Conclusion

It seems sensible in the light of high treatment morbidity, that focus on therapies that prevent recurrence and improve progression free survival would be both cost effective and beneficial and is the current target of research for treatment for ovarian cancer. The role of angiogenesis in ovarian cancer development and progression is beginning to be understood and treatments that inhibit tumour angiogenesis have been developed and show promising improvements in PFS both after primary treatment and in the context of disease recurrence. The predominance for angiogenesis in particular tumours, optimal timing of angiogenic inhibitors and identifying which patients may benefit from these therapies are currently unanswered questions. They are key priorities to ensure that the most effective and least morbid treatment is offered to an individual.

1.2.The role of angiogenesis in cancer

In order for tumours to grow beyond 1-3mm[66] and metastasise, new blood vessels must be formed to allow delivery of oxygen and nutrients. This process is known as angiogenesis. The process is controlled by pro and anti-angiogenic factors and is disordered in cancer. When pro-angiogenic factors are dominant the tumour undergoes a process known as the angiogenic switch which leads to the formation of new vessels and tumour progression.[67, 68] Tumour vessels differ to normal vessels in that they are tortuous, often highly permeable and irregular.[69] Angiogenesis in cancer is a complex process with multiple pathways involved. Sprouting from existing vessels, production of factors that destabilise the normal vasculature and formation of new blood vessels from precursor mesodermal cells are mechanisms by which tumour angiogenesis occurs.[69-73]

There are multiple angiogenic activators that stimulate tumour angiogenesis.[69] Metabolic causes such as hypoxia and genetic mutations such as activation of oncogenes or deletion of tumour suppressor genes that control production of angiogenesis regulators are also important.

1.2.1.Angiogenic factors

1.2.1.1.Vascular Endothelial Growth Factor

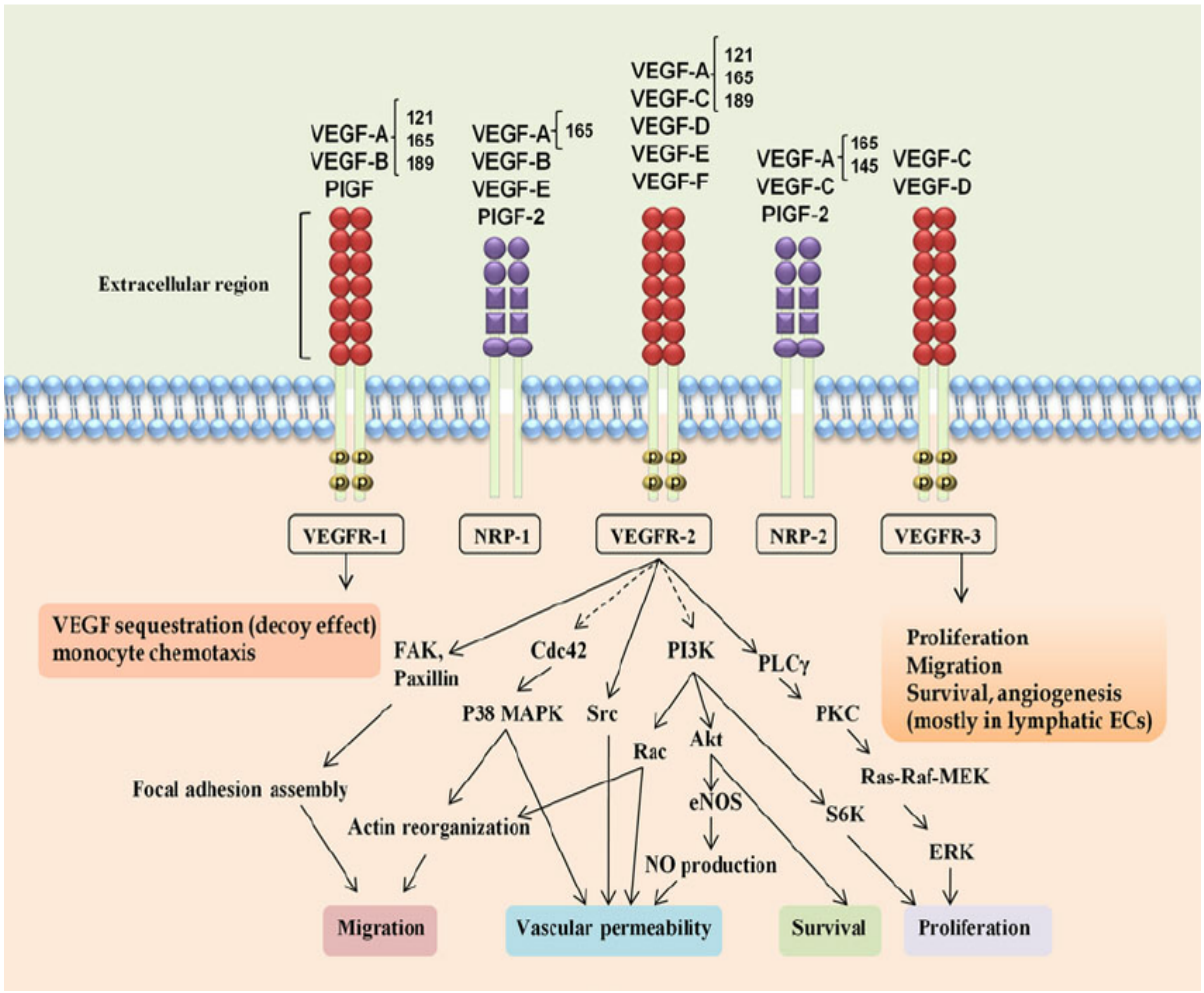
The vascular endothelial growth factor family comprises a group of growth factors VEGF A-D and placenta growth factor (PIGF). VEGF-A (VEGF) is a key molecule in the regulation of angiogenesis. VEGF promotes the survival and proliferation of endothelial cells and increases

vascular permeability.[66] VEGF activity is enhanced by hypoxia[74], oncogenes[75] and levels of VEGF have been widely demonstrated to be raised in tumours.

The human VEGF gene is mapped to chromosome 6p21.3.[76] The gene product is a heparin binding glycoprotein and there are multiple isoforms that make up the VEGF family, which are produced by alternative splicing from an eight-exon VEGF gene.[76] Alternative exon splicing results in the generation of four main VEGF-A isoforms, which have respectively 121, 165, 189, and 206 amino acids after the signal sequence is cleaved ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$).[77] $VEGF_{189}$ and $VEGF_{206}$ are bound to heparin-like structures in the cell surface or in the extracellular matrix, whereas $VEGF_{121}$ is a freely diffusible protein. $VEGF_{165}$ has intermediate properties in terms of heparin-binding and bioavailability.[78-80]

VEGF acts by interacting with a set of cell surface tyrosine kinase receptors.[81, 82] These receptors initiate signal transduction cascades in response to VEGF binding. The receptors have an extracellular portion consisting of 7 immunoglobulin like domains, a single transmembrane spanning region and intracellular portion containing a split tyrosine-kinase.[82] The receptors consist of VEGFR-1 (Flt-1), VEGFR-2 (Flk-1) and VEGFR-3 (Flt-3).[83-85] Whilst VEGFR-3 leads to lymphangiogenesis, VEGFR-1 and VEGR-2 are important in regulating angiogenesis and VEGFR-2 mediates most of the known cellular responses to VEGF.[86, 87] VEGFR-1 mobilises endothelial progenitor cells[88, 89] and its soluble form sVEGFR1 appears to have a negative impact on angiogenesis when bound to VEGF.[90]

Figure 1.1 Vascular endothelial growth factor and receptors[91]



Preclinical studies have demonstrated an excess of endothelial cells and disorganised tubules in mice lacking VEGFR-1 and a sparsity of endothelial cells and inability to develop a vasculature in the absence of VEGFR-2.[92, 93] On stimulation of VEGFR2, intracellular tyrosine kinase residues become phosphorylated and this results in downstream activation of signalling cascades including protein kinase C, RAS and ERK as well as P13-K/AKT/mTOR leading to endothelial proliferation, migration and survival.[91, 94] Various mechanisms mediate VEGF expression including inflammatory cytokines interleukin 1 (IL-1), 6 and 8 and hypoxic inducible factors alpha (HIF-alpha).[78]

Despite some conflicting results, the majority of studies investigating serum VEGF levels have demonstrated an increase in those with malignant compared to benign ovarian tumours.[95-97] Additionally, high levels have been predictors of advanced disease, poorly differentiated tumours, increased metastasis, large volume of ascites and poor survival.[87, 97-103].

1.2.1.2.Other angiogenic factors in ovarian cancer

There is complex interaction between the numerous growth factors mediating angiogenesis. Epidermal growth factor receptor, a member of the human epidermal growth factor (HER) family of tyrosine kinase membrane receptors is expressed on surface of epithelial cells.[104] It has an extracellular domain (sEGFR) that is shed and released into the circulation.[105] The main ligands for EGFR are epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and amphiregulin.[106] Overexpression of EGFR and has been associated with poor prognosis in ovarian and multiple other cancers[104, 106-108] and levels of its ligands are significantly higher in cancer patients compared to controls. Various tyrosine kinase inhibitors such as lapatinib and erlotinib targeting this pathway are licensed for treatment of lung cancer[109] and it has been postulated that these biomarkers could be a potential maker of response to treatment for these patients.[106] There is a sparsity of evidence however for both the significance of these biomarkers and treatment in ovarian cancer.

Hypoxia inducing factor alpha (HIF-alpha) appears to upregulate VEGF expression in hypoxic conditions[110] and correlates to microvessel density in the tumour.[111] High levels of HIF-alpha in tumour have been linked to longer PFS in both ovarian and renal cell carcinoma.[112, 113] Additionally, levels of HIF-alpha have been correlated inversely with sensitivity of ovarian cancers to chemotherapy.[112]

Endothelin-1 acts as an angiogenic factor through its interaction with ET(A) receptor. This receptor has found to be expressed in over 85% of ovarian tumours[114] and high concentrations of ET-1 in ovarian cancer cells has been found in ascites.[115] Binding of ET-1 to ET(A) modulates cell proliferation, apoptosis, migration, epithelial-to-mesenchymal transition, chemo resistance and neovascularization.[116]

Interleukin 8 is a small chemotactic cytokine that induces neovascularisation.[117] IL-8 is secreted by tumour cells to induce tumour growth and has a proangiogenic effect in ovarian cancer.[118] IL-8 levels are raised in women in ovarian cancer[119] high levels in tumour tissue, ascites and serum have been linked to poor prognosis.[120] Similarly, Interleukin 6 (IL-6) mediates tumour growth and angiogenesis in ovarian cancer[121] and high levels of IL in the serum and ovarian cancer tissue are associated with shorter OS and DFS.[69, 122]

Amongst the largest group of receptor tyrosine kinase families is the Eph receptors. There are 16 Eph receptors identified and they along with their ligands play a role in tumour angiogenesis.[111] Specifically, high levels of EphB4 and B2 expression in ovarian tumours correlated with poor OS and may be an independent predictor of chemotherapy response in patients.[123]

Horala *et al*. measured a panel of angiogenic markers in women with malignant ovarian tumour, benign tumours and healthy controls to ascertain their efficacy as a screening tool to detect ovarian cancer.[124] Basic fibroblast growth factor (FGF), follistatin, hepatocyte growth factor (HGF), osteopontin (an extracellular structural protein) and platelet derived growth

factors AB/BB (PDGF-AB/BB) were significantly increased (p=<0.03) in those with malignant disease in comparison to the control group.[124]

Platelet derived growth factor (PDGF) is another important growth factor involved in angiogenesis. Platelet derived growth factors are a disulfide-linked dimers of two polypeptide chains. Four different chains PDGF A-D have been identified.[89] PDGFRs are expressed on pericytes; contractile cells that line and stabilise new endothelial cells to create stable tumour vasculature.[89]

High levels of PDGF have been found in ovarian cancer cells and is related to disease progression.[125] High serum levels of PDGF-AA and PDGF-BB have been associated with residual disease after primary cytoreductive surgery and have a prognostic significance in women with recurrent ovarian cancer who were treated with Bevacizumab. It has also been suggested that PDGR plays a role in tumour evasion in anti-angiogenic treatment.[126]

Fibroblast growth factor (FGF) affects endothelial cell migration, proliferation and is an important proangiogenic factor.[127] There are over 20 FGF ligands and 5 receptors[128] and FGF-2, 3, 1 and 7 are expressed in ovarian cancer models.[128-132] More specifically FGF 1 and 3 are associated with poor prognosis.[129] It is likely that FGF, PDGF and VEGF work in synergism to stimulate tumour angiogenesis and high levels of various angiogenic markers correlate in various studies.[87]

Angiopoietins 1 and 2 (Ang-1, Ang-2) are cytokines which contribute to tumour angiogenesis. Ang-1 and Ang-2 both bind to the tyrosine kinase reception Tie 2.[97] Ang-1 stabilises blood

vessels by promoting adhesive interactions between endothelial cells and pericytes and smooth muscle cells and contributes to tumour dissemination and metastasis.[133, 134] Ang-2 is an antagonist of Ang-1 and has a more prominent role in cancer. In the presence of VEGF, Ang-2 stimulates tumour angiogenesis and destabilises the normal vasculature.[135] High levels of Ang-2 have been demonstrated in hepatocellular and gastric cancer and associated with poor prognostic and advancing tumour stage.[136, 137] In ovarian cancer, increased levels of VEGF produced by the tumour have been shown to increase expression of Ang-2 in the endothelial cells.[138]

Raised serum levels of both Ang-1 and Ang-2 have been demonstrated in women with ovarian cancer compared to controls and elevated Ang-2 levels >2.7ng/ml was a significant predictor of poor OS and PFS.[139]

Zhang *et al* found that VEGF induces Ang-2 transcription via VEGFR-2 and high levels of Ang-2 were found in endothelial cells located in ovarian cancer tumours.[138] A more recent study attached to the AGO-OVAR2.11 study investigating multityrosine kinase inhibitor sunitinib which targets platelet derived growth factor receptors (PDGRF) and VEGRs, in platinum resistant ovarian cancer attempted to assess molecular biomarkers to be used to demonstrate response to the treatment.[140] It demonstrated a trend for prolonged PFS (8.4 months vs 2.7 months p=0.089) with those women who had decreasing serum Ang-2 levels during treatment. There was no significant benefit in prolonged PFS or OS demonstrated with the use of Sunitinib.[140]

Sallinen *et al*. demonstrated significant raised levels of Ang-1, Ang-2, VEGF and Ang-2/sVEGFR-2, VEGF/sVEGR-2 ratios in women with ovarian cancer compared to controls. Elevated Ang-2, VEGF, VEGF/sVEGFR-2 ratio and low sVEGFR-2 levels were also predictors of poor OS and PFS in univariate survival analysis and all reached statistical significance.[97]

Endoglin is a transmembrane receptor that is upregulated in proliferating endothelial cells.[141] A soluble form of endoglin has been detected in patients with pre-eclampsia and is raised in patients with colorectal and breast cancer.[141] Sol-endoglin impairs endothelial cell proliferation and capillary formation and is also implicated in vascular resistance and been demonstrated to induce hypertension in vitro due to its effect on endothelial cell function.[142]

In a series of 113 cases of ovarian cancer samples, a positive stain for p53 was associated with a complete response to treatment.[143] Overexpression of p53 is associated with reduced TPS-1, an inhibitory angiogenic factor. Thrombospondin 1 (TPS-1) inhibits growth of new blood vessels[144] and is regulated by p53. Overexpression of p53 has been demonstrated in ovarian cancer cells and thus may indirectly influence angiogenesis.[143]

1.2.2.Angiogenic inhibitor therapy

The theory of anti-angiogenic medications to prevent tumour growth and metastasis was first postulated in 1971 by Folkman[145] and has been the subject of intensive investigation since. The mechanism by which angiogenic inhibitors are effective is complex. It seems obvious that preventing tumour angiogenesis thus depriving the tumour of oxygen and nutrients would

prevent tumour growth and spread.[94, 145, 146] However, angiogenic inhibitors used as a monotherapy have not resulted in a significant improvement in outcomes in terms of overall survival. In contrast when angiogenic inhibitors have been used in addition to chemotherapy regimens improved PFS has been demonstrated in not only ovarian cancer but colorectal, breast and renal cell carcinoma.[94] Jain *et al* argues that angiogenic inhibitors in fact normalise tumour vasculature by stabilising the balance between pro and anti-angiogenic factors thus making the tumour more sensitive to chemotherapy.[147]

The structure of blood vessels in tumour tissue are poorly formed, tortuous and lack a coat of pericytes.[148] As a result, poor blood flow occurs leading to poor delivery of oxygen, nutrient supply and chemotherapy. Inhibition of VEGF and subsequent normalisation of vasculature can improve this and thus increase efficacy of these drugs.[149]

Pre-clinical studies have demonstrated that an angiogenic inhibitor Bevacizumab decreases tumour perfusion and micro vessel density in rectal cancer.[150] Furthermore, when used with chemotherapy it increased tumour oxygenation and an increased uptake of chemotherapy was noted.[151] This has led to the development of many studies to investigate the addition of angiogenic inhibitors to standard treatment or as a maintenance therapy after primary treatment.

1.2.2.1.Bevacizumab for ovarian cancer

Bevacizumab is a recombinant humanised monoclonal IgG antibody that targets VEGF. Its use as an angiogenic inhibitor has demonstrated benefit in patients when given as a maintenance treatment after primary treatment for ovarian cancer. Inhibition of VEGFA has been demonstrated in pre-clinical studies to reduce ascites formation and inhibits growth of dissemination cancer.[152]

Regarding first line treatment in ovarian cancer, Burger *et al* randomised patients with Stage III/IV ovarian cancer who had undergone debulking surgery to receive standard chemotherapy (control group), chemotherapy with the addition of Bevacizumab (Bevacizumab initiation group) or chemotherapy plus bevacizumab which continued alone for an additional 14 cycles (Bevacizumab throughout group).[153] They demonstrated a lower hazard of progression or death in the Bevacizumab throughout group (HR 0.717 [CI 0.625-0.824]P= <0.001). A significant increase in PFS of 3.8 months was seen in this group once adjusted for various other prognostic factors (14.1 months vs 10.3 months p<0.001). There was no significant difference in progression free survival between the control and Bevacizumab initiation group. There was no difference in OS between the groups.[153]

The study reported an increased risk of grade 2-3 hypertension (22.9% in Bevacizumab throughout group vs 7.2% controls p= 0.05) and GI events including perforations (2.6% vs 1.2%) although there was no reduction in quality of life reported in those treated with Bevacizumab.[153]

ICON are a group of trials by the Gynaecologic Cancer InterGroup (GCIG) designed to determine the most effective treatments for ovarian cancer. The ICON 7 trial has shown similar results of the addition of Bevacizumab to standard treatment in a wider population as this included women with early stage disease.[35] A lower hazard of progression or death (HR 0.81(CI 0.70-0.94) p= 0.004) was seen in women with maintenance Bevacizumab treatment for an additional 12 cycles. An increase in PFS after 42 months was shown compared to those in the standard chemotherapy group (24.1 months vs 22.4 months). Furthermore, these benefits were greater in those women at higher risk of disease progression where 3.6 month improvement in PFS was seen (14.5 months vs 16.1 months HR 0.73 p=0.002).[35] Most significantly, improvement in OS of 7.8 months (28.8 months vs 36.6 months HR 0.64 (CI 0.48-0.85) p=0.002) was seen in those women most at risk of progression: stage 4 or residual disease after surgery.[35] The results of these trials led to Bevacizumab being approved by the EMA in first line treatment in 2011 and is available in England via Cancer drugs fund.

ICON 8b is an ongoing international randomised (1:1:1 ratio), three arm, three stage phase III trial designed to evaluate the safety and efficacy of bevacizumab in combination with dose dense, dose fractionated chemotherapy compared to either strategy alone for first line treatment of ovarian cancer.[154] Bevacizumab continues alone as a maintenance therapy 11 months after primary treatment. Primary outcome measures are OS and PFS. Secondary outcomes will include comparative toxicity, impact on quality of life and cost effectiveness of the additional treatment.

In regards to second line treatment in ovarian cancer; similar efficacy of the addition of Bevacizumab to standard chemotherapy was initially demonstrated by Burger *et al* where

increased progression free and overall survival was seen after the addition of Bevacizumab in patients with persistent or recurrent ovarian cancer.[155] This effect was echoed in patients with platinum resistant disease although significant side effects of hypertension and GI perforation occurred in around 10% of patients. Median progression free survival was superior (12.4 vs 8.4 months) for those treated with Bevacizumab (HR 0.48 p= <0.001).[156]

The Aurelia trial also demonstrated an improvement in PFS in women with platinum resistant ovarian cancer when Bevacizumab was added to standard chemotherapy regime (6.7 vs 3.4 months HR 0.48 p=<0.001).[157]

Despite these multiple studies demonstrating an improved PFS, albeit by only a few months, there has been no evidence of improvement in OS. There was vast heterogeneity of women included in these studies in terms of disease stage, treatment and surgical outcomes. There also remains debate about the optimal length and dose of maintenance Bevacizumab treatment and there is no method of selecting patients likely to benefit from the addition of Bevacizumab to current treatment of chemotherapy.

1.2.2.2.Alternative angiogenic inhibitors in ovarian cancer

Cediranib is an oral tyrosine kinase inhibitor which targets all VEGFRs. In relation to recurrent disease ICON 6 demonstrated an improved PFS (HR 0.56 [CI 0.44-0.72] P<0.0001) of women given Cediranib alongside chemotherapy with maintenance treatment after follow up of 19.5 months (8.7 months vs 11.0 months).[158] This benefit was greater for those who continued Cediranib for maintenance compared to those who received it only alongside chemotherapy.

More follow up data to investigate impact on OS is awaited. Tolerance for the drug however during maintenance treatment was poor due to increased toxic effects namely diarrhoea, hypothyroidism and voice changes.[158]

Nintedanib is an oral multikinase inhibitor of VEGR, FGFR and PDGFR. LUME-OVAR1 trial involving the addition of nintedanib to carboplatin/paclitaxel chemotherapy resulted in a statistically significant prolongation of PFS compared with placebo plus chemotherapy (HR 0.84 (95% CI 0.72, 0.98) p = 0.0239). Median PFS was 17.2 months in the nintedanib arm and 16.6 months in the placebo arm.[159]

Pazopanib is an oral, multikinase inhibitor of VEGFR -1/-2/-3, PDGFR -α/-β. When pazopanib was used as maintenance therapy in patients with ovarian cancer whose disease did not progress during first-line chemotherapy had a median prolonged PFS of 5.6 months compared to those treated with placebo (HR 0.77 (95% CI 0.64 to 0.91) p=0.0021). However, there was no demonstrable increase in OS.[160]

Trebananib is a peptibody that binds to Ang1 and Ang-2 to prevent interaction with Tie -2 receptor. TRINOVA-1 study involved the addition of trebananib to chemotherapy for women with recurrent ovarian cancer. Trebananib significantly improved median PFS (HR 0.85 [CI 0.74-0.98] P=0.024) compared with placebo (12.5 versus 10.9months) but had no significant impact on OS.[161]

In summary there are many large studies as summarised in Table 1.2 that have now investigated the benefit of a number of angiogenic inhibitors although none of these have had an impact on OS and carry additional morbidity to standard treatment.

The NHS has finite funds and the economic impact of the routine use of these therapies must be considered. The cost of Bevacizumab per month is £1800 per patient. It has been stipulated in the literature that the additional annual treatment costs for even 50% of new patients diagnosed each year with ovarian cancer will cost over £20M in the UK.[162]

As these drugs have shown no benefit in increased OS it is imperative that these therapies are targeted to those who will benefit and a biomarker that may predict response to treatment is urgently needed.

Table 1.2. Angiogenic factors in cancer treatment

Antiangiogenic inhibitor	Mechanism of action	Setting	Trials
Bevacizumab	Humanised monoclonal antibody directed against VEGFA. Binds and neutralises VEGFA.	Adjuvant in primary ovarian cancer	GOG 218 (2011)[153] ICON 7 (2011)[35]
		Recurrent ovarian cancer	OCEANS (2012)[156] Aurelia (2014)[157]
Sorafenib	VEGFR tyrosine kinase inhibitor	Recurrent ovarian cancer	Matei *et al* (2011)[163]
Sunitinib	VEGFR tyrosine kinase inhibitor PDGF tyrosine kinase inhibitor	Recurrent ovarian cancer	Biagi *et al* (2011)[164] AGO-OVAR2.11 Baumann *et al* (2012)[165]
Cediranib	VEGFR tyrosine kinase inhibitor	Maintenance recurrent ovarian cancer	Matulonis *et al* (2009)[166] ICON 6 (2011)[158]
Nintedanib (BIBF 1120)	VEGFR/PDGFR/FGFR tyrosine kinase inhibitor	Maintenance recurrent ovarian cancer	Ledermann *et al* (2011)[159]
		Adjuvant primary ovarian cancer	Kristensen *et al* (2014)[167]
Pazopanib	VEGFR/PDGFR tyrosine kinase inhibitor	Maintenance primary ovarian cancer	Friedlander *et al* (2010)[168] AGO OVAR 16 (2014)[160]
AMG 386	Pedtide-Fc fusion protein. Prevents interaction of Ang-1/2 with Tie receptor	Adjuvant recurrent ovarian cancer	TRINOVA-1 (Monk *et al* 2014)[161]
Erlotinib	EGFR tyrosine kinase inhibitor	Maintenance primary ovarian cancer	EORTC55041/OVO7 (2013)[47]

1.3.Microvessel density as a surrogate marker of angiogenesis in the tumour

Microvessel density (MVD) of the tumour is used as a surrogate marker of angiogenesis.[169] MVD in tumours has been studied to ascertain the relationship of angiogenesis with tumour progression and areas of increased MVD in tumours has been demonstrated to have an adverse impact on prognosis and are associated with metastasis.[170]

Numerous studies in a variety of cancers have demonstrated that tumour MVD is a predictor of PFS.[171, 172] Alvarez *et al* demonstrated improved median survival when MVD count was <10 (7.9 years vs 2.7 years p=0.03). This was still the case when adjusted for stage, grade of the tumour and age of the patient.[171] There have been several further studies in ovarian cancer that have demonstrated an inverse relationship between MVD and both PFS and OS and demonstrated that high grade and stage tumours were also associated with increased MVD.[69, 171, 173-180] Lastly a meta-analysis of 22 studies investigating MVD and prognosis in ovarian cancer concluded that high MVD measured with CD34 antibody was associated with poor OS (HR 1.83 (CI 1.33-2.35) and PFS (HR1.36 CI 1.06-1.66).[181]

1.3.1.Methodology of MVD

Methods to quantify tumour angiogenesis by MVD are well established. Sections of tumour are stained immunohistochemically for various endothelial markers such as CD34[181-183] , CD31[181, 184] and 117/13) or factor VIII.[181] The choice of antibody in studies to measure MVD varies. CD34 and CD 31 are glycosylated transmembrane proteins that are expressed on immature haemopoietic cells and on luminal endothelial cells and have a higher specificity

and sensitivity for detection of tumour angiogenesis compared with factor VIII and are the most widely used.[185]

Once stained Weidner's method is most commonly used to measure MVD. Areas of high microvessel density named 'hot spots' are ascertained at low power (x200 magnification) and then vessels in these areas are counted at high power (x400 magnification) through a Chalkley count.[186, 187] This involves a 25 point grid placed onto a scanned image and all points coinciding with marked vessels are counted.[187]

An alternative method for MVD analysis involves systematic, uniform, random sampling (SURS) of up to 10 regions of interest (ROI) from the whole slide images in an attempt to avoid observer dependent sampling variation.[188, 189] Manual or computer assisted counting methods can be used for quantification.[188-190] The number of CD31 or 34 positive vessels /mm2 of tumour tissue thus indicates the MVD.

1.3.2. Microvessel density as a biomarker of angiogenic inhibitors

Although MVD is established as a prognostic factor for untreated ovarian cancer, until recently it was not demonstrated to be a viable biomarker of response to treatment. It seems reasonable that if MVD is a surrogate marker for angiogenic activity in the tumour then it may be a useful biomarker for both predicting and monitoring response to anti angiogenic activity. MVD in cancer models has been shown to decrease after anti angiogenic therapy due to apoptosis of tumour endothelial cells[69] thus the use of MVD as a biomarker of response to treatment with these therapies is possible.

A recent retrospective biomarker analysis of the GOG -0218 trial where patients with incompletely resected stage III or stage IV ovarian cancer were randomised to receive Bevacizumab alongside chemotherapy and for maintenance has been published.[188] The authors revealed that the effect of Bevacizumab on PFS was improved in both women with high and low MVD but was greater in patients with higher (above median) MVD compared to those with low MVD.(HR 0.40 in high MVD (0.29-0.54) vs 0.80 in low MVD CI 0.59-1.07). In the subgroup of women with higher MVD, there was also a gain in OS seen and no benefit in OS to those with low MVD. (HR 0.67 (CI 0.51-0.88 in high MVD vs HR 1.10 (CI 0.84-1.44) in low MVD). [188]

There has been no assessment of MVD pre and post chemotherapy and its relationship with chemotherapy response scores (CRS), nor in association with the use of angiogenic inhibitors.

1.3.3.Alternatives to micro vessel density

Due to some inconsistencies with the value of MVD in some studies and prediction of response to angiogenic inhibitors, there is a growing need to establish an alternative method that measures immature, newly developing` vessels as a measure of active tumour angiogenesis.

It has been argued that quantification of vessels that are immature may be more specific in assessing tumour angiogenesis. These new immature vessels require VEGF-A signalling for survival and are thus the targets of antiangiogenic inhibitors.[135, 188, 190, 191] The ratio of Ang-2:Ang-1 as discussed earlier may go some way to reveal this but other markers associated with the formation of new vessels are being investigated.

Stabilisation of newly formed vessels is a key factor in successful tumour angiogenesis. Covering of endothelial cells by pericytes is part of this process. Pericytes are contractile cells that line endothelial cells and are important for vessel wall stability and endothelial cell survival.[192] PDGFR ß and alpha SMA are expressed on pericytes and have been shown to have a role in metastasis.[193] Covigno *et al* recently demonstrated that high expression of PDGF ß in perivascular cells in ovarian tumours in women who underwent primary debulking surgery was significantly associated with shorter OS.[194] Alpha SMA expression produced by pericytes was also increased. These findings have been reiterated in colorectal and renal cell, the later also demonstrating a positive correlation between increased PDGF ß expression in advanced stage of disease.[193, 195]

Resistance to angiogenic inhibitors in other cancers such as melanoma has been postulated to be due to tumour vessel stabilisation and normalisation by coverage of mature pericytes.[196] PDGF ß and alpha SMA as markers of pericytes coverage are therefore potential biomarkers to target angiogenic therapies more suitably.

1.3.4. Proliferative markers in cancer

Unregulated cellular proliferation is a fundamental hallmark of cancer behaviour. Loss of response to proliferative control distinguishes cancer cells from normal and leads to invasion and metastasis. Ki67 is a marker of cell proliferation. It is localised in the nucleus of the cell and is expressed at all stages of the cell cycle apart from the rest phase (G0). Pathologists usually describe Ki67 as a percentage of nuclei that stain positive and typically high-grade

serous carcinoma of the ovary is associated with a high (>40%) and diffuse pattern of staining for Ki67.[197]

There is a lack of consensus regarding Ki67 and its association with outcomes or role as a biomarker to target treatment and studies show conflicting results. Some investigations suggest that high Ki67 is associated with reduced OS and PFS due to high proliferative nature of the tumour.[198] Nonetheless others have demonstrated improve PFS due to improved sensitivity of the tumour to chemotherapy.[197] A large cohort of 318 patients showed a low Ki67 (<40%) was significantly associated with resistance to platinum based chemotherapy (HR 2.85, 95% CI 1.43–5.98, $P < 0.001$) and reduced OS (HR 1.74, 95% CI 1.38–5.01, $P = 0.003$).[197]

1.4.Biomarkers of angiogenesis

A biomarker is defined as any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease.[199] The marker CA125 (a glycoprotein) is currently widely used as a monitoring tool in ovarian cancer treatment in relation to chemotherapy and relapse although early treatment based on rising CA125 in the absence of symptoms is controversial.[200]

At present there is no biomarker in clinical use with relation to angiogenesis in cancer. As discussed, the process of tumour angiogenesis is multifactorial and complex making it difficult to identify a specific maker. Nonetheless identification of a reliable biomarker is paramount for many reasons. Identification of those patients who will benefit from targeted therapies,

monitoring response to treatment and identifying those at risk of adverse events are essential for achieving a personalised medicine approach to cancer treatment and cost effectiveness for the NHS.

Many biomarkers are assessed after starting treatment or are studied in recurrent disease and are therefore unhelpful in determining suitable patient selection for treatment. Robust predictive markers for response to treatment or for appropriate patient selection are lacking in ovarian cancer.

1.4.1.Biomarkers in response to angiogenic inhibitor therapy

1.4.1.1. The role of VEGF as a biomarker

There are many studies that have measured VEGF to help predict outcome of Bevacizumab treatment with conflicting results. A large meta-analysis of 1816 patients confirmed that pre-treatment VEGF levels were prognostic rather than predictive of response to treatment in patients treated with Bevacizumab for colorectal, lung and renal cell carcinoma.[201] However, sVEGFR1 levels have been shown to be inversely related to outcome of treatment.[202] In those studies which have measured a specific VEGF isoform VEGF121, high baseline levels have consistently demonstrated an association with improved PFS and OS after treatment with Bevacizumab.[202-205]

There are a few studies which have monitored VEGF in women treated with antiangiogenic inhibitors. 2 studies demonstrated that a low baseline VEGF was associated with increased response and survival, one of these studies showed a significant association (60% increased

chance of response when VEGF was below median vs 0% response above median p=>0.0007).[103, 206] However, both studies looked at patients with recurrent disease and included less than 40 patients. In a study of 60 patients again with recurrent disease, high VEGF levels and increased MVD at baseline were associated with decreased OS. This study also measured thrombospondin-1 (a glycoprotein and inhibitor of angiogenesis) where high levels were associated with decreased OS and PFS.[100]

A retrospective analysis of the GOG 0218 trial found that tumours with a high expression of tumour VEGF-A were found to have an increased OS advantage compared to low levels of expression HR 0.72 (CI 0.56-0.94) in high expression vs HR 1.06 (CI 0.81-1.39) in low expression.[207] Since the primary target of Bevacizumab therapy is VEGF-A, it makes sense that tumours expressing high levels of VEGF-A may be the most sensitive to its effect.

1.4.1.2. VEGF isoforms (VEGF165b)

VEGF pre-mRNA undergoes alternative splicing to produce both pro angiogenic (VEGFxxx) and anti angiogenic (VEGFxxxb) families of isoforms.[208] Serine-arginine protein kinase 1 (SRPK1) is a kinase that phosphorylates SR- proteins and modulates their activity.[209]

A study involving patients with metastatic colon cancer demonstrated that only those patients where low levels of VEGF165b were expressed in the tumour gained benefit from Bevazizumab.[210] The balance of VEGFxxx:VEGFxxxb is important in regulation of tumour angiogenesis[208] and a study which demonstrated that immuno-histochemistry (IHC) staining of tumours for VEGFxxxb predicted disease free survival in patient treated with Bevacizumab and chemotherapy.[211] Bunni *et al* demonstrated that circulating VEGFxxxb levels correlated

to IHC staining and thus could be a surrogate marker to help stratify patients with colorectal cancer patient for Bevazizumab.[208] Furthermore, in ovarian cancer reduction in SPRK1 expression was associated with reduced cell proliferation rate and enhanced sensitivity to cisplatin suggesting a potential new target for ovarian cancer treatment.[212]

VEGF165b is a spliced variant and differs from VGEFA in 6 amino acids at the carboxy terminal 3 end. This switch from argine to aspartic acid and lysine is postulated as the mechanism behind the change in function to an antiangiogenic variant due to the different function and binding to VEGF R 2.[213] Recently Ganta *et al* has demonstrated that VEGF165b inhibits VEGF receptor 1 related Signal Transduction and Activator of Transcription 3 (STAT3) signalling thus causing a decrease in VEGF A mediated activation of VEGFR1 resulting in reduced angiogenesis.[214, 215] STAT3 pathways have been shown to be important in metastasis and resistance to cancer treatments. This suggests that VEGF165b may pay an important role which so far has not been investigated in ovarian cancer.

1.4.1.3. Alternative serum and tissue biomarkers

In relation to serum and plasma biomarkers which are inevitably more convenient to obtain, further retrospective analysis of pre-treatment plasma in the GOG-0218 trial were also investigated. Authors demonstrated that IL6 was predictive of therapeutic benefit in terms of prolonged PFS, HR 0.76 (CI 0.48-0.94) for high levels vs HR 0.87 (CI 0.70-1.08) for low levels and OS HR 0.79 (CI 0.65-1.16) for high levels vs HR 1.07 (CI 0.84-1.37) for low levels from the addition of Bevacizumab in addition to standard treatment. IL6 and osteopontin were negative prognostic markers for both PFS and OS (p=<0.001).[216] High Ang-2, IL-8 and low

VEGFR2 were also demonstrated to be poor prognostic factors in the retrospective analysis.[216]

A translational retrospective analysis of 92 patients enrolled in the ICON 7 study investigated 15 circulating angio-biomarkers in order to predict response to Bevazicumab. Changes in Tie 2 and Ang 1 were restricted to those receiving Bevacizumab and although levels did not predict response to treatment, a rise corresponded to progression of disease.[217]

In patients with recurrent disease who were treated with Bevacizumab in addition to standard chemotherapy patients genotyped for the A/A or A/T for the IL-8 T-251A gene polymorphism had a lower response rate to treatment compared to those who were homozygous.[218] Authors have postulated that this could be a potential biomarker for response but this has not been further validated.[94, 218] High baseline levels of PDGF-BB and FGF2 are associated with lower OS in women treated with Bevacizumab in recurrent ovarian cancer as demonstrated by Madsen *et al*.[219] Levels of these markers were collected after each cycle of treatment but there was no significant difference in the levels detected in those responding to treatment compared to those with refractory disease.[219]

No trial has monitored these angiogenic markers throughout treatment as yet and there is a need to ascertain trends to help predict response.

In normal tissues endothelial cells divide infrequently however rapid proliferation occurs during angiogenesis.[69] Levels of circulating endothelial cells are raised in patients with cancer compared to control.[69] Both mature circulating endothelial cells (CEC) and circulating endothelial progenitor cells (CEP) which are released from the bone marrow under VEGF influence have been identified. The use of antiangiogenic therapy reduces the amount of

CEPs[220] which is further reflected in reduction of vascularity in the tumour. Additionally, levels of CEPs have been shown to fall with the use of metronomic chemotherapy[221] where chemotherapy agents are given at shorter intervals at a lower dose suggesting these methods of chemotherapy administration has a negative effect on angiogenesis. This is currently being investigated in ovarian cancer as part of the ICON8b trial.

Circulating endothelial cells (CEC) and circulating progenitor cells (CEP) have been found to be elevated in women with ovarian cancer and are a potential dynamic biomarker.[168, 222] High levels of CEP are associated with advanced stage and poor prognosis.[222] VEGF has been shown to mobilise CECs and levels fell in a preclinical study where ovarian cancer mouse models were treated with a VEGF inhibitor.[168, 220]

1.4.2.Clinical biomarkers

Multiple clinical and molecular biomarkers have been investigated in other cancers such as renal cell carcinoma where the use of angiogenic inhibitors is common for primary and recurrent disease.[223] So far only clinical related biomarkers, the Heng criteria[224] are used to risk stratify and counsel patients. Heng *et al* demonstrated that pre-treatment low haemoglobin, raised corrected calcium, low performance status, raised neutrophil and platelet counts were associated with lower OS for patients treated with angiogenic inhibitors.[225] No such work regarding clinical related biomarkers is used in ovarian cancer as yet.

There are known side effects as a result of the use of antiangiogenic inhibitors, the most common being hypertension. There have been studies in patients with renal cell carcinoma that have demonstrated a link between treatment induced hypertension and a higher response rate and improved PFS after treatment with antiangiogenic inhibitors including Bevacizumab and Sunitinib.[226-230]

Hypertension may represent a biomarker of tumour response in other cancer sites.[231] In a small cohort of patients with colorectal cancer treated with Bevacizumab, partial remission was seen in 75% of patients who developed hypertension compared to 32% of those who did not (p=0.04).[232] Similar findings have also been seen in breast cancer treatment where women who developed hypertension had an improved OS (38.7 vs. 25.3 months, p=0.002).[233]

A small study has linked a reduction in skin capillary density (SCD) associated with treatment[234] induced hypertension thus it is a reasonable hypothesis to consider that SCD may be an independent clinical biomarker of response to treatment with antiangiogenic inhibitors. Whether it can also be used to predict prognosis or useful to target treatment with antiangiogenic inhibitors is also an interesting unanswered question.

The role of molecular biomarkers in ovarian cancer is complex. The ultimate aim of understanding the relationship between various markers and prognosis is to provide a targeted therapy and ability to monitor or predict response to such a treatment.

1.5.Skin capillary density

1.5.1.Definition

The number of capillaries per unit of skin is defined as skin capillary density (SCD). Assessment of SCD is a non invasive efficient technique that is easily accessible and requires a minimal amount of training. It is an easily repeatable measure that can be collected in the outpatient setting and is well tolerated by patients.

1.5.2.Factors that affect skin capillary density

At present there is no agreement on a value for 'normal' capillary density in a healthy individual. Due to the variance in some methodological techniques in studies it is difficult to be certain. Nonetheless many studies have reported no statistical difference in SCD between children and adults, white and non-white populations and males and females.[235] There is evidence however that capillary density decreases as part of the normal aging process in line with reduction of angiogenic factors such as VEGF, FGF and Angiopoietins.[236, 237]

Certain disease states have also been shown to affect SCD. Foremost, most work has been conducted in patient with rheumatological skin conditions to ascertain SCD is a biomarker that may predict onset of disease and response to treatment.[237] Well recognised changes in the morphological appearance of the capillaries occur in conditions such as systemic sclerosis and Raynaud's phenomenon.[238]

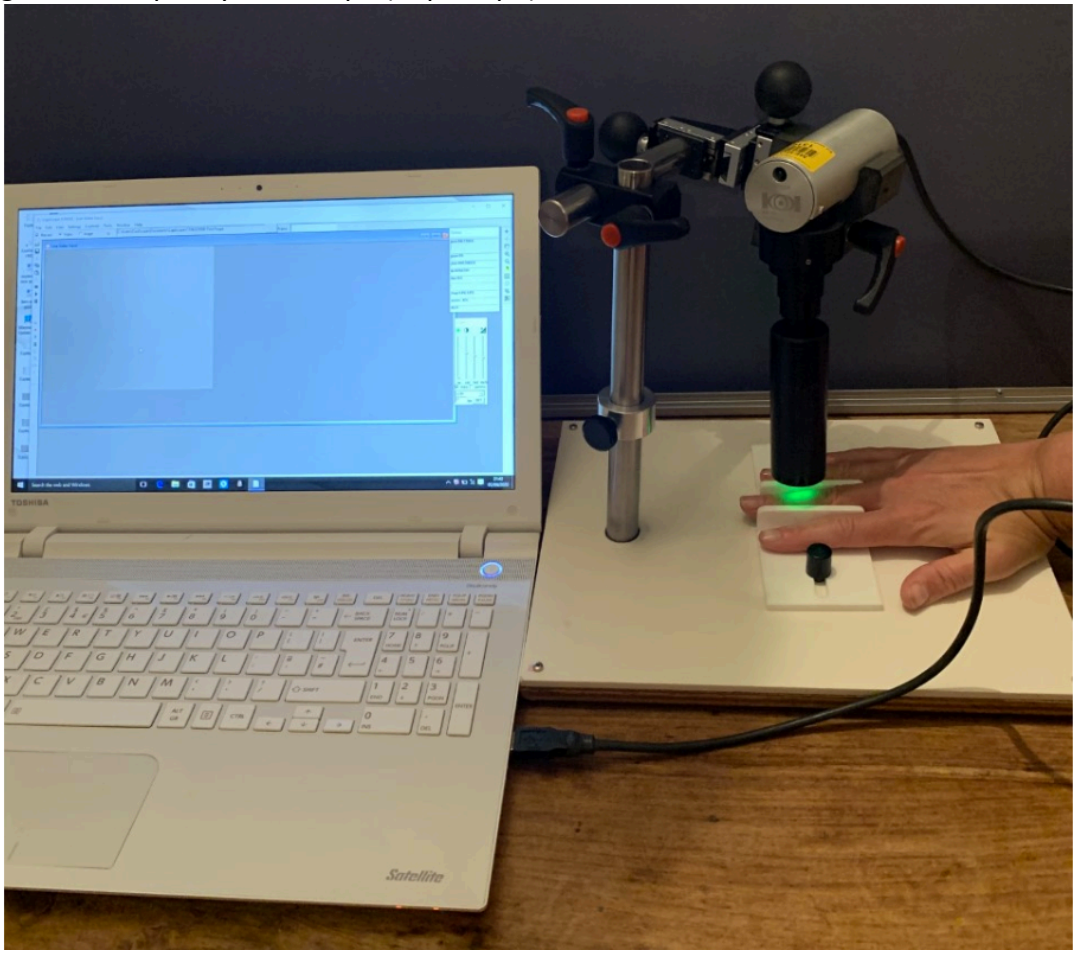
1.5.3.Methodology

Capillaries are visualised by capillary microscopy or capillaroscopy which provides a 2D image of a 3D network of capillaries. The number of capillaries are then counted to provide a skin capillary density.

A capillary microscope in combination with a computer screen allows high contrast images that can be frozen or recorded. The capillary microscope is mounted on a focusing block which is attached to an arm that allows the microscope to move up and down and back and forth over the patient's skin.

A light sensitive black and white camera is placed over a lens system and the skin illuminated by a 50 or 100W mercury vapour light. The emission spectrum of mercury vapour is similar to the absorption spectrum of haemoglobin (370-450nm) so blood cells appear as black dots on the screen. A green filter enhances the contrast to allow better interpretation of the images for analysis. Images are magnified 400 fold and stored on a laptop as still pictures or videos.

Figure 1.2 Capillary microsope (CapiScope)



Images are best achieved with the lens at 90 degree angle to the patient skin. Due to the magnification small movements in the patient can distort images thus the patient's finger is immobilised in a support. A thin layer of oil is placed over a central dot that is to be examined marked on the dorsum of the middle finger of the left hand. The oil prevents scattering of light to improve the image.[235] In order to calculate SCD, four 1mm^2 microscopic fields centred around an ink spot are recorded for 2 minutes continuously. A video can be recorded for each quadrant with focusing level adjusted throughout to capture all capillaries and then capillaries counted during video replay in each quadrant and mean taken to calculate the SCD.

Capillaries counted at rest must be perfused and this count is known as the functional count. In some conditions, not all capillaries may be perfused, hence they must be filled in order to provide a structural count. To achieve this venous occlusion is provoked at the base of the finger using a sphygmomanometer cuff at 60mmHg for 2 minutes then the same area recounted. In healthy individuals the increase in capillary density is 12-18%.[237] Functional rarefaction is thought to be the first step in the development of structural rarefaction as persistent non perfusion will lead to involution and irreversible loss of capillaries[239] thus both measurements are important to consider. This standardised method of measuring skin capillary density is well documented in the literature and the inter and intra observer variability has been quoted to be 4-6%.[240, 241]

1.5.4.Capillary rarefaction

Skin capillary density has been shown to decrease (capillary rarefaction) in patients with hypertensive disease compared to normotensive patients or those with well controlled and treated hypertension.[237, 242] It has been demonstrated that the reduction in SCD is structural due to the absence of capillaries rather than functional non perfusion of them.[242] This decrease is thought to precede the onset of hypertension and young individuals with a familial predisposition to hypertension have been found to have structural capillary rarefaction prior to developing hypertension themselves.[243, 244]

Furthermore, this microvascular rarefaction has been demonstrated in women in pre-eclampsia compared to normotensive pregnant controls.[245] In fact capillary rarefaction was

identified to be a prognostic marker of pre-eclampsia, again preceding the onset of symptoms.[246]

Insufficient angiogenesis has been suggested as a possible mechanism for capillary rarefaction but associations between angiogenic markers such as VEGF and capillary density have yet to be fully addressed. In a preclinical study of rats, capillary density in striated muscle and heart was increased along with VEGF levels after prolonged exposure to hypoxia, thus reaffirming the effect of HIF-alpha on angiogenic factors such as VEGF. Furthermore, the increase in capillary density was associated with normalisation of blood pressure in pre-hypertensive rats.[247] There is also evidence that chronic VGEF inhibition causes capillary rarefaction in preclinical models. These effects in capillary regression are seen as early as 24 hours after initiation of VEGF inhibitors.[231]

It is clear therefore that skin capillary density is a dynamic variable that not only may be a causative factor in the disease process but may alter in response to pathology. Skin capillary density may also act as a surrogate marker of angiogenic activity.

1.5.5.Skin capillary density as a marker of disease

Capillary density impacts on the total resistance of the capillary bed and thus capillary pressure.[237] Capillary pressure is vital to tissue fluid homeostasis and trans capillary exchanges.[237] It is well recognised that clinical presentation of ovarian cancer is heterogeneous with some women presenting with massive volumes of ascites. Additionally, post-operative recovery courses vary considerably. The assessment of SCD at presentation

and post operatively in relation to these clinical aspects may provide an insight into the microvascular status that may then help to predict those more at risk of post operative complications.

A recent study has demonstrated an association between skin capillary rarefaction and the presence of albuminuria.[248] This association was present after control for confounding variables such as diabetes and cardiovascular risk factors. Although the assumption that skin capillary rarefaction is a reflection of the rarefaction in the kidney (a reason for the development of albuminuria)[248] was recognised as a limitation it will be interesting to see whether SCD may also be associated with the presence of ascites and post-operative complications occurring due to increased capillary permeability and hypalbuminaemia.
We have seen that SCD reduces with age due to the reduction in angiogenic factors so it can be argued that capillary density assessed in the skin may alter as VEGF alters throughout the cancer journey.

A common side effect to angiogenic inhibitor therapy is hypertension. The mechanism by which this occurs is not fully understood but 9-16% of patients treated with bevacizumab develop grade 3-4 hypertension.[249]

It has been postulated that hypertension is a result of reduction in structural skin capillary density.[249] Additionally, this rarefaction may be the cause of bevacizumab induced enteric perforations.[250] A small study of 16 patients with metastatic renal cell carcinoma treated with an angiogenic inhibitor sunitinib had a significant decrease in skin capillary density associated with rise in blood pressure.[234] This effect was reversible once the therapy stopped. An

additional two small studies in patients with metastatic colorectal and breast disease found a decrease in SCD from baseline after 6 months of treatment angiogenic inhibitors and, with the effect reversing after cessation of treatment.[249, 251]

A study of 37 patients with colorectal cancer receiving Bevacizumab recently demonstrated a reduction in skin capillary density during treatment. There was no association with capillary rarefaction and response to treatment and the authors were not able to demonstrate correlation to hypertension.[252]

In animal models, regression and apoptosis of endothelial cells leading to reduction in capillaries has been demonstrated after VEGF inhibition and it may be that similar mechanisms are occurring in dermal capillaries in patients treated with anti angiogenic inhibitors.[249]

1.5.6.Conclusion

Ovarian cancer is a complex diverse disease with a poor survival rate and complex treatment that carries significant morbidity. The role of angiogenesis in ovarian cancer progression is well recognised and the area of research into angiogenic inhibitors as additional therapies is novel and exciting. However, none of these treatments have demonstrated a survival benefit as yet and carry potential serious complications and high economic cost to the NHS. The era of personalised medicine is developing and biomarkers are needed to target these treatments to those who will benefit at the optimal time.

There has been no work as yet investigating SCD and angiogenic markers in cancer patients. As we have discussed, hypertension is a consequence of angiogenic inhibitors and potential

marker of response. We have established that reduction in SCD occurs prior to the clinical development of hypertension thus it is reasonable to hypothesise that SCD may be the first thing to change in response to angiogenic inhibitors.

Since many complications from bevacizumab treatment carry high morbidity and mortality, monitoring of response to allow early prediction of complications and therefore early intervention with a simple non-invasive measurement is appealing. Furthermore, Bevacizumab is used in other malignancies and in the palliative setting hence the potential patient impact is large.

1.6. Hypothesis

After consideration of the literature I have constructed the following hypotheses that I aim to address in my thesis.

- Skin capillary density is a surrogate marker of angiogenesis
- Skin capillary density reduces as a result of cancer treatment
- Capillary rarefaction occurs with Bevacizumab treatment and correlates with change in blood pressure
- Skin capillary density can predict response to treatment and cancer outcomes
- Skin capillary density correlates with vascular markers and response in tumour tissue

1.7.Aims

I will now address the following aims in my subsequent chapters to address my hypotheses.

- To identify changes in skin capillary density throughout treatment in ovarian cancer
- To identify changes in angiogenic factors throughout treatment in ovarian cancer
- To identify any association between skin capillary density and angiogenic factors
- To investigate whether SCD at baseline predicts OS, PFS or surgical cytoreduction
- To investigate whether the change SCD before and after 3 cycles of treatment can predict surgical cytoreduction or surgical morbidity in women undergoing neoadjuvant chemotherapy
- To investigate whether tumour proliferation and vascular markers at baseline or at surgery can predict OS, PFS
- To investigate whether tumour proliferation markers at baseline or at surgery can predict surgical cytoreduction, post-operative complications and response to chemotherapy (radiological CT response and histological chemotherapy response score)
- To investigate the longitudinal changes in tumour proliferation and vascular markers after chemotherapy
- To investigate the association between tumour vascular markers and skin capillary density
- To assess the change in SCD in a specific population of patients on anti angiogenic therapy
- To assess the effect of anti angiogenic therapy on blood pressure and association with skin capillary density
- To assess the prognostic role of SCD in patients on Bevacizumab treatment

Chapter 2. Methods

2.1. Study design and population

2.1.1. Study design

This was a prospective longitudinal cohort study conducted over a 2-year period. Ethical approval was granted in 2016. (Appendix 1).

2.1.2. Study setting

Women were recruited from the primary cancer centre, St Michael's Hospital, University Hospitals Bristol and a secondary site Weston General Hospital. Patients were recruited from November 2017 until May 2018 and were followed up until January 2020.

2.1.3. Population

During the recruitment period, I attended weekly Multidisciplinary Meetings where new patients were discussed and screened cases for suitable participants. I approached women who were suspected of having a high-grade serous carcinoma and discussed the study and provided patient information leaflets. If they were agreeable, I contacted them after 1 week over the telephone to see if they would like to participate. I then met these women at their new patient chemotherapy talk or pre-operative assessment for the first data collection.

Some of the women who were thought to have ovarian cancer had benign disease after histological examination of the tissue. A small number of women with no pathology were approached to measure baseline skin capillary density as part of validation of skin capillary measurements.

2.2. Inclusion criteria

- All patients undergoing primary treatment with suspected or confirmed high grade serous ovarian cancer.

2.3. Exclusion criteria

- Ovarian cancer patients with co-existing tumours
- Patients who have received anti-angiogenic chemotherapy previously (e.g. bevacizumab)
- Patients who have had radiotherapy, as the systemic effects of radiotherapy on angiogenesis are unknown

2.4. Data collection

Patients who had 3b disease or less were offered primary surgery unless there was a clinical reason to offer neoadjuvant chemotherapy. Those with more advanced disease or clinical contra indications for primary surgery underwent neoadjuvant chemotherapy. Each patient was seen 5 times during their treatment as described in detail below. I recorded basal and maximal skin capillary density and collected serum samples at each visit. At each visit I also recorded blood pressure and skin temperature. Tissue was collected to make the diagnosis

(biopsy sample) and during major debulking surgery as part of routine care and details about preparation of tissue to count vessels is described in more detail below (section 2.9 page 66).

There is a potential psychological impact and burden of taking part in a study in addition to a new diagnosis and treatment of cancer. Additionally, for many women because of the intensity of the treatment, often feeling unwell and the arduous number of hospital visits, I did not ask women to attend on additional occasions for my data collection. Instead, all tests were performed at the time of normal hospital attendance and I collected blood samples at the same time as chemotherapy-related investigations to avoid unnecessary repeat tests. The visit 48 hours post operatively was conducted on the ward. In order to maintain consistency amongst patient visits, scrupulous organisation and follow up was required to ensure I did not miss data collection visits and that women were not required to attend more frequently. This required a significant number of hours per week for me to monitor various hospital databases to keep a record of follow up clinics, potential complications from treatment, changes in treatment plans and outpatient and chemotherapy appointments.

Patients reported enjoyment and enhancement in their care by taking part in the study and I was able to provide some continuity of care over the course of treatment and help to offer a greater understanding of their treatment pathway and access to information and support. On one occasion I was able to recognise a significant side effect of Bevacizumab treatment as the patient reported an episode of weakness during conversation. I was able to organise urgent oncological review and the patient was diagnosed as having had a transient ischaemic attack. I also took great enjoyment in seeing patients regularly and being able to follow up on clinical progress over the course of many months.

2.5. Details of research visits

Figure 2.1 summaries visits and treatment.

2.5.1. First visit:

The first visit was scheduled prior to starting any treatment. In some women who were having neoadjuvant chemotherapy, a diagnosis of high grade serous (HGS) ovarian cancer had been made by combination of histology or cytology and imaging. The remaining women had suspected disease based on the aforementioned investigations. In all cases women were seen prior to treatment. Women were allowed to rest in a temperature-controlled room for 20 minutes and blood pressure was measured. Skin capillary density was measured as described later and 10ml of venous blood in a plain tube was collected alongside pre chemotherapy or pre-operative routine blood investigations. The processing of blood samples is described below (section 2.8 page 62).

2.5.2. Second visit

The second visit was scheduled either after 3 cycles of chemotherapy before interval debulking surgery or 48 hours after surgery in those women undergoing primary surgery. Bloods pressure, skin capillary density was measured and blood taken alongside pre-operative bloods or routine post-operative bloods for those who were inpatients. For those patients due to have surgery, information on mid treatment CT scan was recorded according to RECIST criteria. RECIST criteria provides a standardised system to evaluate measurable lesions and response to treatment. They are defined as the following: Complete response (CR): Disappearance of all target lesions. Partial response (PR): At least 30% decrease in the sum of the target lesions. Stable disease (SD): Neither sufficient shrinkage to qualify PR nor

sufficient increase to qualify for PD. Progressive disease (PD): At least a 20% increase in the sum of the larger lesions or the appearance of one or more new lesions.[253]

2.5.3. Third visit

The third visit was scheduled for 48 hours or 2 weeks post operatively depending on the treatment pathway. For those 2 weeks post-surgery, blood was collected from patients alongside pre chemotherapy bloods as a joint surgical and oncology appointment was usually scheduled for this time. Skin capillary density and blood pressure was recorded in all patients. Information regarding surgery and surgical debulking outcome was also collected at this visit.

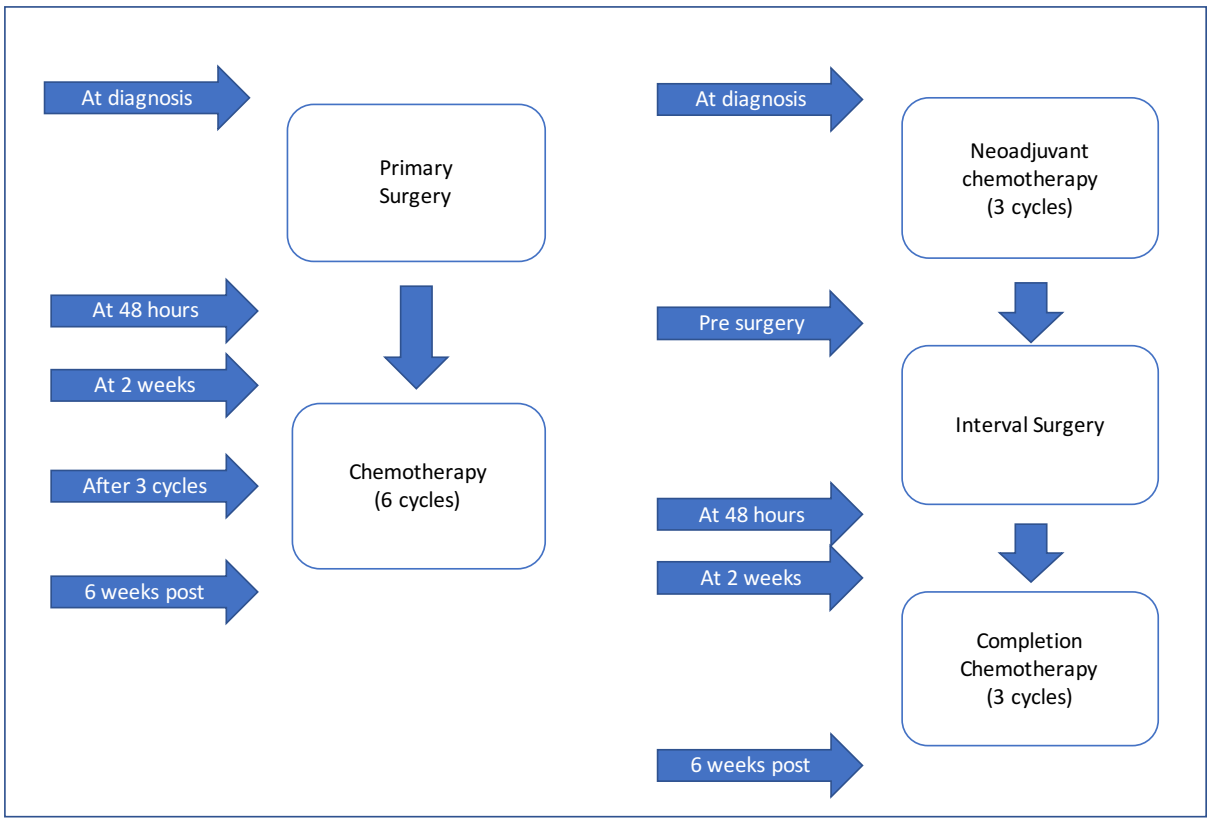
2.5.4. Fourth visit

The fourth visit was scheduled for 2 weeks post-surgery or after 3 cycles of chemotherapy for those patients having primary surgery. Blood pressure, skin capillary density and blood were measured in the same way as previously.

2.5.5. Fifth visit

The fifth visit was scheduled for end of treatment for all patients. Skin capillary density and blood pressure was recorded as well as outcome criteria of end of treatment CT scans. This visit was conducted 4-6 weeks after completion of chemotherapy in correlation with routine follow up. Blood was taken alongside routine bloods as part of end of treatment and baseline CA 125 measurements for those women embarking on routine follow up.

Figure 2.1. Flow chart of visits and treatment.



2.6. Outcomes

2.6.1. Variables of interest

Skin capillary density (number of vessels/mm^2), CD 31 (number of vessels/field), PDGFR (number of vessels/field), VEGF (pg/ml), Ang 1 (pg/ml), Sol Endo (ng/ml): These was recorded in absolute values. Change was calculated in absolute value change.

Ki67: This was recorded as a percentage.

Death and recurrent events were recorded. Overall survival (OS) was calculated from time of diagnosis to time of death and was recorded in months. Progression free survival was calculated from time of diagnosis to time of recurrence and was recorded in months.
Surgical outcome was defined as optimal (complete or <1cm residual disease) or suboptimal (>1cm disease remaining).

2.6.2. Demographic details

Patient demographics were gathered from clinical notes and clinic questionnaires to include clinical presentation, histology, initial computed tomographic (CT) findings, age, medical history, drug history, proposed chemotherapy. All patients receiving chemotherapy for ovarian cancer had CT scans performed before, mid- and post-treatment. Data from the CT scans was recorded from the MDT reports during their follow up visits or from hospital data base systems and disease response characterised by the Response evaluation criteria in solid tumours (RECIST) criteria to Complete Response, Partial Response, Stable disease and Progressive Disease.[253]

I collected this information in a database which was held securely on a gynaecological oncology folder, located on the Hospital server. Anonymised data was backed up on another drive which was stored in a secure research office. Data protection guidelines and confidentiality was respected at all times.

2.6.3. Definitions

Surgical complications were also recorded and procedure scores were calculated depending on complexity of surgery. Surgery included total abdominal hysterectomy, bilateral salpingo-oophorectomy, appendicectomy, omentectomy, and excision of peritoneal disease. Extended procedures were those that included bowel resection, splenectomy, diaphragm resection and excision of lymph nodes. Data from intra operative notes was collected and intra operative complications such as haemorrhage, visceral injury, blood transfusion and high dependency or intensive care admission. Surgery was scored 0-3: 0 for standard surgery, 1 for one additional extended procedure, 2 for two additional extended procedures and 3 for three additional extended procedures.

2.6.4. Subgroups

Patients were categorised into groups for analysis according to the treatment they received.

Neoadjuvant chemotherapy: These women had advanced disease at presentation so were treated with 3 cycles of chemotherapy followed by surgery and then 3 further cycles of chemotherapy.

Primary surgery: These women had debulking surgery followed by 6 cycles of chemotherapy.

Neoadjuvant chemotherapy plus Bevacizumab: These women had bevacizumab added to the 3 cycles of chemotherapy after debulking surgery. This was because of stage 4 disease or a suboptimal surgical outcome.

ICON8b: These women were enrolled in an international trial and received neoadjuvant chemotherapy but had bevacizumab alongside chemotherapy from the start of treatment.

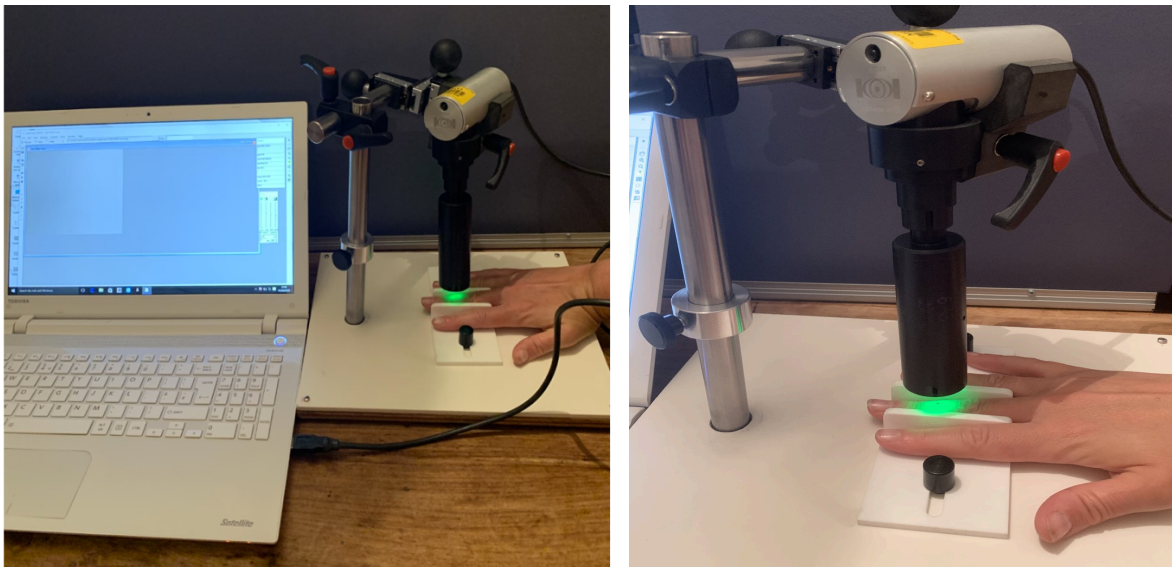
Chemotherapy only: These women had neoadjuvant chemotherapy but did not undergo surgery after 3 cycles due to extensive disease or because they were medically unfit. They continued with chemotherapy and received 6 cycles.

2.7. Methodology of Skin capillary density

Capillaroscopy allows a 2D visualisation of a 3D capillary network in real time and software enables the images to be recorded as videos or stored as image files.

Capillaroscopy was performed using a standardised validated method.[237] Images and videos were obtained with a CapiScope camera and I analysed images using the computer software (KK Technology).

Figure 2.2. Set up of Capiscope and computer software



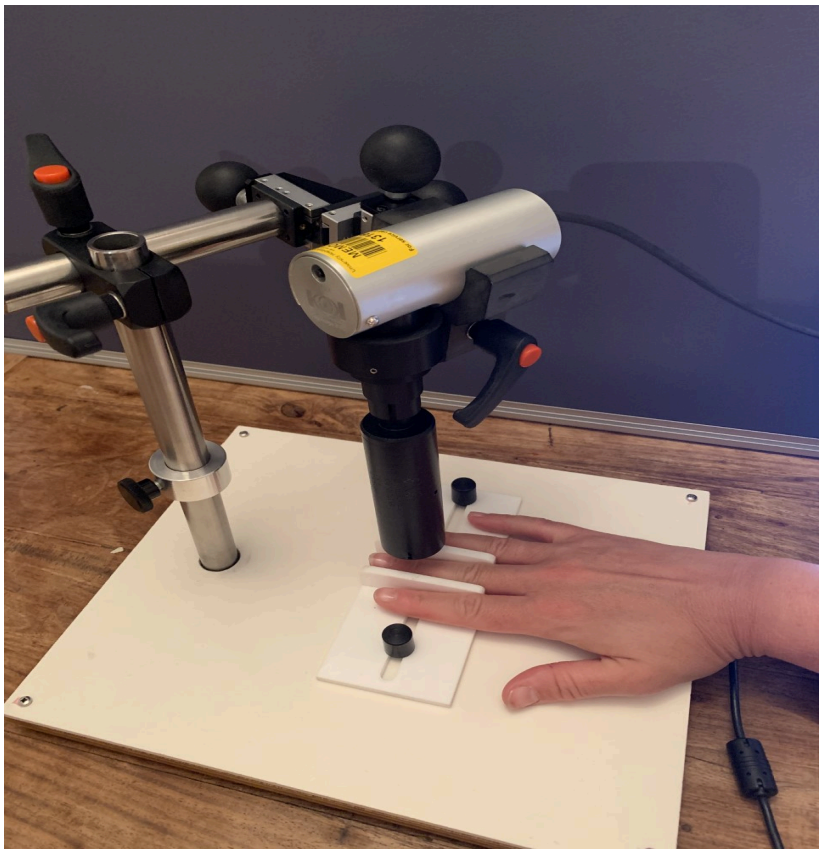
A light sensitive black and white camera is mounted over a lens system and the skin is illuminated. A green filter enhances the contrast to improve images for analysis. The microscope can be moved over the patient skin on the standard mounting block. Once over

the correct point and perpendicular to the skin, it can be moved up and down to improve focus.

Stringently controlled conditions for analysis are vital to ensure standardised and comparable examinations as capillary flow is susceptible to temperature, sympathetic drive and venous pressure.[237] I therefore examined all patients in a temperature-controlled room of 21-24°C after 15-20 minutes of acclimatisation. Patient were seated with hand at heart level during this period of acclimatisation. I took this opportunity to discuss treatment plans and make notes of complications, CT scans and surgical information. Skin temperature was also recorded.

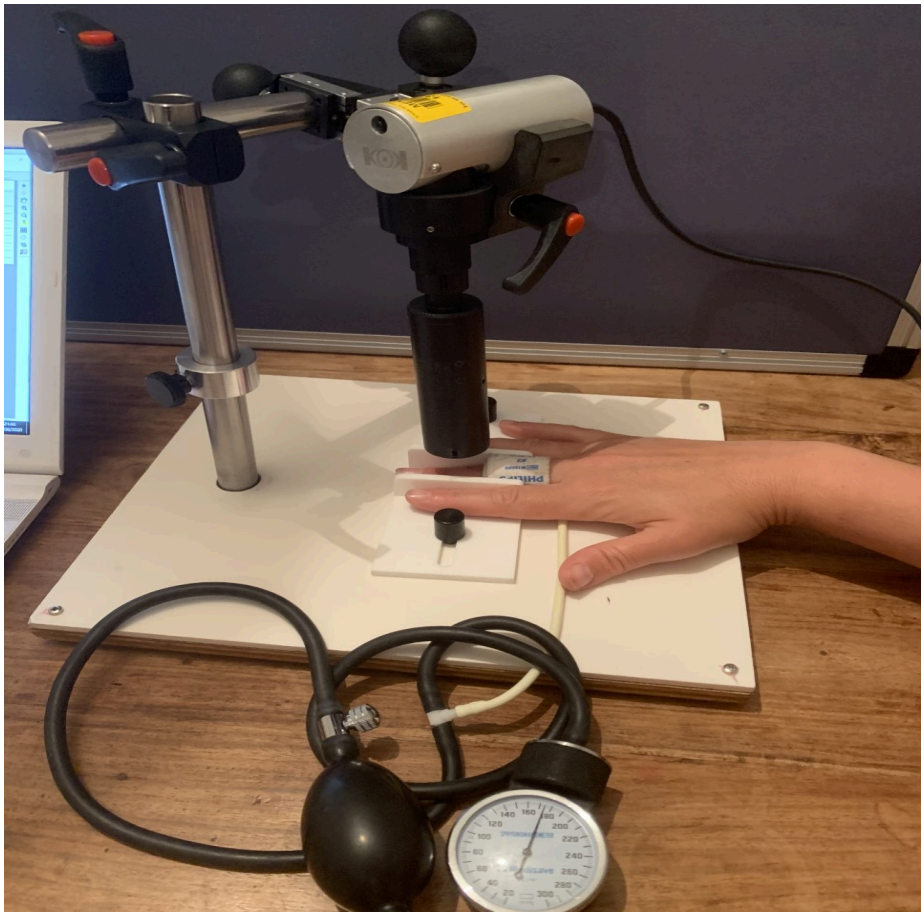
The middle finger of the left hand was used for each patient and four microscopic fields (1mm^2 each) around a central ink mark placed on the dorsum of the middle finger. The finger was placed horizontally and immobilised between two plastic stabilisers to improve image quality. The microscope was focused on to the ink spot and a drop of oil placed on the ink spot in order to reduce scattering of light and improve image quality.

Figure 2.3. Set up of finger immobilised for capillaroscopy. Capiscope is seen mounted on a block which allows movement and focus to improve image quality.



Images were recorded continuously for 5 minutes to permit detection of all perfused capillaries (basal skin capillary density) to provide functional skin capillary measurement. In order to ascertain maximal capillary density, I recounted vessels after venous occlusion (paediatric sphygmomanometer cuff placed at base of finger and inflated to 60mmHg for 2 minutes). The number of capillaries were counted in one of the four microscopic fields chosen at random after the cuff inflation for 2 minutes. The image during this point was focused at difference depths. The increase of capillary density is reported around 12-18% in health in the literature.[237]

Figure 2.4. Measurement of maximal (structural) skin capillary density with sphygmomanometer cuff placed at base of finger and inflated to 60mmHg for 2 minutes

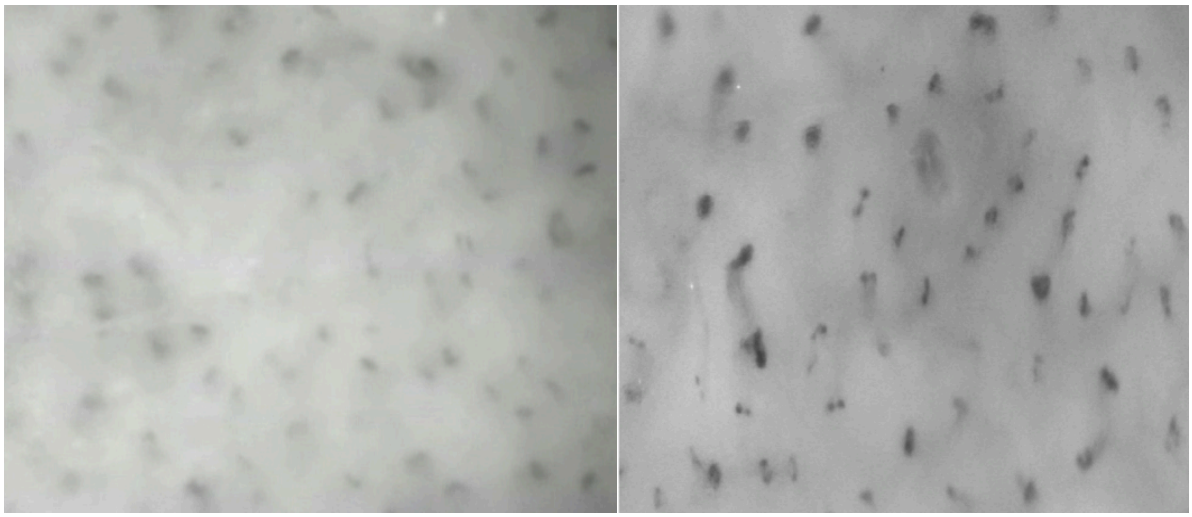


Initially I attempted to count the capillaries in live time however this took a considerable amount of time for the patient and so I chose to record the images and then analyse and count these off-line later the same day. This also allowed me time to pause, rewind and recount areas to improve consistency and accuracy. Recording videos also allowed for some to be recounted by me at a later date and by my supervisor to assess intra and interobserver variability of the technique. 20 videos were examined for intra and inter observer variability with 3% and 5% variability respectively. This is consistent with the literature where reproducibility of capillaroscopy using these methods is reported at 5-8%.[237]

For each patient at each visit, the mean basal SCD was calculated as the mean count from the four microscopic fields. Maximal SCD for then recorded for each patient at each visit. Each video required around 30 minutes to count and calculate the mean.

Figure 2.5. Typical appearance of 1mm^2 image of capillaries. A: Basal SCD (functional). B: Maximal SCD (structural). Each black dot represents a capillary and is counted as part of calculating skin capillary density

A. Basal SCD B. Maximal



2.8. Measurement of angiogenic factors

2.8.1. Collection and storage of serum

I collected 10ml of blood in a plain Vacutainer tube at each visit. As some patients had treatment at a hospital around 30 miles away I decided to leave samples to clot for 2 hours prior to centrifugation to allow time for me to return to the laboratory and process the sample. They were then centrifuged at 3000g for 5 minutes. After centrifugation serum was stored in 500 µl aliquots in Eppendorf tubes at -80°C. Each study sample was given a number and I coded and recorded this in the laboratory book which was securely stored anonymously. All samples were stored and disposed of in accordance with the Human Tissue Act 2004.

2.8.2. ELISA technique

Angiogenic factors were measured by enzyme-linked immunosorbent assays (ELISAs). I used validated quantikine ELISA kits that had been described in the literature as I wanted the most reliable assay in view of the limited and essentially timed serum samples. All of the ELISA techniques required smaller aliquots of 50-100 µl so in order to avoid repeat freeze-thaw cycles samples were split into smaller aliquots when ELISAs were carried out. Each sample used for my analysis only underwent 1 freeze thaw cycle.

All ELISAs employed the quantitative sandwich enzyme immunoassay technique. In order to avoid cross contamination pipette tips were changed between additions of each standard

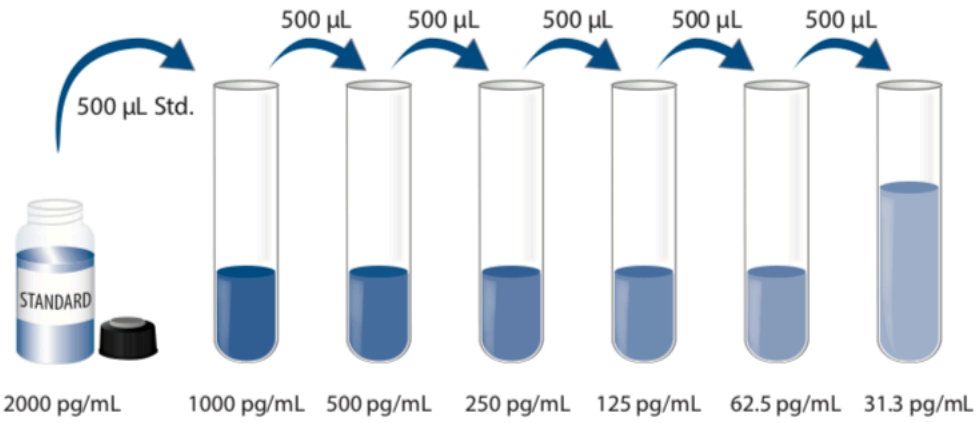
level, between sample and reagent additions. All substrate solutions were protected from the light and an automatic pipette was used to help with accuracy.

The r value of the standard curve was recorded for every plate reading and if >0.95 was thought to be acceptable. The plate was repeated if the r value was too low. Every sample was measured in duplicate and if the readings differed by more than 30% they were repeated. Raw data collected from the ELISA plates was processed using excel software into a concentration in the units of the standard.

2.8.2.1. VEGF (DVE00 R&D systems)

Instructions for reagent preparation and assay technique were followed according to the protocol. All reagents and samples were bought to room temperature before use. A 96 well plate was used which was pre coated with a monoclonal antibody specific for human VEGF. 100 μl of assay diluent was added to each well. 100μl of the standard or sample were then added and incubated at room temperature for 2 hours. A plate layout was used to recorded standards and samples assayed. Each well was then aspirated and washed with wash buffer 4 times, ensuring complete removal of any liquid at each wash and inverting the plate and blotting against paper towels. 200μl of the human VEGF Conjugate was added to each well and incubated for 2 hours at room temperature. Wells were then aspirated and washed as previously described. 200μl of the substrate solution was added to each well and then incubated for 25 minutes at room temperature away from light. 50μl of the Stop Solution was added to each well and colour change from blue to yellow was observed. A microplate reader set to 450nm was used to determine optical density within 30 minutes.

Figure 2.6. Demonstration of the process of dilution of the standard. The stock solution is used to produce the dilution series. 500µl of calibrator diluent RD6U is pipetted into each tube. Each tube is thoroughly mixed before the next transfer. The 2000pg/ml standard serves as the high standard. The calibrator diluent serves as the zero standard (0pg/ml).



2.8.2.2. Angiopoeitin-1 (DANG10 R&D systems) and Soluble Endoglin (CD105 R&D systems)

All reagents and samples were bought to room temperature before use. A 96 well plate was used which was pre coated with a monoclonal antibody specific for human Angiopoietin- 1 or soluble endoglin. The reagent and assay preparation was again prepared as per protocol and Table 2.1 describes any differences from the VEGF ELISA procedure described above.

Table 2.1. Description of procedure for ELISAs

	VEGF (DVE00 R&D systems)	Angiopoeitin-1 (DANG10 R&D systems)	Soluble Endoglin (CD105 R&D systems)
Assay diluent	100 µl	100 µl	100 µl
Standard/Sample	100 µl	50 µl	50 µl
First incubation	2 hours	2 hours	2 hours
Wash buffer	4 times	4 times	4 times
Conjugate	200 µl	200 µl	200 µl
Second incubation	2 hours	2 hours	2 hours
Substrate solution	200 µl	200 µl	200 µl
Third incubation	25 minutes	30 minutes	30 minutes
Stop solution	50 µl	50 µl	50 µl

2.8.2.3. VEGF 165b (DY3045 R&D systems)

There are limited validated ELISA kits for this protein, and I contacted various researchers across the country in order to gauge opinion on those that had produced reliable results. Unfortunately, many researchers had unsuccessful attempts at trying to measure VEGF 165b in an ELISA. The ELISA that was used was also by R&D and had previously been developed at the University of Bristol and validated in other studies.

Despite this it was difficult to measure. Consistently producing an accurate standard curve with the kit used was not achievable despite a good technique and following the protocol. For some plates a curve was achieved but the levels were negative hence undetectable. For this reason they were not included in the analysis due to the sparsity of results.

2.9. Tissue preparation and tumour vasculature and proliferative markers

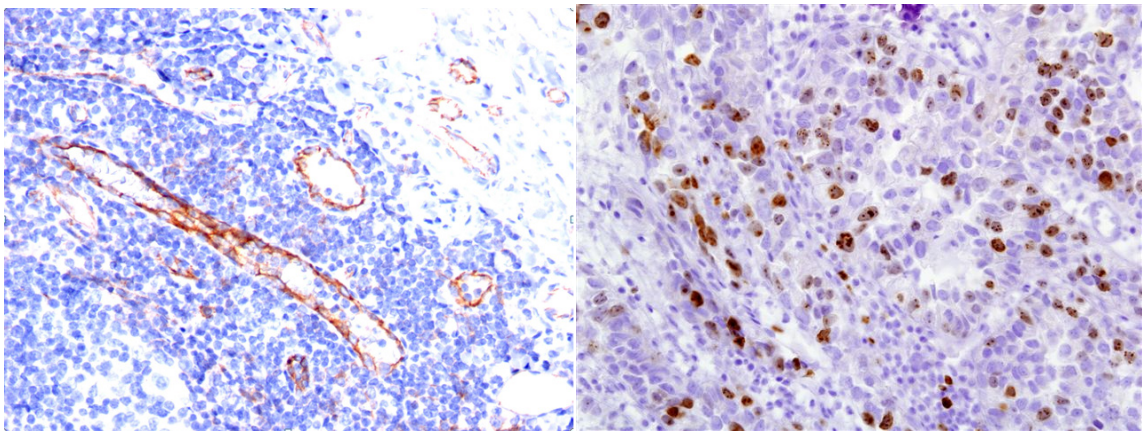
2.9.1. Slide preparation

Protein expression of the tumour vasculature and proliferation markers of interest were detected by antibodies using immunohistochemistry (IHC) staining techniques.

Slides were prepared by a biomedical scientist. At the time of biopsy and at primary or interval debulking surgery, tissue was removed and immersion fixed in formaldehyde and paraffin embedded (FFPE blocks). Tissue blocks of areas representative of the invasive component of the cancer from metastatic tissue, either peritoneum or omentum were selected from the sections stained with haematoxylin and eosin by myself and an experienced Gynaecologic Consultant Pathologist. The relevant 4-um tissue sections were cut from FFPE block using a microtome and mounted onto slides. Slides were rehydrated and primary antibody detection was performed for Ki67, PDGFR and CD31 using validated protocols and detection kits from companies Abcam and R&D systems. The figures below give examples of staining with the various markers.

Figure 2.7A. Paraffin embedded tissue stained with CD 31 antibody. 2.7B Paraffin embedded tissue stained with Ki67 antibody. Brown staining represents a blood vessel in the case of CD 31 antibody or nuclei staining for ki67.

A. CD 31 antibody B. Ki67 antibody



2.9.2. Calculation of vascular and proliferative markers

Ki67 was calculated as a percentage of nuclei stained. This was done independently by myself and a specialised Gynaecologic Consultant Pathologist to improve accuracy and reliability. Microvessel density using CD31 and PDGFR was calculated using the validated Weidner method.[187] We selected areas with the highest density of microvessel (hotspots) at low magnification. All the vessels in the region were then manually counted in these areas at high magnification (200x-400x). Any brown or blue staining endothelial cell or cluster that was seen separately from tumour cells and other connective tissue element was considered a single vessel. Vessels were counted by myself and the Pathologist on double headed light microscopes concurrently to ensure agreement and consistency. Each count took around 10 minutes and we were blinded to patient outcomes or information.

2.9.3. Chemotherapy response score

Chemotherapy response score (CRS) was calculated for each specimen taken at interval debulking surgery by an experienced tertiary specialist Pathologist in Gynaeoncology. CRS is a simple and reproducible grading system to assess response to neoadjuvant chemotherapy.[254] The International Collaboration on Cancer Reporting (ICCR) has recommended clear guidance on the assessment of CRS which involves assessment of a single block of omental tissue that shows the least response to chemotherapy.[255] The amount of viable tumour is assessed and graded on a three tier system; >95% of tumour should be viable for a score of 1 and <5% for a score of 3.[255] Table 2.2 is taken from the ICCR to describe the difference scoring mechanisms for CRS.

Table 2.2. Characteristics for chemotherapy response scoring: recommendations from the International Collaboration of Cancer Reporting (ICCR)[255]

Score	Criterion	Tumour regression grading
1	Mainly viable tumour with minimal regression-associated fibro-inflammatory changes limited to a few foci	No or minimal tumour response
2	Multifocal or diffuse regression-associated fibro-inflammatory changes, with viable tumour ranging from diffuse sheets, streaks or nodules, to extensive regression with multifocal but easily identifiable residual tumour	Partial tumour response
3	Mainly regression, with few irregularly scattered individual tumour cells or cell groups (all measuring <2 mm), or no residual tumour identified	Complete or near-complete response

2.10. Statistical considerations

I received guidance from a University of Bristol statistician, Dr Sofia Kavanou, throughout my analyses to improve my understanding of methods and ensure that I processed my data correctly.

In order to ensure enough participants were recruited to detect a correlation between variables we used R software package to perform the calculation. A cohort of 50 patients with full data on 90% (45) of the participants would give 88% power to detect correlations as low as 0.45 as statistically significant at the 5% level between capillary density or VEGF and PFS, OS. This would be sufficient to provide proof of concept required to take this study further and use the data to inform plans for subsequent studies.

Repeated measures ANOVA revealed non-linear trends in longitudinal analysis. To adjust for the heterogeneity present between treatment groups, a mixed model with unstructured covariance was fit. For pairwise comparisons we used Tukey's HSD post-hoc adjustment to account for Type-I error inflation.

I have detailed descriptive statistics for demographics and clinical characteristics for the entire cohort and specifically for those studied in each chapter. Continuous variables were reported as mean and standard deviation.

Continuous variables were dichotomised to above and below means when compared with outcomes in logistic regression and Kaplain Meier models. ROC curves were constructed to predict optimal cut off points.

Associations between continuous variables were analysed with linear regression models. Logistic regression models were used to determine associations between continuous variables and outcomes. Comparison of means was assessed with non-parametric t tests (Mann Whitney) and categorical variables used Spearman's Rank and Kendall tau test. ANOVA was used to compare differences between groups. Kaplain Meier analysis was used to estimate the survival outcomes and construct progression free survival (PFS) and overall survival (OS) curves. To assess the prognostic value of variables to OS and PFS curves were compared using the log rank test. Univariate and multivariate cox proportional hazards modelling was used to test associations between variables and outcomes. Where appropriate models were adjusted for age, performance status, stage of disease, treatment and surgical outcomes. Statistical significance was defined as $p<0.05$.

I used IBM SPSS Statistics version 25, PRISM 8 and Microsoft® Excel for Mac to perform the analyses.

2.11. Ethics

The study was granted ethical approval on 15th February 2016 by REC Nottingham Research Committee (ref: 15/EM/0489) and was sponsored by Universities Hospital Bristol. Consent processes were consistent with GMC guidance on 'Good Medical Practice in Research'.

Participants confidentiality was maintained throughout the study and all documents were stored securely and only accessible by myself. These were kept in a Site file secured on premises. The study complied with the Data Protection Act and Human Tissue Act and all data was anonymised.

2.12. Funding

I achieved funding from David Telling Trust for £11,309 to buy the capillary microscopy and £14, 440 from Above and Beyond to fund analysis of serum and tissue samples. Insurance was arranged by the sponsor (University Hospitals Bristol NHS Foundation Trust).

Chapter 3. Validation of skin capillary density

3.1. Introduction

Absolute values for skin capillary density in the healthy population are heterogenous. Values in the literature range from 40 to 85 (vessels/mm^2).[239, 240, 242-246, 248, 256] when SCD has been investigated in health and in the presence of cardiovascular disease, hypertension, diabetes, rheumatological disease and pregnancy. However there is not a specific range or value that denotes health or disease[265]. Skin capillary density has been shown to decrease in patients with hypertensive disease compared to normotensive patients or those with well controlled and treated hypertension.[237, 242]

In order to ensure my methods of measuring skin capillary density were reliable I carried out measurements in healthy patients and those with benign disease to help validate my methodology prior to recruitment of my study cohort.

3.2. Methods

I measured mean basal and maximal SCD in 10 healthy age matched volunteers. I also measured SCD in 2 subjects at 3 points throughout the day in order to assess the time on possible variations in SCD measurements. 10 women who were undergoing primary surgery for an ovarian mass with low risk of malignancy were also included. All these women had benign disease confirmed on histological examination. Differences in mean skin capillary density were compared using Wilcoxon test

3.3. Aims.

- To validate methodology of capillaroscopy

3.4. Results

3.4.1. Demographics of cohort

The mean age and BMI of patients amongst all groups were comparable. No patients had uncontrolled hypertension or history of rheumatological disease which may have affected SCD values. Table 3.1 describes the demographics in the groups.

Table 3.1. Skin capillary density (vessels/mm^2) and demographics of all patients

	Malignant (n=50)	**Benign (n=10)**	**Healthy control (n=10)**	**P value**
Age (years) Mean (SD)	69.8 (11.2)	69.0 (4.4)	67.0 (5.6)	0.53
BMI (kg/m^2) Mean (SD)	25.6 (4.7)	28.0 (4.1)	27.5 (5.7)	0.33

3.4.2. Skin capillary density variation throughout the day

Skin capillary density was measured in 2 healthy controls on 3 occasions 15 minutes apart at 9am, 12pm and 3pm. Mean basal SCD did not vary significantly throughout the day (p=0.60) and this was the case for both control patients. Similarly maximal SCD values remained stable at both 9am, 12pm and 3pm (p=0.40). Table 3.2 records the values of skin capillary density at different time points throughout the day.

Table 3.2. Variation in skin capillary density (vessels/mm^2) during the day

Mean basal	**9am**	**12pm**	**3pm**	**P value**
Control 1	58, 59	60, 60	56, 61	0.600
Control 2	63, 66	60, 61	66, 60	0.400
Maximal				
Control 1	61, 69	70, 67	70, 71	0.733
Control 2	70, 71	69, 67	71, 69	0.467

3.4.3. Differences in skin capillary density in malignant, benign and control group

I compared the baseline values of those women recruited in the study with malignant disease, with those who had benign disease and the control cohort. Mean basal SCD at baseline varied between the groups and was 62.9, 57.6 and 65.2 vessels/mm2 in the benign, control and malignant cohort respectively (p=0.018). Variation between the groups for maximal SCD was not significant (p= 0.258) and was 68.2, 66.0 and 70.2 vessels/mm^2 for the benign, control and malignant cohort.

Figure 3.1 and 3.2 demonstrate the mean and maximal SCD according to each group.

Figure 3.1. Box plot of mean basal SCD (vessels/mm^2) at baseline for the different groups

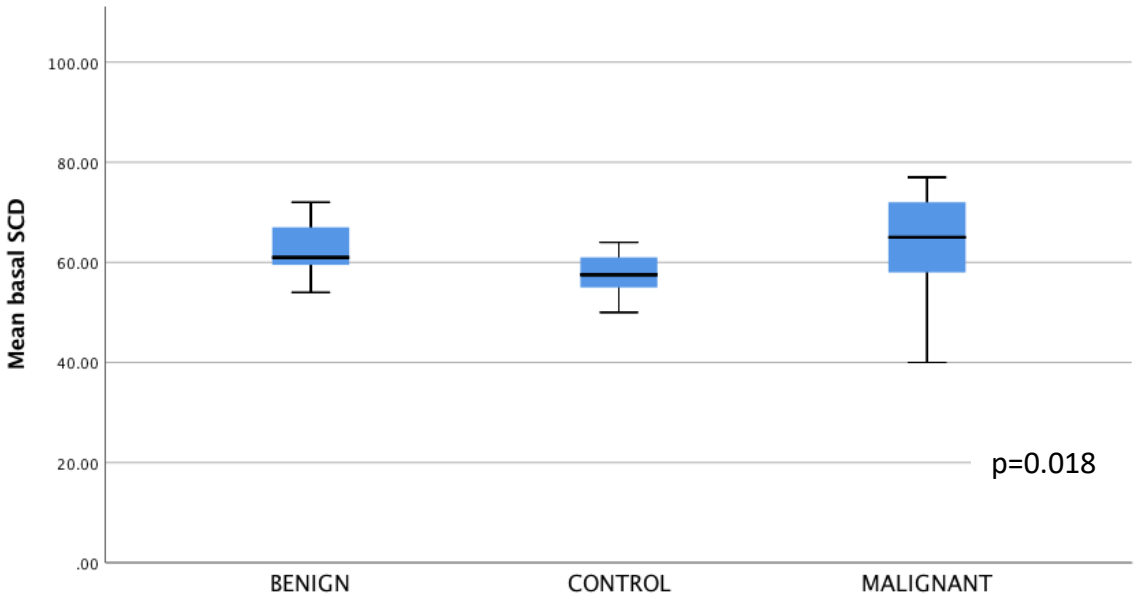
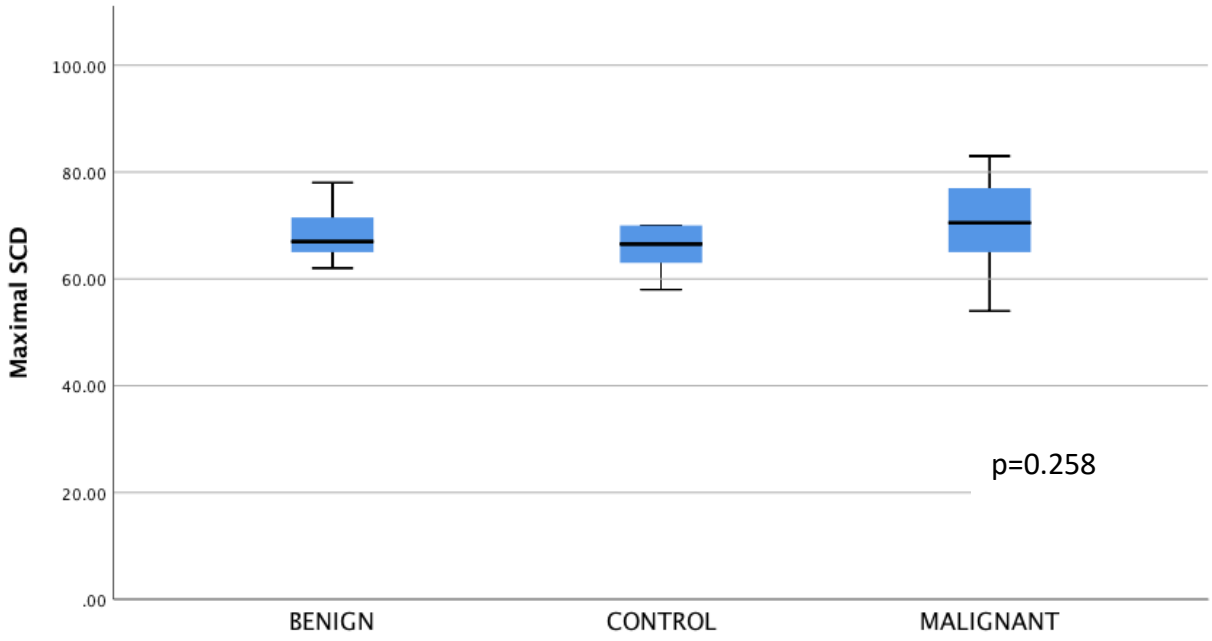


Figure 3.2. Box plot of maximal SCD (vessels/mm^2)at baseline for the different groups



When those with benign disease were compared with patients who had malignancy there was no significant difference in SCD in either mean basal or maximal SCD values (62.9 vs 65.2 vessels/mm^2 p=0.313 and 68.2 vs 70.2 vessels/mm^2 p= 0.411 respectively).

There was a significant difference between the control group and those who had malignancy in regards to mean basal SCD (65.2 vs 57.6 vessels/mm^2 p=0.0061) but this was not seen in maximal SCD values (70.2 vs 66.0 vessels/mm^2 p=0.124).

The difference between SCD in the control and benign group was not significantly different for mean basal or maximal values (57.6 vs 62.9 vessels/mm^2 p=0.062 and 66.0 vs 68.2 vessels/mm^2 p =0.638 respectively).

Table 3.3 records mean basal and maximal skin capillary density (vessels/mm^2) in patients in the control and malignant disease cohorts.

Table 3.3. Skin capillary density (vessels/mm^2) in control and malignant cohort of patients

	Control (n=10) **Mean (SD)**	**Malignant (n=50)** **Mean (SD)**	**P value**
Mean basal SCD	57. 6 (4.3)	65.2 (9.4)	0.0061
Maximal SCD	66.0 (4.1)	70.2 (9.1)	0.124

3.5. Discussion

Skin capillary density did not appear to vary throughout the day which suggests that measuring at a consistent time point (early morning) as was done with my study cohort is reliable. This is also alongside ensuring the appropriate measures were taken as described in the main methods section in order to standardise the technique.

There is no data for skin capillary density in ovarian cancer to date. Furthermore it has not be studied as a marker to determine cancer from benign ovarian lesions or healthy controls. In this cohort of patients mean basal skin capillary density was significantly higher in the malignant group compared to healthy controls whereas there was no significant difference in maximal capillary density. This suggests that structural capillary density is unchanged but perfusion of those capillaries may be altered in the presence of malignancy. A limitation is that the number of controls in this cohort was smaller than that in the malignant group and further work is necessary on a larger scale if conclusions are to be drawn regarding the value of SCD in predicting malignancy.

3.6. Conclusion

The aim of this work was to ensure my methods of measuring skin capillary density were reliable and those values I obtained in the benign and control group are in keeping with the ranges published in the literature. The paucity of data of SCD in ovarian cancer makes it hard to be confident of a 'normal range' but I feel assured that my technique is validated and my methods are robust and reliable.

Chapter 4: Longitudinal changes in skin capillary density and angiogenic factors

4.1 Introduction

Angiogenesis is a key component of cancer growth and metastasis.[66] The development and maintenance of capillaries is also dependent on angiogenesis. VEGF is a potent promotor of angiogenesis and chronic VEGF inhibition leads to capillary regression in animal models.[257] Ang 1 stabilises new vessels and acts synergistically with VEGF to promote tumour angiogenesis.[133, 134] Endoglin is expressed on proliferating endothelial cells that undergo neovascularisation and form disorganised leaky vessels, those classic of tumour vessels.[258] A soluble form (soluble endoglin) has been shown to be raised in colorectal and breast cancer and has not been studied longitudinally in ovarian cancer treatment before. It is also implicated in vascular resistance and been demonstrated to induce hypertension in vitro due to its effect on endothelial cell function.[142] It would be interesting to see whether higher soluble endoglin levels are associated with capillary rarefaction.

Skin capillary density is a dynamic variable and has been studied widely in the context of vascular disorders such as cardiovascular disease, hypertension and ageing. Insufficient angiogenesis has been suggested as a possible mechanism for capillary rarefaction[249] however associations between angiogenic markers such as VEGF and capillary density have yet to be fully addressed. No work has been conducted before investigating skin capillary density in ovarian cancer.

Treatment for ovarian cancer consists of chemotherapy and surgery although therapies are beginning to become personalised alongside the introduction of genetic testing in patients. Anti angiogenic drugs are being developed and used in conjunction with standard treatments such as chemotherapy where they have demonstrated an increased in PFS[35, 153, 155] however these are not yet widely used or funded for all patients with ovarian cancer in both the first line and recurrence setting.

Personalised medicine requires targeted therapies to achieve the best outcome for an individual. Identifying patterns of disease and clinical responses to treatment can help to determine the most effective interventions for an individual.

Skin capillary density and angiogenic markers are dynamic variables that may be helpful in identifying patients who are responding to treatment in ovarian cancer or who may benefit from additional therapies, particularly anti angiogenic drugs. In order to investigate this hypothesis, skin capillary density and angiogenic markers were studied longitudinally throughout treatment for ovarian cancer to determine any impact treatment had on these variables.

4.2 Aims

- To identify changes in skin capillary density throughout treatment in ovarian cancer
- To identify changes in angiogenic factors throughout treatment in ovarian cancer
- To identify any association between skin capillary density and angiogenic factors

4.3 Methods

Participants were recruited as described in the Methods section (section 2.1 page 49). Written informed consent was obtained from each participant. Mean basal and maximal skin capillary density were recorded at each visit along with angiogenic markers. Every subject had a visit before starting treatment known as the baseline visit. Measurements were then taken after 3 cycles of chemotherapy, 48 hours and 2 weeks post operatively in those who underwent surgery and finally at the end of treatment.

I initially analysed all treatment groups up to the first five visits as these included the treatment duration for all groups, visit 5 being at the end of standard treatment. Those in ICON8b or those who were given Bevacizumab post operatively continued on maintenance therapy after this time. I conducted pair wise comparisons of maximal and basal capillary density and angiogenic markers between each visit and in comparison to baseline.

Those women who had chemotherapy only and no surgery were followed up for 3 visits. Those who had chemotherapy and surgery with or without the addition of Bevacizumab were followed up for 5 or more visits. Those who were on maintenance Bevacizumab either post surgery or from the start of treatment (ICON8b) were followed up an additional 2 visits in order to monitor the effect Bevacizumab may have had on factors (Table 4.1)

Table 4.1. Summary of observations at each visit according to treatment

Treatment	Time (visit)							Total
	1	2	3	4	5	6	7	
Chemotherapy only	10	7	7	0	0	0	0	24
Icon8b	6	6	6	6	5	4	4	37
Neoadj. chemotherapy	20	20	20	19	16	11	6	112
Neoadj. chemotherapy + bevacizumab	9	9	9	9	9	8	5	58
Primary surgery	5	5	5	5	5	0	0	25
Total	**50**	**47**	**47**	**39**	**35**	**23**	**15**	**256**

*No patients were lost to follow up. Missing values were due to death of the patient.

4.3.1. Statistical analysis

Univariate and multivariate analysis was performed to predict the effect of variables on overall survival and progression free survival. OS was defined as time from diagnosis to death and PFS from diagnosis to first recurrence. Both were reported in months.

In regard to longitudinal analysis, I focused on the comparison of neoadjuvant chemo, neoadjuvant chemo plus Bevacizumab and ICON 8b patients to visit 5 as these three groups were the most comparable at visit time points.

The longitudinal changes in capillary density and angiogenic markers were analysed using repeated measures ANOVA which revealed non-linear trends in the treatment groups. To adjust for the heterogeneity present between the groups, a mixed model with unstructured covariance was fit. For pairwise comparisons Tukey's HSD post-hoc adjustment was used to

account for Type-I error inflation. Statistical significance was declared when the p value was <0.05.

Women having primary surgery were analysed separately by repeated ANOVA and those who received only chemotherapy were also not included in the model and were also analysed separately. This was because their number of visits were less and I felt that the model was too heterogenous to include this later two treatment groups due to where in time they received various aspect of treatment.

4.4 Results

4.4.1. Summary

All 50 (100%) women had high grade serous disease at histological examination of the tumour. Stage ranged from 2b to 4 disease. All 45 (90%) of those who had neoadjuvant treatment prior to surgery had stage 3 or 4 disease. All 5 (10%) of those with stage 2 disease radiologically underwent primary surgery.

In regard to treatment, 20 (40%) women had neoadjuvant chemotherapy. The treatment consisted of 3 cycles of chemotherapy followed by a mid-treatment CT. This CT was reported according to RECIST criteria which is further described in the main methods section (section 2.5.2 page 52). Those with a partial response to chemotherapy and suitable fitness underwent debulking surgery followed by 3 more cycles of chemotherapy. An additional subgroup of 9 (18%) women had Bevacizumab treatment added to the 4^{th} and 5^{th} cycles of

chemotherapy. This was either because of stage 4 disease or suboptimal surgical debulking outcome. 6 (12%) women in the study were enrolled into the ICON8b trial which involved having Bevacizumab in addition to chemotherapy in cycles 1, 2, 5 and 6. This trial is described in detail in the main methods section. 10 (20%) patients had chemotherapy only, either because they were not fit for surgery or had too extensive disease thought suitable for resection by the multidisciplinary team. The remaining 5 (10%) patients had primary debulking surgery followed by 6 consecutive cycles of chemotherapy.

Table 4.1 A and B describes the demographics in all patients. There were no significant difference in age, BMI or CA 125 between the treatment subgroups.

Table 4.1 A Demographics of all patients

	N= 50
Characteristic	
Age at diagnosis (years)	69.5 (10.8)
<65	16 (32%)
65-70	8 (16%)
>70	26 (52%)
BMI (kg/m^2)	26.1 (4.76)
History of hypertension (yes)	4 (9.5%)
Ca-125	553 (69, 40000)
Performance status at diagnosis	
0	10 (20%)
1	32 (64%)
2	8 (16%)
Tumour stage	
2b	5 (10%)
3b	7(14%)
3c	22 (44%)
4	16 (32%)
Treatment	
Primary surgery	5 (10%)
Neoadjuvant chemotherapy	39 (78%)
Chemotherapy only	10 (20%)

Neoadjuvant chemotherapy (Bev post treatment)	9 (18%)
Icon8b	6 (12%)
Type of surgery	
Complete/optimal	33 (66%)
Suboptimal	8 (16%)
Unfit/died/inoperable	9 (18%)
Chemotherapy response score	
1	11 (26%)
2	18 (48%)
3	7 (17%)
Bevacizumab treatment (Yes)	15 (36%)
Death (Yes)	20 (48%)
Recurrence (Yes)	34 (81%)

Table 4.1B. Characteristics of patients according to treatment pathways

			Mean	**SD**		
	Neoadjuvant chemotherapy	Neoadjuvant chemotherapy plus Bev	ICON8b	Chemotherapy only	Primary surgery	Pvalue
Age (years) Mean (SD)	67.9 (12.5)	67.4 (9.7)	65 (9.1)	77.2 (7.6)	66.2 (10.1)	0.123
BMI (kg/m^2) Mean (SD)	24.9 (5.3)	27.4 (4.9)	26.5 (4.3)	25.1 (2.7)	29.8 (4.9)	0.254
CA 125 Mean (SD)	1320 (1448)	6552 (12948.2)	918 (1471.7)	853 (959.2)	284 (238.5)	0.136
No of women N (%)	**20 (40)**	**9 (18)**	**6 (12)**	**10 (20)**	**5 (10)**	
Performance status at diagnosis						
0	5 (25)	0 (0)	2 (33)	0 (0)	3 (60)	
1	12 (60)	9 (100)	4 (67)	5 (50)	2 (40)	
2	3 (15)	0 (0)	0 (0)	5 (50)	0 (0)	
Bevacizumab treatment	0 (0)	9 (100)	6 (100)	0 (0)	0 (0)	
Dead						
Yes	8 (40)	4 (44)	2 (33)	9 (90)	0 (0)	
No	12 (60)	5 (56)	4 (67)	1 (10)	5 (100)	
Recurrence						
Yes	16 (80)	9 (100)	3 (50)	7 (70)	0 (0)	
No	4 (20)	0 (0)	3 (50)	3 (30)	5 (100)	

Outcome of surgery					
Complete	17 (85)	3 (33)	6 (100)	0 (0)	5 (100)
Optimal	1 (5)	1 (11)	0 (0)	0 (0)	0 (0)
Suboptimal	2 (10)	4 (45)	0 (0)	0 (0)	0 (0)
Unfit/extensive disease	0 (0)	1 (11)	0 (0)	10 (100)	0 (0)

4.4.2. Survival analysis

Median overall survival was 21 months. Median progression free survival was 19 months. Univariate analysis was carried out to see the impact of various variables on overall survival (table 4.2). When comparing treatments, those women who had chemotherapy only with no surgery had a significant worse overall survival. The outcome of surgery had a significant impact on overall survival (HR 0.231 (0.099-0.537) p= <0.001). Patients who had optimal surgery had a 76.9% reduction in risk of death compared to those with suboptimal outcome. Patients were excluded if they were unfit for surgery.

Performance status also impacted on survival and those women with a performance status of 2 were estimated to have a higher risk of death compared to those with status 1 or 0 (HR 0.008 (CI 0.113-0.713) p = 0.009 and HR 0.050 (CI 0.006-0.418) p= 0.006 respectively).

Table 4.2. Summary of univariate Cox models for Overall survival

Number of patients: 50 **Number of events: 23**			
Characteristics	**Hazard ratio**	**95% CI**	**p-value**
Treatment ***(Primary surgery group omitted)***			
Neoadjuvant chemo vs Chemo only	0.211	(0.079, 0.0562)	0.0019
Neoadjuvant chemo with Bevacizumab vs Chemo only	0.208	(0.062, 0.692)	0.0105
Icon8b vs Chemo only	0.154	(0.032, 0.730)	0.0185
Addition of Bevacizumab in chemotherapy			
No addition vs post-op	1.580	(0.619, 4.037)	0.339
Surgery outcome			
Optimal vs suboptimal	0.231	(0.099, 0.537)	<0.001
Age at diagnosis	1.021	(0.980, 1.064)	0.324
Performance status at diagnosis			
0 vs 2	0.050	(0.006, 0.418)	0.006
1 vs 2	0.008	(0.113, 0.713)	0.009
Chemotherapy Response Score (CRS)*			
Good vs Poor	0.281	(0.088, 0.895)	0.032
Cancer stage			
Stage-IV vs Stage-III	2.072	(0.911, 4.709)	0.082

*CRS 1:poor CRS 2/3: good.

Univariate analysis was also carried out for progression free survival (Table 4.3). It showed that treatment appeared to have a less significant impact on PFS compared to OS. However patients who had optimal surgery had a 58.3% reduction in risk of recurrence compared to those with suboptimal surgery (HR 0.417 (CI 0.210, 0.828) p= 0.012). A good chemotherapy response score (CRS) score of 3 was estimated to be a less hazard of recurrence by 92.4% compared to those with a mediocre or poor score (score of 2 or 1) (HR 0.076 (CI 0.010-0.564) p=0.012).

Table 4.3. Summary of univariate cox models for Progression free survival

Number of patients: 50 **Number of PFS events: 35**			
Characteristics	**Hazard ratio**	**95% CI**	**p-value**
Treatment ***(Primary surgery group omitted)***			
Neoadjuvant chemo & surgery vs Chemo only	0.914	(0.375, 2.227)	0.130
Neoadjuvant chemo with Bevacizumab & surgery vs Chemo only	1.157	(0.429, 3.124)	0.773
Icon8b vs Chemo only	0.348	(0.089, 1.364)	0.130
Addition of Bevacizumab in chemotherapy			
No addition vs post-op	1.036	(0.510, 2.103)	0.923
Surgery outcome			
Optimal vs suboptimal	0.417	(0.210, 0.828)	0.012
Age at diagnosis	1.005	(0.975, 1.037)	0.736
Performance status			
0 vs 2	0.380	(0.115, 1.254)	0.112
1 vs 2	0.531	(0.211, 1.335)	0.178
Chemotherapy Response Score (CRS)*			
Good vs Poor	0.039	(0.005, 0.314)	0.002
Cancer stage			
Stage-IV vs Stage-III	1.478	(0.750, 2.915)	0.259

*CRS 1:poor CRS 2/3: good.

Multivariate analysis was performed and model was adjusted for age, performance status, stage and surgical outcome. Those who received chemotherapy only had an increased risk of recurrence compared to those who had debulking surgery with or without Bevacizumab (HR 7.98 (CI 1.085-58.75) p=0.041). Optimal debulking and good chemotherapy response score remaining positive factors for improved OS and PFS. (Table 4.4, 4.5).

Table 4.4. Summary of multivariate cox models for progression free survival

Number of patients: 50 **Number of PFS events: 35**			
Characteristics	**Hazard ratio**	**95% CI**	**p-value**
Treatment ***(Primary surgery group omitted)***			
Chemo only vs Neoadjuvant chemo	2.318	(0.578, 9.290)	0.235
Chemo only vs Neoadjuvant chemo with Bevacizumab	7.984	(1.085, 58.750)	0.041
Icon8b vs Chemo only	0.665	(0.108, 4.077)	0.659
Surgery outcome			
Optimal vs suboptimal	0.242	(0.061, 0.969)	0.045
Chemotherapy Response Score (CRS)			
Good vs Poor	0.121	(0.017, 0.838)	0.032

*CRS 1:poor CRS 2/3: good.

Table 4.5 Summary of multivariate cox models for overall survival

Number of patients: 50 **Number of PFS events: 35**			
Characteristics	**Hazard ratio**	**95% CI**	**p-value**
Treatment ***(Primary surgery group omitted)***			
Chemo only vs Neoadjuvant chemo	2.713	(0.418, 17.593)	0.295
Chemo only vs Neoadjuvant chemo with Bevacizumab	3.072	(0.315, 30.009)	0.334
Chemo only vs ICON 8b	2.968	(0.352, 24.993)	0.317
Surgery outcome			
Optimal vs suboptimal	0.169	(0.026, 1.107)	0.064
Chemotherapy Response Score (CRS)			
Good vs Poor	0.538	(0.140, 2.068)	0.367

*CRS 1:poor CRS 2/3: good.

4.4.3. Longitudinal analysis

A total of 256 measurements were analysed across all treatment groups (table 4.6). Skin capillary density was recorded at every visit. Blood was also taken at every visit however during ELISA experiments and data analysis, some unreliable values for VEGF were obtained which I considered inaccurate and therefore omitted. This led to a 51 VEGF values being lost so 209 (80.4%) were analysed.

Table 4.6. Summary of observations per time point

Time (Visit)	Frequency	Percent	Cumulative Frequency	Cumulative Percent
1	50	19.53	50	19.53
2	47	18.36	97	37.89
3	47	18.36	144	56.25
4	39	15.23	183	71.48
5	35	13.67	218	85.15
6	23	8.98	241	94.13
7	15	5.87	256	100.00

Table 4.7 summarises the effect of time and treatment on the various factors of interest. Both VEGF and Ang 1 decreased over the 5 visits in all treatment groups and maximal and mean capillary density also decreased over time. There was no difference in soluble endoglin over the course of treatment. The model was adjusted for treatment and there was no effect of treatment on the changes observed.

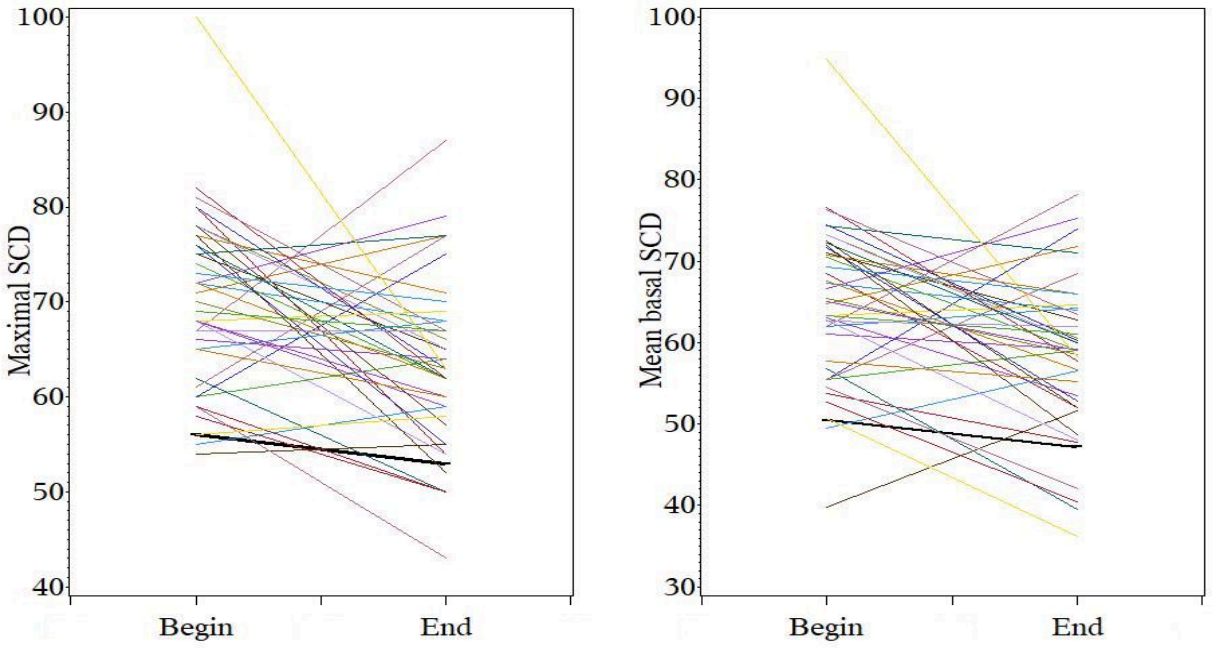
Table 4.7. Type 3 tests of fixed effects

Effect	p-values	VegF	Ang1	SolEndo	Maximal SCD	Mean basal SCD
Treatment		0.922	0.019	0.2289	0.1089	0.2437
Time		0.022	<0.001	0.6330	<.0001	<.0001
Treatment*time		0.726	0.3552	0.8889	0.1106	0.4169

4.4.3.1 Skin capillary density

Figure 4.1 demonstrates the individual profile plots of changes in skin capillary density (vessels/mm^2) from start to end of treatment for all patients.

Figure 4.1. Change in SCD for all individual patients. Each line represents an individual patients SCD from the start and end of treatment.



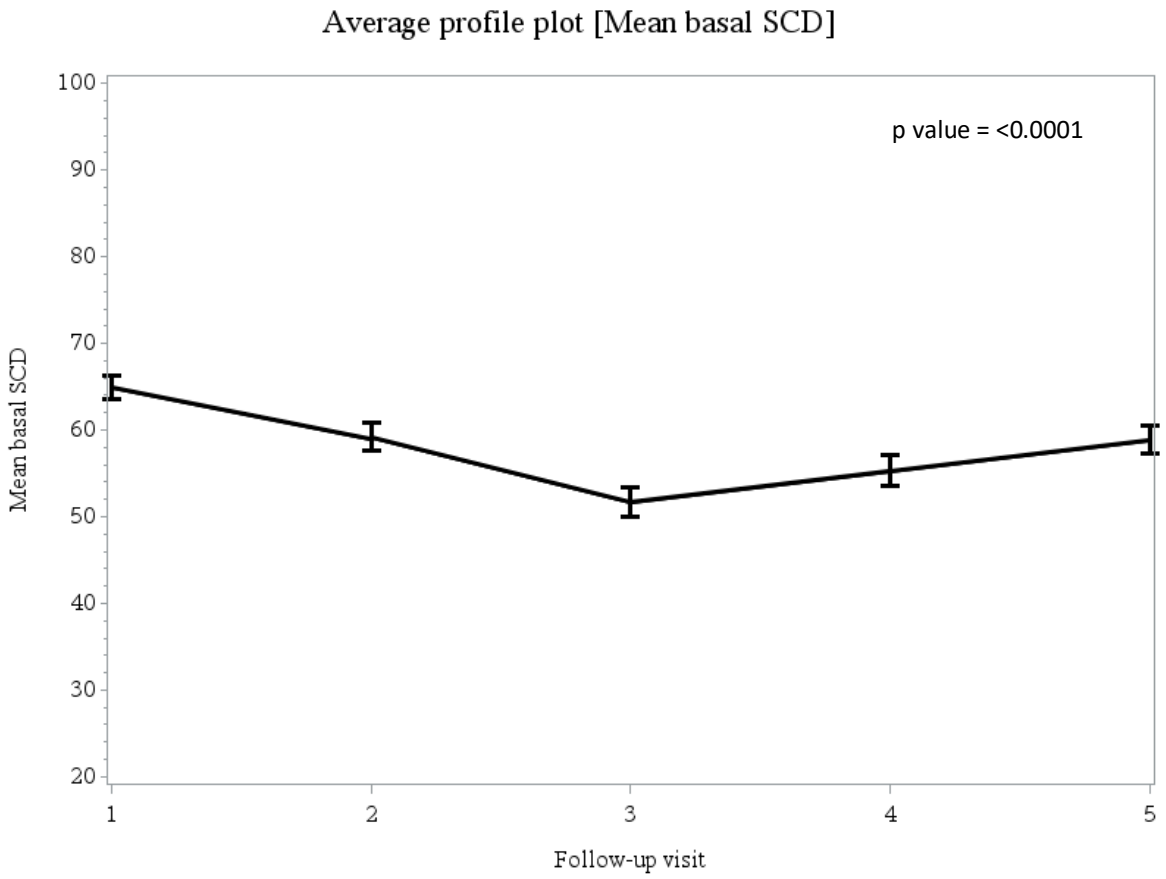
The mean basal skin capillary density had a gradual decline during treatment in all groups. Mean basal skin capillary density fell significantly at each visit compared to baseline (Table 4.7). The most significant drop was from baseline to the visit immediately after surgery. Although it then increased it never returned to baseline. Overall the trend in mean changes in basal skin capillary density from baseline were statistically significant (p= <0.001) (Figure 4.1).

Table 4.8. Pairwise comparisons of mean basal SCD (vessels/mm^2)measurements over time

Visit	**1**	**2**	**3**	**4**	**5**
1	-				
2	**-7.09** **(1.96)** **p=0.008**	-			
3	**-16.56** **(2.95)** **p=<0.0001**	**-9.47** **(3.27)** **p=0.047**	-		
4	**-11.51** **(2.55)** **p=0.0007**	- 4.41 (2.33) p=0.34	5.06 (3.19) p=0.52	-	
5	**-7.68** **(2.20)** **p=0.012**	- 0.59 (2.30) p=0.99	**8.88** **(2.48)** **p=0.009**	3.82 (2.22) p=0.43	-

Estimated difference of the means and standard deviation (SD).

Figure 4.2. Model adjusting for treatment demonstrating changes in mean basal SCD (vessels/mm^2) during treatment.



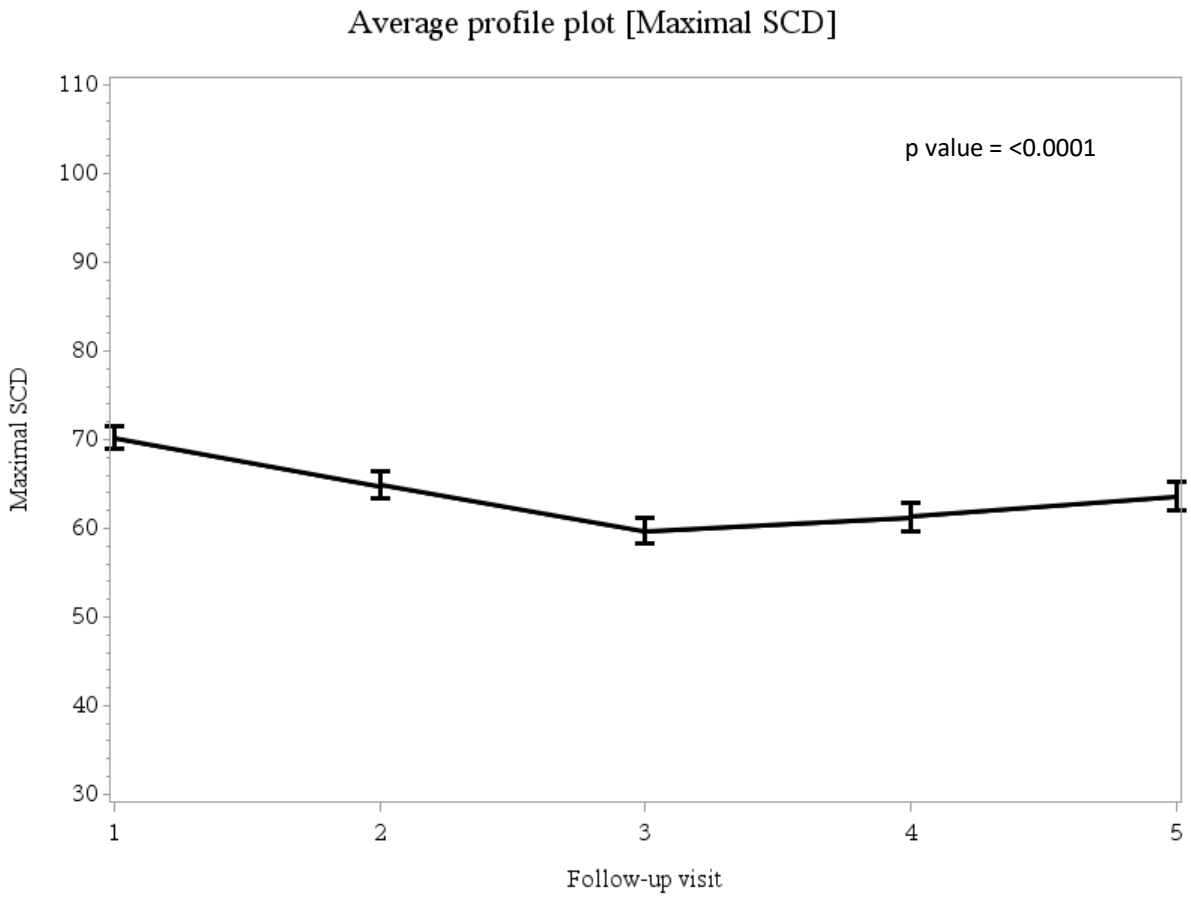
Similarly mean maximal capillary density significantly fell at each visit compared to baseline (Table 4.8) The most significant drop was from baseline to visit 3 immediately after surgery. Overall the trend in mean changes in maximal skin capillary density from baseline were statistically significant (p=<0.0001) (Figure 4.3).

Table 4.9. Pairwise comparisons of mean maximal SCD (vessels/mm^2) measurements over time

Visit	**1**	**2**	**3**	**4**	**5**
1	-				
2	**7.39** **(1.70)** **p=0.0012**	-			
3	**13.51** **(2.57)** **p=<0.0001**	6.12 (2.76) p=0.19	-		
4	**10.91** **(2.47)** **p=0.0009**	3.52 (1.99) p=0.41	-2.60 (2.64) p=0.86	-	
5	**9.04** **(2.32)** **p=0.004**	1.65 (2.04) p=0.93	-4.47 (2.05) p=0.21	-1.87 (2.05) p=0.89	-

Estimated difference of the means and standard deviation (SD).

Figure 4.3. Model adjusting for treatment demonstrating changes in mean maximal SCD (vessels/mm^2) during treatment.



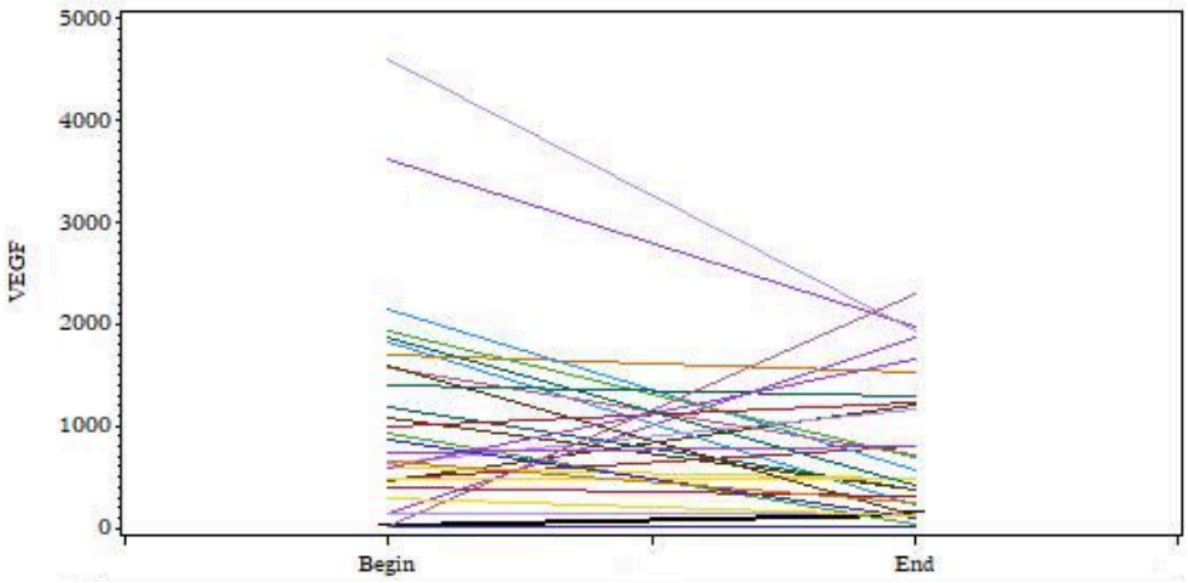
The effect of treatment on mean basal SCD over time was significant for all 3 groups (Icon8b p-value=0.004; neoadjuvant chemotherapy p-value =0.001; neoadjuvant chemotherapy plus Bevacizumab p-value= 0.018) , after comparison of measurements recorded during the first 5 follow-up visits.

4.4.3.2 Angiogenic markers

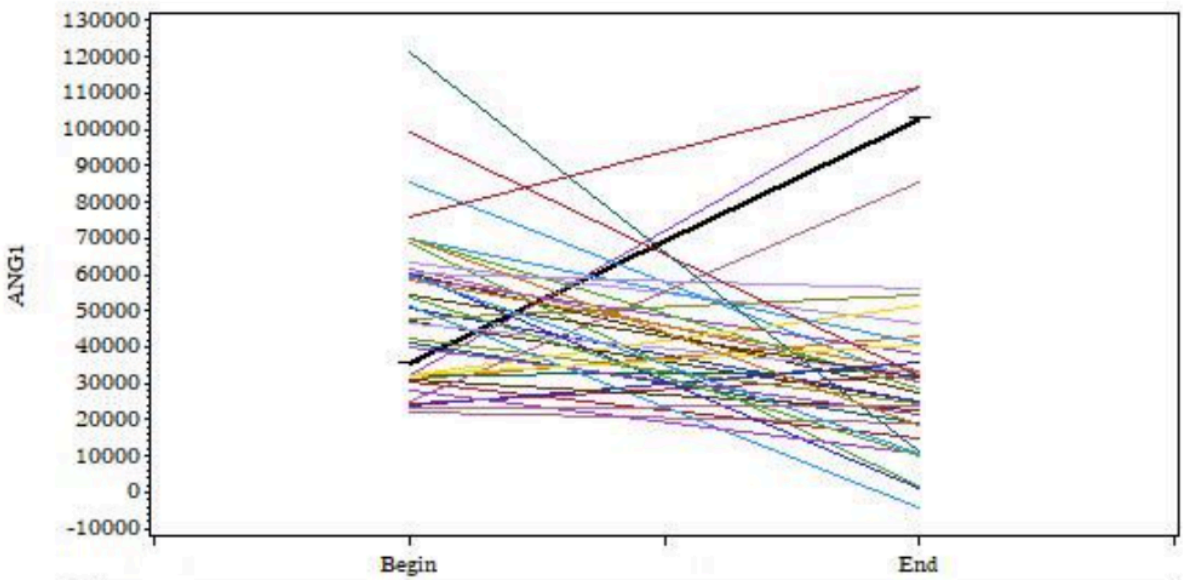
Figure 4.4 (a, b, c) below demonstrates the individual profile plots of changes in angiogenic markers from start to end of treatment for all patients.

Figure 4.4. Markers in all patients from start to end of treatment. 4.4a VEGF. 4.4b Ang 1. 4.4c Sol endoglin

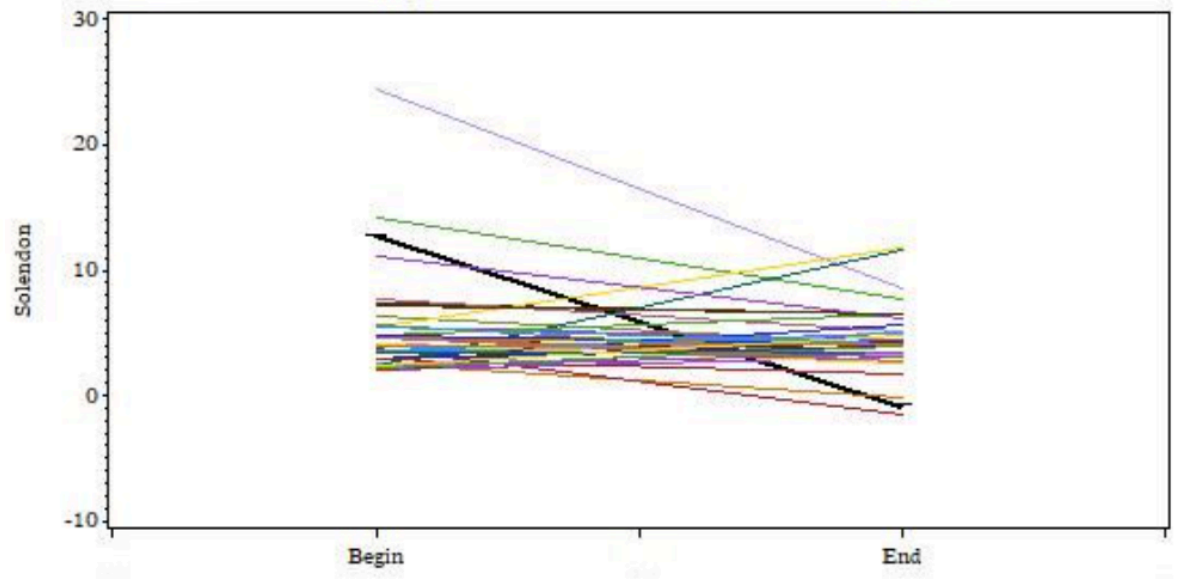
4.4a VEGF



4.4b Ang 1



4.4c Sol endoglin



4.4.3.2.1 VEGF

The levels of mean VEGF reduced at each visit compared to baseline. The largest and most significant decline in VEGF occurred from baseline to 2 weeks following surgery (visit 4, p=0.04) (Table 4.9)

There was no difference in the change of VEGF according to treatment and whether women received Bevacizumab.

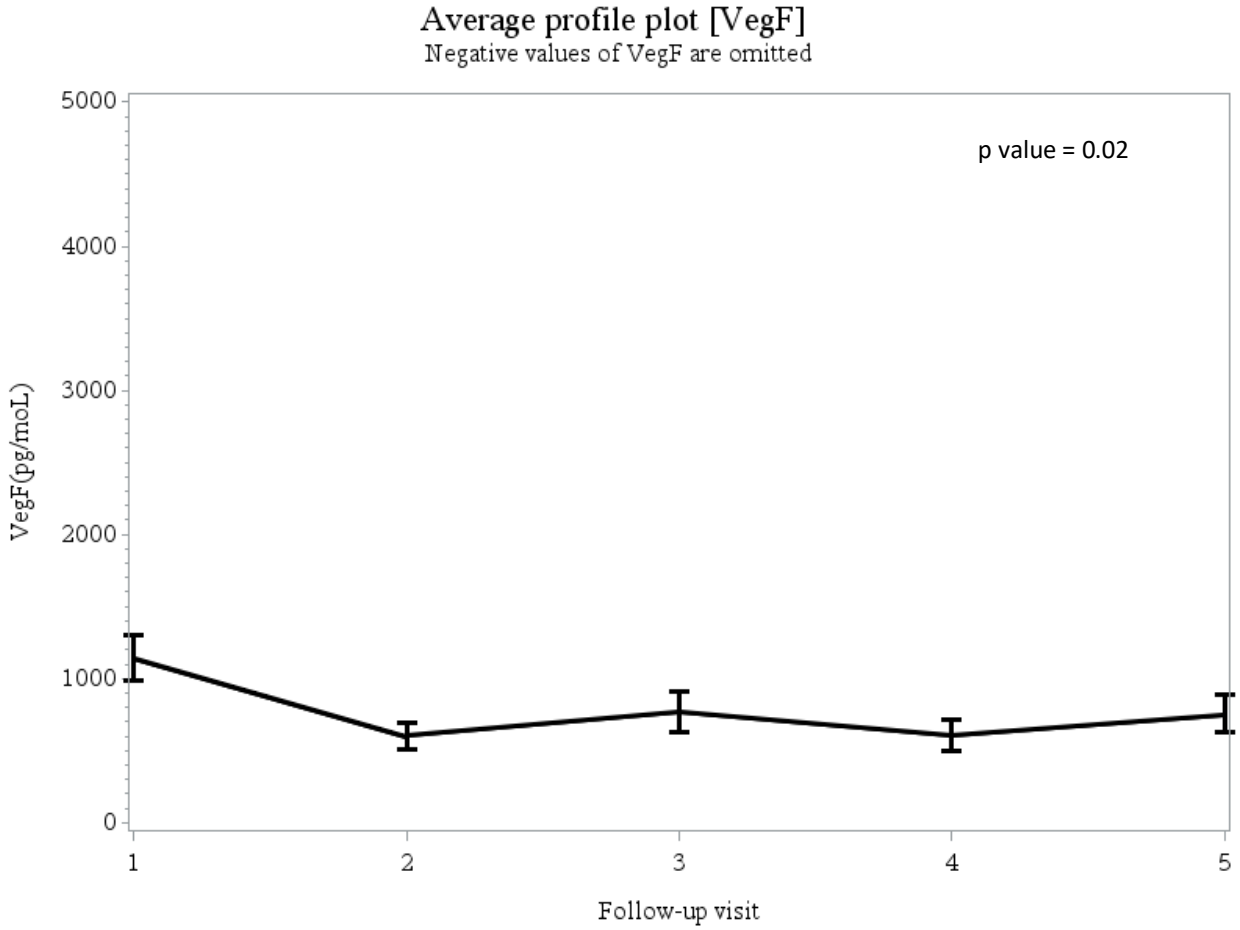
Overall there was a decline in VEGF throughout treatment and this was statistically significant (p=0.02) (figure 4.5).

Table 4.10. Pairwise comparisons of mean VEGF measurements over time using Tukey's HSD.

Visit	1	2	3	4	5
1	-				
2	-438.3 (220.5) p=0.29	-			
3	-399.0 (202.4) p=0.30	39.26 (189.6) p=0.99	-		
4	**-571.9** **(197.7)** p=0.04	-133.59 (163.1) p=0.92	-172.85 (184.27) p=0.88	-	
5	-277.8 (240.7) p=0.78	160.52 (157.84) p=0.85	121.26 (175.86) p=0.96	294.11 (158.27) p=0.36	-

Estimated difference of the means and standard deviation (SD).

Figure 4.5. Model adjusting for treatment demonstrating changes in VEGF during treatment.



4.4.3.2.2 Ang 1

The levels of mean Ang 1 reduced at each visit compared to baseline. The largest and most significant decline in Ang 1 occurred at the start of treatment from baseline to visit 2 (p=0.0002) (Table 4.10).

There was no difference in the change of Ang 1 according to treatment and whether women received Bevacizumab.

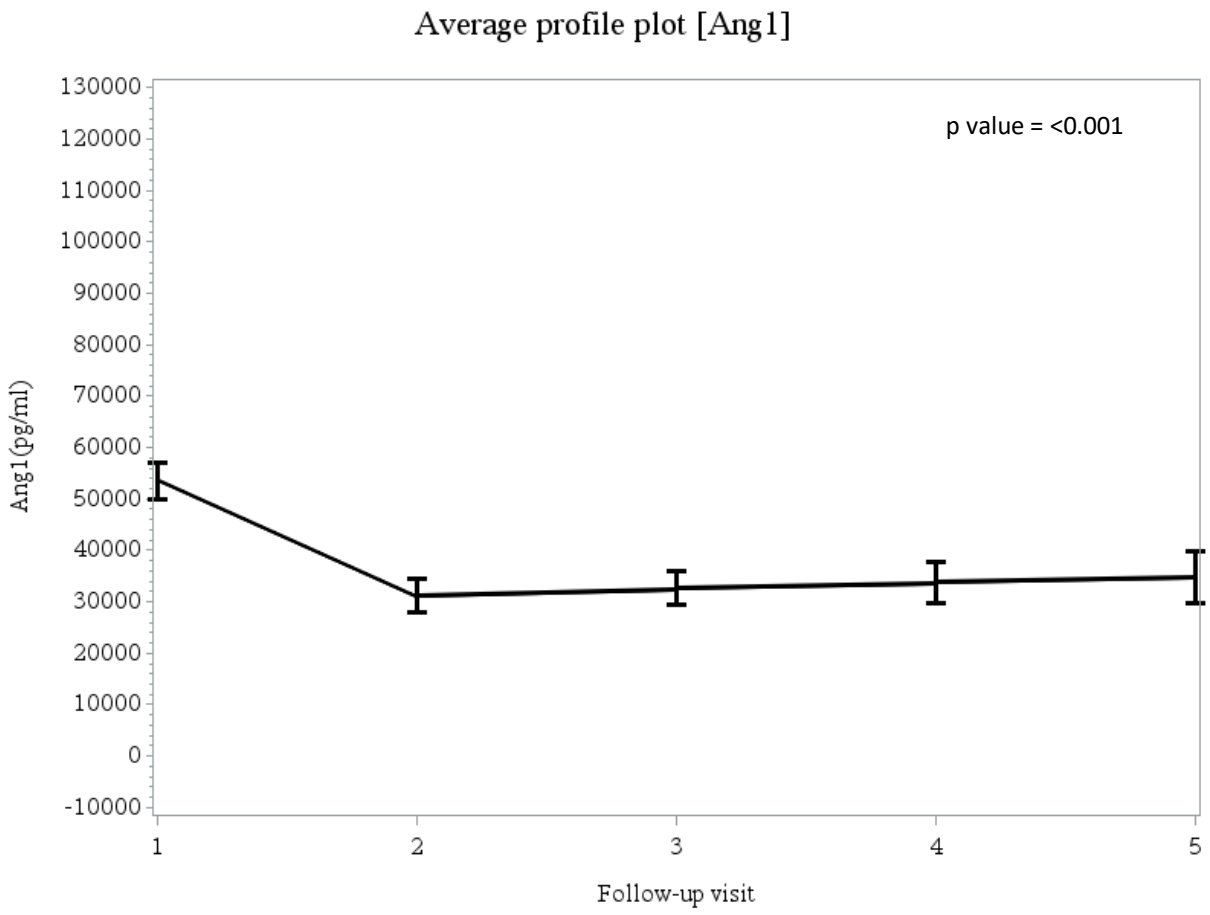
Overall there was a decline in Ang1 throughout treatment and this was statistically significant (p=<0.001) (figure 4.6).

Table 4.11. Pairwise comparison of mean Ang1 measurements over time.

Visit	1	2	3	4	5
1	-				
2	**-23149** **(4717.8)** **p=0.0002**	-			
3	**-18286** **(4188.0)** **p=0.001**	4862.84 (4572.4) p=0.82	-		
4	-15894 (6153.5) p=0.098	7254.83 (5152.3) p=0.63	2392.0 (6107.8) p=0.99	-	
5	-12577 (6930.1) p=0.38	10572 (5078.0) p=0.25	5709.07 (7239.1) p=0.93	3317.1 (7179.3) p=0.99	-

Estimated difference of the means and standard deviation (sd).

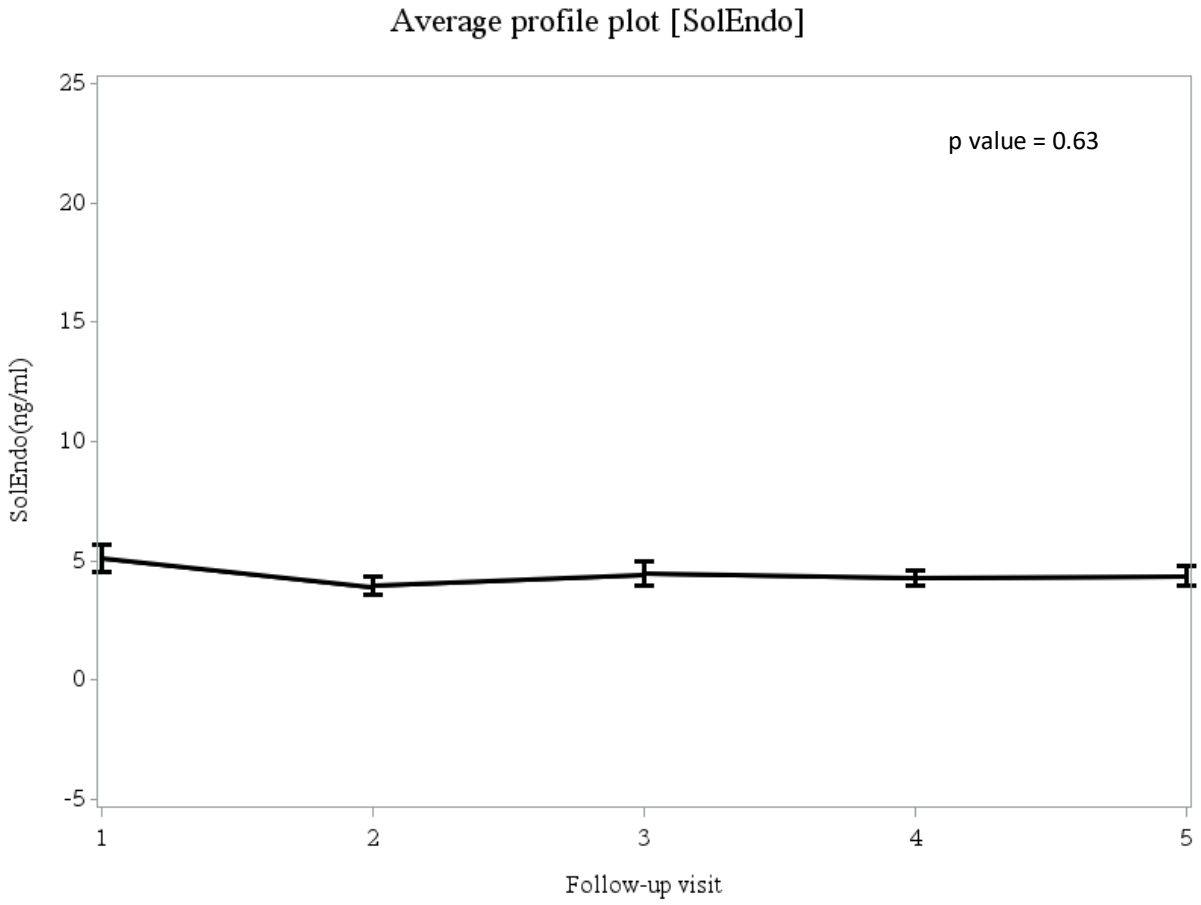
Figure 4.6. Model adjusting for treatment demonstrating changes in Ang 1 during treatment.



4.4.3.2.3. Soluble endoglin

There was no significant differences in the levels of soluble endoglin during treatment (p=0.63).

Figure 4.7. Model adjusting for treatment demonstrating changes in Soluble Endoglin during treatment.



4.4.3.3. Chemotherapy only

Table 4.11 below demonstrates the fall in factors in the chemotherapy only group. There was a significant drop in baseline to visit 2 and from baseline to visit 3 for mean basal and maximal SCD (p=<0.001). A significant drop in VEGF and Ang 1 was also seen from baseline to visit 2 and 3 (p=<0.001)

Table 4.12. Pairwise comparison of mean markers over time for patients receiving chemotherapy only

	Visit	Visit	Difference Mean (SD)	P value
Mean basal SCD	1	2	-6.79 (1.56)	0.0005
	1	3	-12.3 (2.27)	<0.001
	2	3	-5.49 (2.50)	0.141
Maximal basal SCD	1	2	-6.2 (1.30)	0.0001
	1	3	-10.28 (1.94)	<0.0001
	2	3	-4.06 (2.06)	0.216
VEGF	1	2	-623 (131)	<0.001
	1	3	-102.9 (194)	<0.001
	2	3	4.06 (2.07)	0.056
Ang 1	1	2	-22277 (4251.8)	<0.0001
	1	3	-21821 (3832.3)	0.0001
	2	3	456.26 (4565.1)	0.999

4.5 Discussion

This the first study investigating skin capillary density in ovarian cancer. It is also the first study where angiogenic markers have been measured at time points throughout treatment. I discovered that capillary rarefaction occurred in all women irrespective of nature and duration of treatment ($p = <0.001$). Furthermore, there is a significant reduction between baseline and visit one which was measured after 3 cycles of chemotherapy with or without Bevacizumab. A further significant drop is also demonstrated between visit 2 and 3 which was measured after surgery. The levels then increase but never return to baseline. This is demonstrated in both mean and maximal skin capillary density suggesting not only a functional drop in skin capillaries but also a structural reduction in the number of vessels. Capillary rarefaction during cancer treatment has not been demonstrated before and I postulate that this may be a reflection of a fall in angiogenic activity. This would correspond with a fall in the angiogenic markers of VEGF and Ang 1 which I have also demonstrated.

There was a fall of VEGF between baseline and visit 1 and then a small rise after surgery but overall there was a downwards trend across the treatment phases. The levels of Ang 1 significantly fell from baseline to visit 1 and that drop was maintained throughout the rest of the treatment. I did not demonstrate any change in soluble endoglin during treatment.

It has previously been documented that serum VEGF increases after major abdominal surgery in patients with colorectal cancer[259] as well as similar reports seen in breast cancer surgery.[260] As VEGF is a potent promotor of angiogenesis it is reasonable to expect an increase after surgery to promote healing and recovery. However, in this cohort of patients, although

immediately after surgery VEGF rises, it then continues to fall again towards end of treatment with an overall trend of reduction from baseline to end of treatment.

Tumour derived VEGF enhances tumour spread, dissemination and high levels of VEGF at diagnosis both in serum and tissue are widely known to correspond to more advanced disease and poor outcomes in ovarian cancer.[87, 97-103] It is logical that reduction in tumour bulk by the end of treatment would mirror reduction in serum VEGF measurements and serum VEGF levels have been shown to reduce after resection of other tumours and completion of chemotherapy.[261, 262] This is the first study to demonstrate this dynamic change in ovarian cancer.

Ang 1 is another potent stimulator of angiogenesis and has been demonstrated to have a synergistic effect with VEGF.[138] In ovarian cancer higher levels of Ang 1 are demonstrated in malignant tumours compared to benign controls and associated with worse overall and progression free survival.[139] In those patients on Bevacizumab, high Ang 1 levels at baseline did predict an improved PFS in a small group of patients.[140] Although this data has not be validated in larger studies, it can be postulated that in those patients with a higher angiogenic drive at baseline may respond best to anti angiogenic inhibitors.

In my cohort of patients, serum levels of Ang 1 fell in all treatment groups particularly at the start of treatment. While there was no significant difference in those who had Bevacizumab, my finding of an effect of treatment on levels is promising.

It must be considered whether the fall in skin capillary density and angiogenic markers in all patients is a consequence of a change in measuring technique over time. However I think this is unlikely as the patients entered and went through the study at different times and care was taken to ensure collection of data was standardised and consistent. The universal feature is that all patients received chemotherapy. Cytotoxic treatment leads to vascular dysfunction and may have a direct impact on angiogenesis[263] and this may be reflected in the reduction in skin capillary density and angiogenic markers in these patients.

Demonstration of an effect of an intervention on a marker is a key part of development of a biomarker in clinical medicine. I have demonstrated that skin capillary density appears to alter after treatment in women with ovarian cancer. It is important not only validate these results with a larger number of women but also ascertain this change in relation to outcomes such as surgical resection, overall survival and recurrence.

4.6. Conclusion

This is the first study to my knowledge that has demonstrated a reduction in angiogenic markers during treatment for ovarian cancer. Skin capillary density also significantly fell during treatment. There is a paucity of data in the literature regarding skin capillary density amongst cancer patients and my data suggest a promising dynamic change that has potential for further investigation as a biomarker of treatment response and prognosis.

Chapter 5: Skin capillary density and cancer outcomes

5.1 Introduction

In my previous chapter I demonstrated that skin capillary rarefaction (fall in skin capillary density) occurred during treatment for ovarian cancer. Capillary rarefaction has not been studied thus far in ovarian cancer and my research contributes this novel finding. However, the implication of these changes must be assessed in order to determine their value.

It is well recognised that most women present with advanced stage disease (stage 3 or 4) and only a third of these women survive beyond 3 years.[1-4] Additionally, recurrence occurs in the vast majority of women.[45] Advanced stage of disease and response to platinum-based chemotherapy are factors that impact on overall survival and recurrence. Surgical outcome also has a significant impact on overall survival for women with ovarian cancer and residual tumour after surgery is an independent prognostic factor for recurrence and survival.[42-46]
The Gynaecological Oncologic Group (GOG) trials demonstrated that those patients with no residual disease had improved overall survival (OS) compared to those with <1cm residual disease (64 months vs 29 months).[44]

Additionally, a Cochrane review demonstrated that maximal surgical effort is a key determinant of survival outcome with the goal of achieving complete cytoreduction.[43] Those women with >1cm macroscopic disease remaining had an increased risk of death compared to those with <1cm disease (HR 1.36 CI 1.10-1.68)[43] suggesting that optimal cytoreduction should still be regarded as a favourable outcome.

In clinical practice, a combination of radiographic opinion of staging CT images, diagnostic laparoscopic findings, co-morbidities, patient's wishes and surgical expertise are used to decide on surgical suitability. Currently, there are no clinically useful biomarkers that predict morbidity from surgery or the likelihood of achieving complete or optimal cytoreduction.

Capillary density impacts on the total resistance of the capillary bed and thus capillary pressure and capillary pressure is vital to tissue fluid homeostasis and trans capillary exchanges.[237] The assessment of SCD at presentation and post operatively in relation to these clinical aspects may provide an insight into the microvascular status that may then help to predict those more at risk of post-operative complications.

We have seen in the previous chapter that SCD falls in response to chemotherapy. The implication for this in clinical practice is important to ascertain particularly as to whether this dynamic change is indicative of response to treatment.

In this chapter I attempt to investigate whether SCD at baseline and during treatment can predict survival, recurrence and surgical outcome and morbidity.

5.2 Aims

- To investigate whether SCD at baseline predicts OS, PFS or surgical cytoreduction
- To investigate whether the change SCD before and after 3 cycles of treatment can predict surgical cytoreduction or surgical morbidity in women undergoing neoadjuvant chemotherapy

5.3. Methods

This was a prospective cohort study and patients with a diagnosis of high grade serous ovarian cancer were invited to participate over an 18-month period. Skin capillary density was measured at diagnosis (baseline) and prior to surgery after the first 3 cycles of chemotherapy (baseline to visit 2). The absolute difference in values between this visit point was calculated. Treatment groups were split into those who had chemotherapy only and those who had chemotherapy and bevacizumab.

A CT scan was conducted after 3 cycles of treatment in time with visit 2. This CT was reported according to RECIST criteria which is further described in the main methods section. Those with a partial response to chemotherapy and suitable fitness underwent debulking surgery followed by 3 more cycles of chemotherapy.

5.3.1 Statistics

In order to determine the impact of change in skin capillary density, the difference was dichotomised into above and below the mean. Wilcoxon test and Kruskal Wallis was used to compare means. Logistic regression was used to determine the effect on surgical outcomes and complications.

Dichotomised variables were tabulated as high and low values above the mean. The associations between the calculated difference in values and PFS and OS were assessed with Kaplan Meier plots stratified by the dichotomised variables. Log rank test was used to

ascertain significance in survival data. Where relevant, statistical significance was described when p = <0.05.

5.4 Results

All women had neoadjuvant treatment and a diagnosis of high-grade serous carcinoma. 50 women were recruited to the study. As I wanted to investigate the association between baseline values and surgical outcome and the difference between baseline and visit 2, only 42 women are included in this analysis. Those who had primary surgery were excluded (n = 5) and those who died after the first cycle of chemotherapy were also excluded (n = 3). As regards to treatment, 36 (79%) had neoadjuvant carboplatin and paclitaxel chemotherapy and the remaining 6 (21%) received Bevacizumab alongside the first two cycles of chemotherapy. 6 of those who had neoadjuvant chemotherapy did not have surgery as they were too unfit or had extensive disease.

Table 5.1 Demographics of patients

	N= 42
Characteristic	
Age (years) at diagnosis	69.8 (11.2)
<65	14 (33%)
65-70	6 (14%)
>70	22 (53%)
BMI (kg/m^2)	25.6 (4.74)
History of hypertension* (yes)	4 (9.5%)
Ca-125	2273 (69, 40000)
Performance status at diagnosis	
0	6 (14%)
1	30 (72%)
2	6 (14%)
Tumour stage	
3b	5(12%)
3c	22 (52%)
4	15 (36%)
Treatment	
Neoadjuvant chemotherapy	33 (79%)
Icon8b	6 (21%)
Type of surgery	
Complete/optimal	28 (67%)

Suboptimal	8 (19%)
Unfit/died/inoperable	6 (14%)
Chemotherapy response score	
1	11 (26%)
2	18 (48%)
3	7 (17%)
Bevacizumab treatment (Yes)	15 (36%)
Death (Yes)	20 (48%)
Recurrence (Yes)	34 (81%)

5.4.1. Skin capillary changes prior to surgery

Mean basal and maximal skin capillary density reduced between baseline and visit 2 but the drop was greater in those who received Bevacizumab (p=0.0023, p= 0.0125) (Table 5.2, figure 5.1, 5.2)

Table 5.2. The difference in skin capillary density (vessels/mm^2) between baseline and visit 2 according to treatment

	Mean basal SCD (vessels/mm^2)		**Maximal SCD (vessels/mm^2)**	
	Mean difference (SD)	P value	Mean difference (SD)	P value
Neoadjuvant chemotherapy (36)	-3.89	0.0023	-4.36	0.0125
ICON8b (6)	-16.33		-14.17	

Figure 5.1. Box plots of difference in mean basal SCD (vessels/mm^2) before and after 3 cycles of treatment

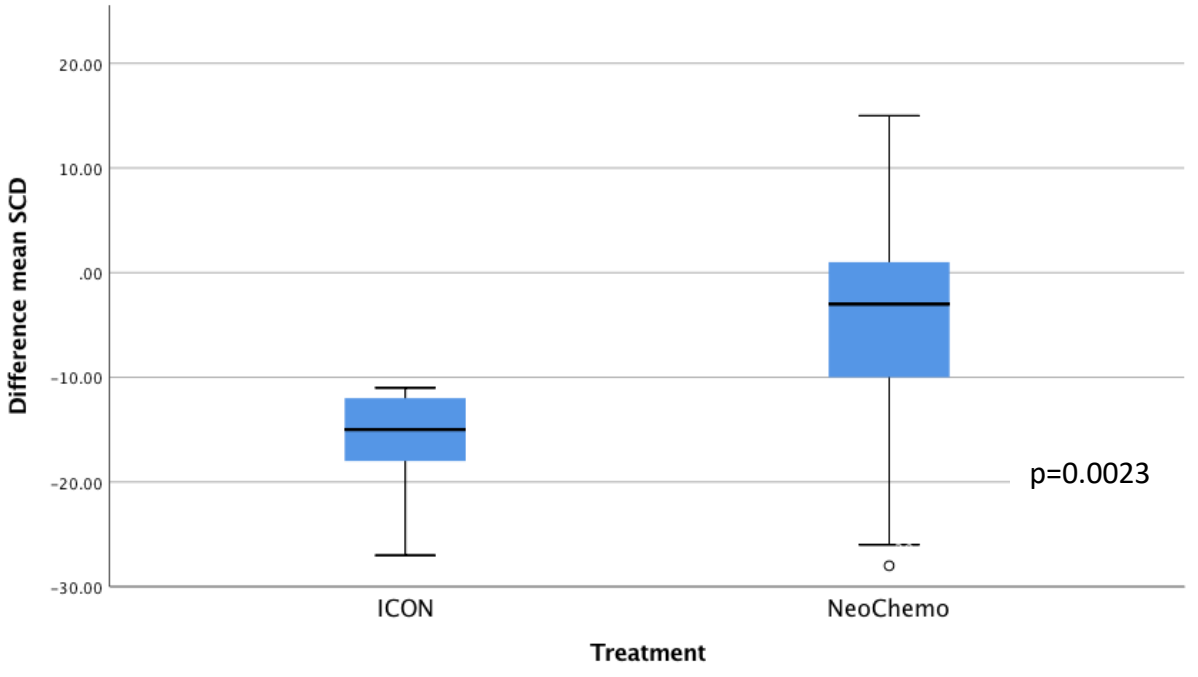
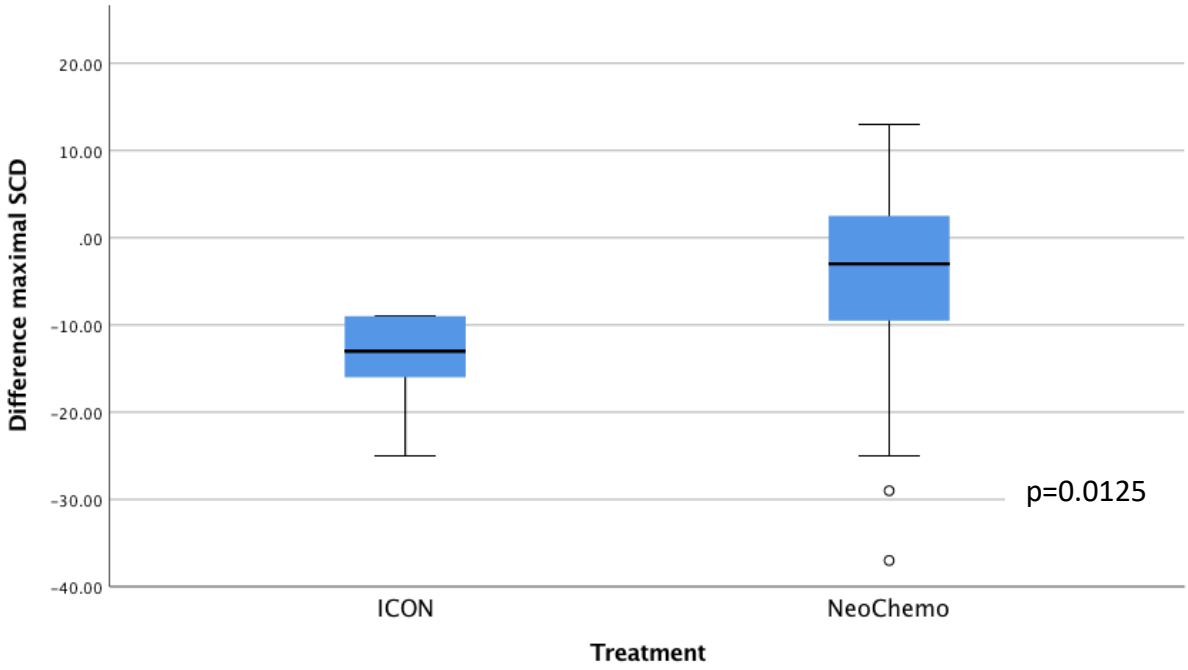


Figure 5.2. Box plots of difference in maximal SCD (vessels/mm^2) before and after 3 cycles of treatment



5.4.2 Skin capillary density at baseline and prediction of outcomes

There was no significant difference in mean basal or maximal skin capillary density at baseline between the 3 groups of response reported at mid treatment CT although the p value approaches significance (Table 5.3).

Table 5.3 Skin capillary density (vessels/mm^2) and CT response

	Mean basal SCD (vessels/mm^2)	**P value**	**Maximal SCD (vessels/mm^2)**	**P value**
	Mean (SD)		Mean (SD)	
Complete response	73.2 (2.9)	0.07	77.0 (3.6)	0.06
Partial response	63.4 (10.1)		68.6 (9.7)	
Stable disease	71.5 (0.7)		77.0 (0)	

5.4.2.1. Surgical resection outcomes

Mean basal and maximal skin capillary density was higher at baseline in patients who had optimal surgery (p= 0.001, 0.0009 respectively) (Table 5.4).

Table 5.4 Mean basal and maximal SCD (vessels/mm^2) at baseline according to surgical outcome

	Mean basal SCD (vessels/mm^2)	**P value**	**Maximal SCD (vessels/mm^2)**	**P value**
	Mean (SD)		Mean (SD)	
Optimal	6.9 (9.0)	0.001	71.67 (8.94)	0.0009
Suboptimal	55.4 (7.1)		61.13 (4.16)	

All women who had Bevacizumab treatment prior to surgery had complete resection. When I excluded those women (n=6) the significance remained the same as demonstrated in table 5.5.

Table 5.5 Mean basal and maximal SCD (vessels/mm^2) at baseline according to surgical outcome (excluding ICON 8b patients)

	Mean basal SCD (vessels/mm^2)	**P value**	**Maximal SCD (vessels/mm^2)**	**P value**
	Mean (SD)		Mean (SD)	
Optimal	66.5 (9.5)	0.002	71.2 (9.2)	0.001
Suboptimal	55.4 (7.1)		61.13 (4.16)	

I used logistic regression to explore the association between surgical outcome and baseline skin capillary density which demonstrated for every increase in mean basal SCD by 1, there was a 19% chance of achieving complete or optimal resection (p= 0.014) (Table 5.6). Similarly, for every increase in maximal SCD by 1, there was a 22% increase in chance of optimal or complete resection (p=0.008).

Table 5.6 Logistic regression of mean basal and maximal SCD (vessels/mm^2) at baseline and surgical outcome

	Odd ratio (95% CI)	**P-value**
Maximal SCD at Baseline (vessels/mm^2)	0.78 (0.58, 0.98)	0.008
Mean SCD at Baseline (vessels/mm^2)	0.81 (0.64, 0.98	0.014

In order to explore the relationship with other factors, I also used logistic regression to explore the association between surgical outcome and the difference from baseline to Visit 2 calculated for each of the angiogenic factors and skin capillary measurement (Table 5.7).

Table 5.7. Logistic regression of change in angiogenic factors and skin capillary density (vessels/mm^2) from baseline to visit 2.

Clinical factors	**Odd ratio (95% CI)**	**P-value**
VEGF[1]	1.048 (0.947, 1.24)	0.241
ANG1[1]	1.00 (0.996, 1.004)	0.912
SOLEND	0.85 (0.566,1.274)	0.430
Mean Basal SCD (vessels/mm^2)	0.91 (0.815, 1.018)	0.099
Maximal SCD (vessels/mm^2)	1.085(0.984, 1.195)	0.101

[1]Odds ratio based on 100ug difference

For every 100-unit increase in VEGF after baseline, the odds of having optimal surgery is 0.08% higher however this is not significant (p-value= 0.241) (table 5.7)

5.4.2.2.Post-operative complications

I used logistic regression to ascertain any association between SCD at baseline and post-operative adverse events (Table 5.8). There were no significant associations seen.

Table 5.8. Logistic regression of skin capillary density (vessels/mm^2) and post-operative morbidity

Clinical variables	**Post-op complications (Yes/ No)**		**ITU admissions (Yes/ No)**		**Extended hospital stay (Yes/ No)**	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
Max SCD	0.97 (0.90, 1.06)	0.530	1.12 (0.99, 1.28)	0.081	0.996 (0.90, 1.10)	0.935
Mean SCD	0.97 (0.90, 1.05)	0.481	1.12 (0.98, 1.28)	0.474	0.998 (0.91, 1.10)	0.974

5.4.3 Prediction of overall and progression free survival

Patients with a smaller reduction in change in mean basal skin capillary density (those above the mean) had a shorter OS and PFS (mean 22.0 vs 22.5 and 16.5 vs 21.1 months) (figure 5.3, 5.4). This was also true of maximal skin capillary density (21.8 vs 23.1 and 15.8 vs 22.3 months) (figure 5.5, 5.6)

Figure 5.3. Overall survival according to change in mean basal skin capillary density (vessels/mm^2)

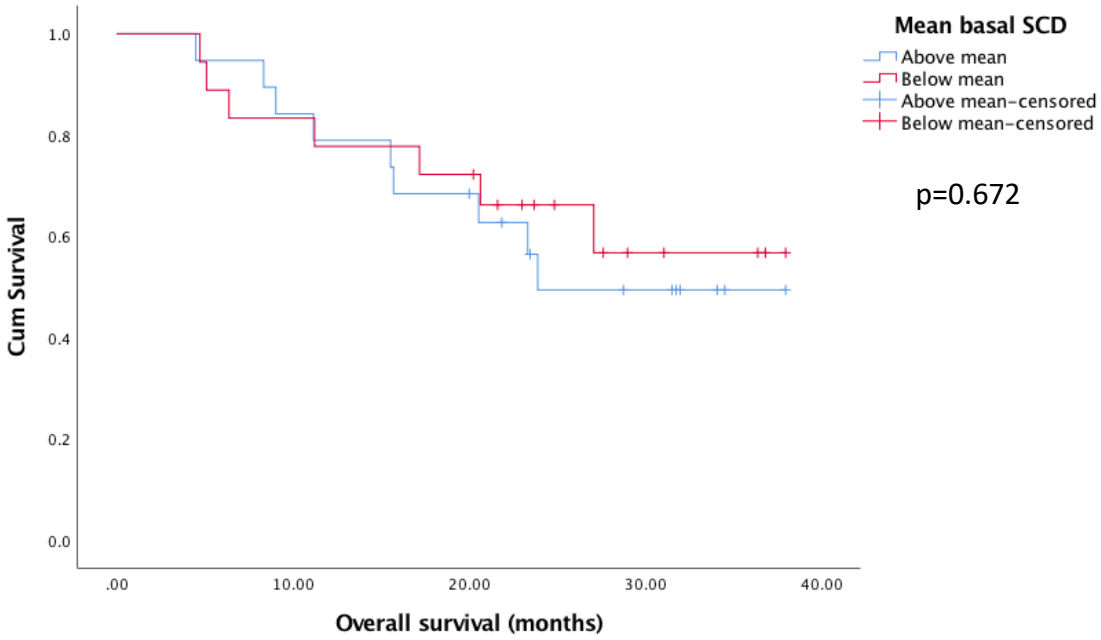
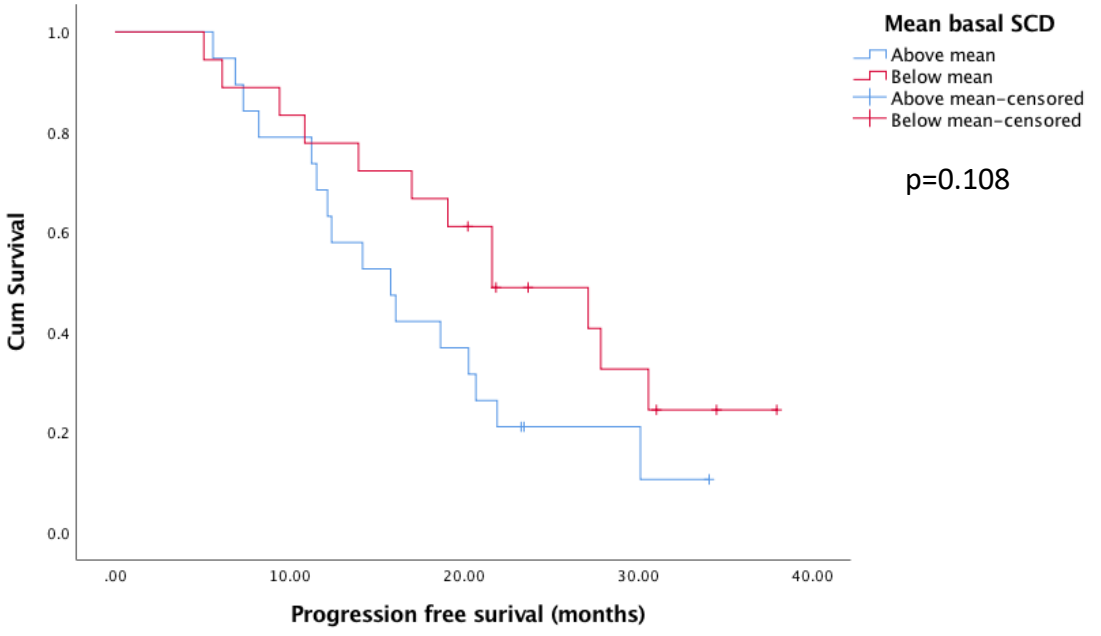


Figure 5.4. Progression free survival according to change in mean basal skin capillary density (vessels/mm^2)



There was a significant difference in PFS according to the change in maximal skin capillary density with those who had a smaller reduction in SCD (change above the mean) having a shortened PFS (15.8 vs 22.3 months p= 0.010) (Figure 5.6).

Figure 5.5. Overall survival according to change in maximal skin capillary density (vessels/mm^2)

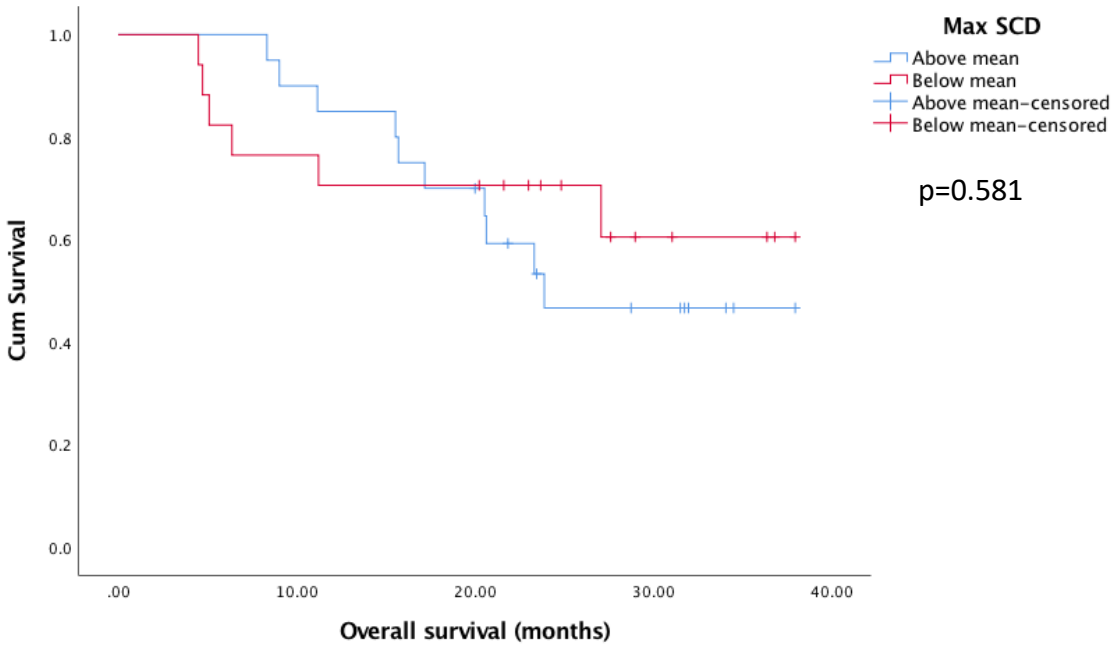
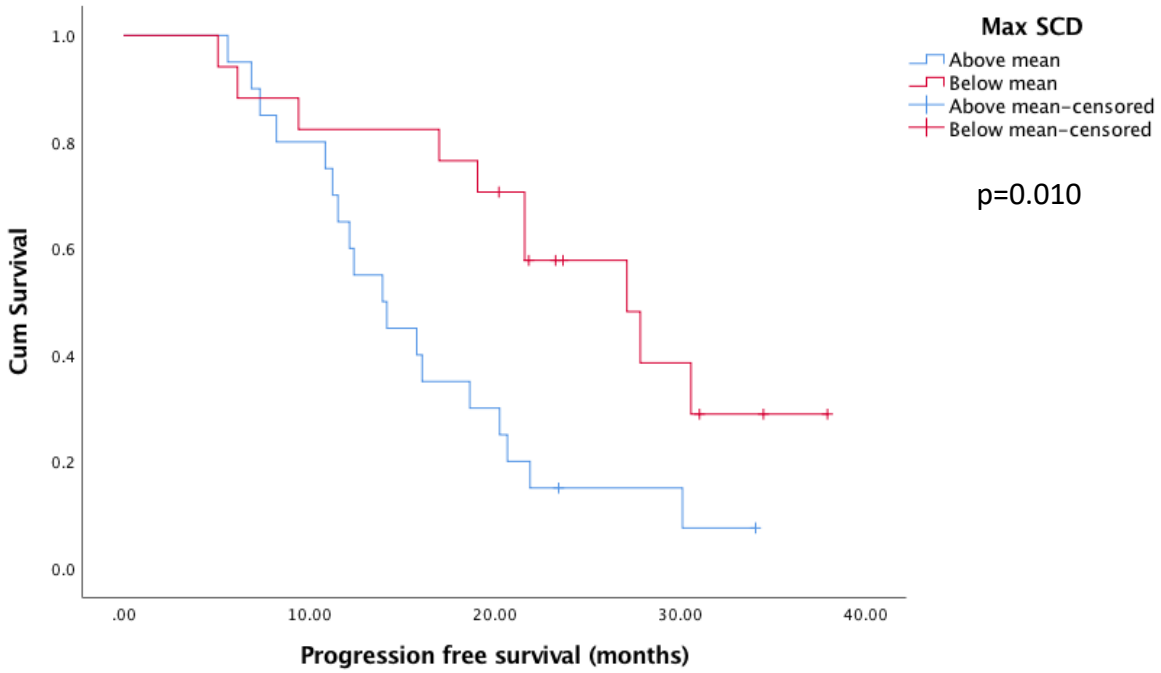


Figure 5.6. Progression free survival according to change in maximal skin capillary density (vessels/mm^2)



5.4.4 Estimation of optimal skin capillary density

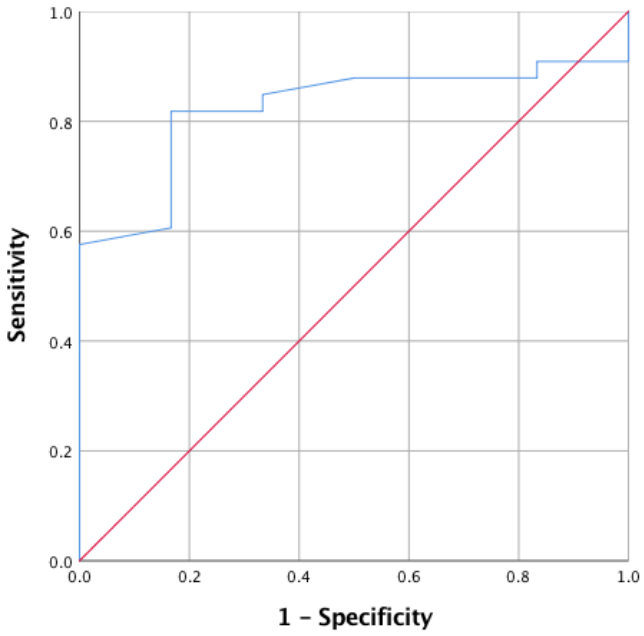
5.4.4.1 Surgical outcomes

I have shown that those who had optimal surgery had higher values of mean basal and maximal skin capillary density. I performed a ROC analysis to define the optimal SCD that better predicts surgical outcome (Table 5.9).

Table 5.9. Optimal values for SCD (vessels/mm^2) and prediction of surgical debulking outcome

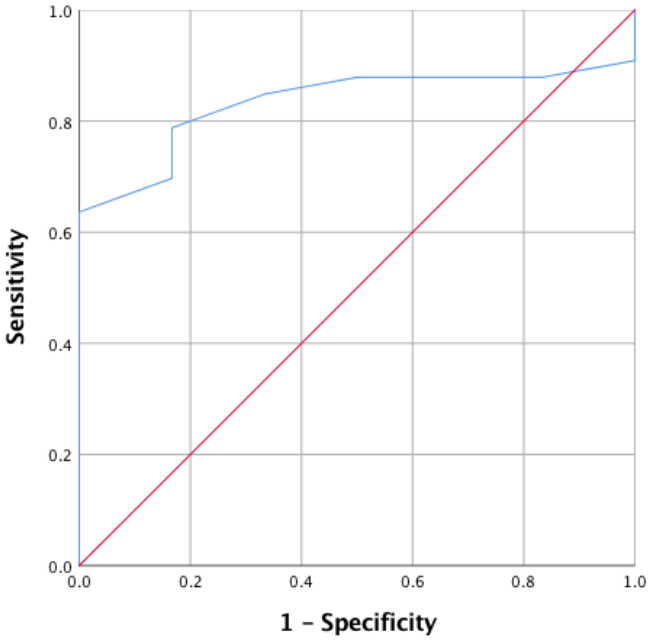
SCD at Baseline (vessels/mm^2)	Optimal value	Sensitivity	Specificity	AUC
Maximal	66	78.8%	87.5%	0.833
Mean basal	61	81.8%	87.5%	0.823

Figure 5.7. ROC curve of mean basal SCD at baseline (vessels/mm^2) and optimal surgical outcome



AUC 0.823
p=0.007 (CI 0.715-0.983)

Figure 5.8. ROC curve of maximal SCD at baseline (vessels/mm^2) and optimal surgical outcome



AUC 0.833
p=0.006 (CI 0.738-0.981)

5.4.4.2 Survival outcomes

I carried out a ROC analysis for survival based on the same optimal cut off values for SCD but the sensitive and specificity were low and therefore not robust (Table 5.10).

Table 5.10. Optimal values for mean basal and maximal skin capillary density (vessels/mm^2) and prediction of overall survival

Overall Survival				
SCD at Baseline	Optimal value	Sensitivity	Specificity	AUC
Maximal	66	76.9	34.6%	0.530
Mean basal	61	76.9%	34.6%	0.577
Progression free survival				
SCD at Baseline	Optimal value	Sensitivity	Specificity	AUC
Maximal	66	59.3%	16.7%	0.427
Mean basal	61	63.0%	16.7%	0.448

5.5 Discussion

A reduction in skin capillary density was seen in all patients overall. However, there was a significant difference in progression free survival in patients who had a smaller reduction (above mean) in maximal density of capillaries. There was a trend to reduced overall survival also. This trend was also demonstrated for both progression free and overall survival and mean basal skin capillary density although in these cases it was not significant.

In regard to radiological tumour response, there was no difference in SCD at baseline and mid treatment CT response. It was not possible to assess associations between SCD in relation to radiological response to Bevacizumab in my cohort as all patients who had Bevacizumab pre surgery had complete surgical resection and a complete response at mid treatment CT.

However, there was a significant association between baseline SCD and surgical resection outcome with a higher maximal and mean basal SCD predicting increased likelihood of complete resection. Maximal SCD of over 66 and mean basal over 61 at baseline predicts the probability of achieving optimal surgery in more than 80% of patients. I was unable to demonstrate that SCD predicts risk of post-operative morbidity.

Although it was not significant, there was a trend of higher baseline VEGF and increased likelihood of optimal debulking. This further adds weight to the association of higher SCD and surgical outcome in that those patients with higher angiogenic activity as measured by SCD were more likely to achieve optimal surgical debulking.

There are no studies investigating capillary density and surgical treatments in cancer. Furthermore, there are none regarding SCD and response to chemotherapy. It is difficult to place my work in the context of scanty literature, however I have established the potential of SCD to be a marker of chemotherapy response and prediction of surgical outcomes.

The lack of demonstrable association between SCD and survival may be explained by the small numbers in the cohort and that data is not fully mature. However, the association between change in SCD and PFS is a promising finding that suggests the role of SCD as a biomarker of response requires further investigation. High micro vessel count within the tumour has been demonstrated to be associated with survival outcomes[171, 172] and correlation between the tumour and peripheral vascular networks requires evaluation to ascertain whether SCD has a role as a surrogate method of assessing tumour angiogenesis.

5.6 Conclusion

Skin capillary density at baseline is associated with surgical debulking outcome. Various models exist to predict optimal debulking, but none are widely accepted in clinical practice and radiological assessment of disease is heavily relied upon. Furthermore, a greater fall in structural skin capillary density during treatment is associated with reduced risk of recurrence.

Chapter 6: Tumour vasculature and proliferation markers

6.1 Introduction

Angiogenesis is key for tumour growth and recurrence.[66] The tumour microvascular environment in ovarian cancer is characterised by disordered blood vasculature comprising of immature and dysfunctional vessels.[69]

CD 31 is a glycoprotein that is expressed in endothelial cells and is a measure of microvessel density in solid tumours[185] and therefore provides an assessment of tumour angiogenesis. In a subgroup analysis of the phase III GOG-0218 trial, women with a high CD 31 at diagnosis had improved PFS and OS (19.9 vs 9.8 months HR = 0.40 95% CI 0.29-0.54 p= 0.0025 and 45.6 vs 35.9 months HR = 0.68 95% CI 0.52-0.89 p=0.0155).[188] OS and PFS were not improved by such a margin in the general non stratified cohort. This is contradictory to many studies which demonstrate poorer OS and PFS in patients with high microvessel density. Further work is needed to validate the potential predictive value of CD31 to target angiogenic treatment. PDGFR is a marker of pericytes which may be indicative of neo vascularised tissue and active angiogenesis. High levels of expression have been linked to shorter OS and high grade tumours.[193-195]

Alongside angiogenesis, uncontrolled cell proliferation is a hallmark of cancer growth. Ki67 is a marker of cell proliferation and high-grade serous carcinoma of the ovary is associated with a high (>40%) and diffuse pattern of staining for Ki67.[197] Low Ki67 has been shown to be

associated with poorer OS in a cohort of ovarian cancer patients, postulated to be secondary to reduced response of these tumours to chemotherapy.[198]

Chemotherapy response score (CRS) is the most reproducible criteria for assessing in vivo tumour response to chemotherapy and prognostic significance of CRS has been demonstrated in small cohorts[264] but has not be related to vascular markers or associated with surgical outcomes.

I have established that skin capillary density is associated with optimal surgical debulking but there are no studies investigating tumour vasculature and proliferative markers to predict surgical outcomes. Furthermore, there is no assessment of microvessel density pre and post chemotherapy and its relationship with chemotherapy response scores, nor have there been studies comparing vascular markers in the tumour milieu to the peripheral microvascular environment.

6.2 Aims

- To investigate whether tumour proliferation and vascular markers at baseline or at surgery can predict OS, PFS
- To investigate whether tumour proliferation markers at baseline or at surgery can predict surgical cytoreduction, post-operative complications and response to chemotherapy (radiological CT response and histological chemotherapy response score)

- To investigate the longitudinal changes in tumour proliferation and vascular markers after chemotherapy
- To investigate the association between tumour vascular markers and skin capillary density

6.3 Methods

As previously outlined, this was a prospective cohort study and patients with a diagnosis of high grade serous ovarian cancer were invited to participate over an 18 month period. Biopsy of metastatic tissue (omentum or peritoneum) was taken prior to treatment. Patients who were suitable had debulking surgery either prior to chemotherapy or after 3 cycles of treatment where disease was too extensive. The details of how tissue was prepared and analysed are described in detail in the main methods section (section 2.9 page 66). CD 31, PDGFR and Ki67 were calculated by myself and a Pathologist in tissue taken at biopsy and at debulking surgery. Chemotherapy response score was calculated by an expert Pathologist according to the International Collaboration on Cancer Reporting guidance as detailed in the main methods section[311] (section 2.9.3 page 68).

6.3.1. Definitions

Surgery included total abdominal hysterectomy, bilateral salpingo-oophorectomy, appendicectomy, omentectomy, and excision of peritoneal disease. Extended procedures were those that included bowel resection, splenectomy, diaphragm resection and excision of

lymph nodes. Surgery was scored 0-3: 0 for standard surgery, 1 for 1 additional extended procedure, 2 for 2 additional extended procedure and 3 for 3 additional extended procedures.

Post-operative complications were defined as a complication that occurred within 30 days of surgery. This included infection (wound, chest, urinary tract) and sepsis, renal failure, cardiopulmonary failure, blood transfusion, return to theatre, ileus, prolonged admission for more than 8 days, visceral injury, venous thromboembolism, ITU admission and death.

All those women who had neoadjuvant treatment had computed tomography (CT) scans after 3 cycles of treatment. These were discussed in the multi-disciplinary team (MDT) in order to determine suitability for debulking surgery. CT scans were reported according to RECIST criteria as partial response (PR), complete response (CR), stable disease (SD) or progressive disease (PD). Further details of the RECIST criteria are described in the main methods section. Chemotherapy response score was categorised to 'good'; CRS 2 and 3 combined and 'poor'; CRS 1.

6.3.2. Statistics

When investigating the change in markers, the difference between baseline and visit two was calculated. Women were assigned into 2 groups, those who had 3 cycles of chemotherapy and those who had 3 cycles chemotherapy plus Bevacizumab (ICON 8b).

Dichotomised variables were tabulated as high and low values above the mean. The associations between tumour markers and PFS and OS were assessed with Kaplan Meier plots stratified by the dichotomised biomarkers and with cox proportional hazards models using

univariate and multivariate models. The degree of association was characterised by hazard ratios and confidence intervals. Logistic and linear regression was used to test association between variables and models were adjusted for treatment, age and stage. Fishers exact test was used to assess the association between CRS and surgical outcome. Kendalls tau-b correlation was used to measure the strength of association between CT response and tumour markers. In order to test the difference between markers in groups Mann Whitney test was used. Where relevant, statistical significance was described when p = <0.05.

6.4 Results

6.4.1. Tumour vasculature and proliferation markers at diagnosis

6.4.1.1. Recruitment

50 women were recruited into the study. 5 (10%) women had primary debulking surgery and were excluded from this analysis. Descriptive data is included in the table 6.1 below.

Table 6.1 Demographics

	N= 45
Characteristic	
Age (years) at diagnosis	69.9 (10.9)
Ca-125	2218 (73, 40000)
Performance status at diagnosis	
0	6 (13%)

1	31 (69%)
2	8 (18%)
Tumour stage	
3b	7(15%)
3c	21 (47%)
4	17 (38%)
Treatment	
Neoadjuvant chemotherapy	39 (87%)
Icon8b	6 (13%)
Biopsy (pre surgery)	31 (69%)
Type of surgery	
Complete/optimal	28 (62%)
Suboptimal	8 (18%)
Unfit/died/inoperable	9 (20%)
Chemotherapy response score	
1	11 (24%)
2	18 (40%)
3	7 (16%)
Bevacizumab treatment (Yes, pre surgery)	6 (13%)
Death (Yes)	22 (49%)
Recurrence (Yes)	35 (78%)

31 (69%) women who had neoadjuvant treatment had a biopsy taken to ascertain diagnosis. The remaining women had the diagnosis based on cytology and radiological imaging. Of those who had a biopsy, tissue was taken from the peritoneum or omentum in all cases. Table 6.2 describes the mean levels of markers in the tissue for those taken at biopsy prior to treatment.

Table 6. 2. Mean (SD) of tumour markers collected at biopsy

	Ki67	**PDGFR**	**CD31**
Biopsy (n= 31)	49.2 (24.6)	4.3 (2.6)	5.3 (2.4)

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

6.4.1.2.Survival outcomes

Ki 67

19 (61%) biopsy samples recorded a high Ki67 (above mean 49.2%). There was no association between high and low Ki and OS (23.0 vs 17.9 month) and PFS (18.7 vs 20.5 months) (p=0.167).

Figure 6.1. Overall survival according to high and low Ki67 at biopsy

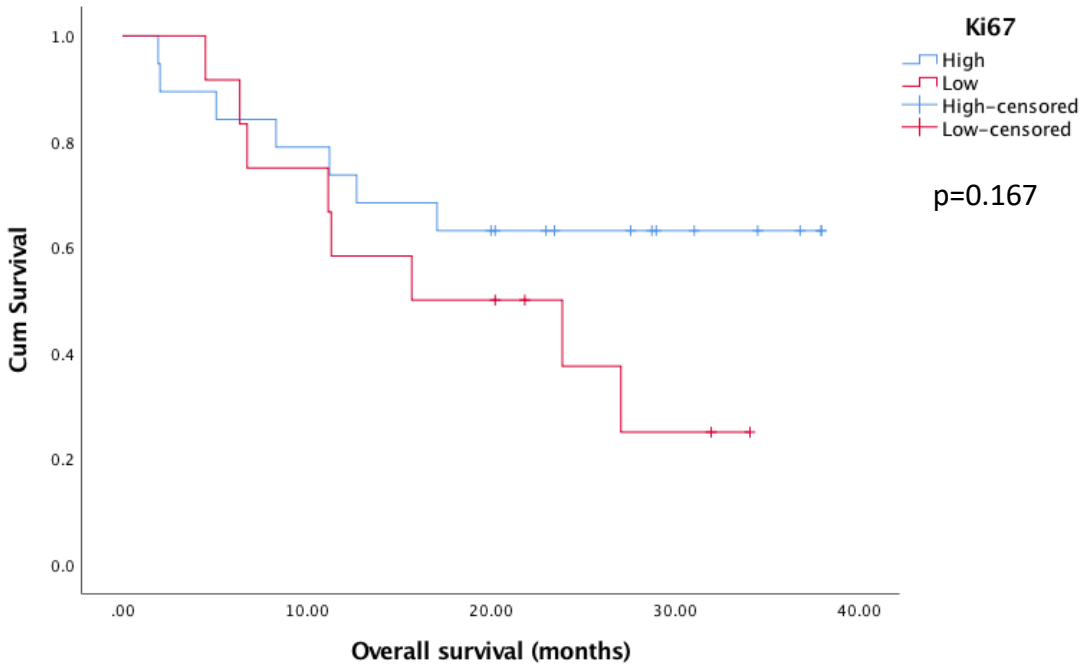
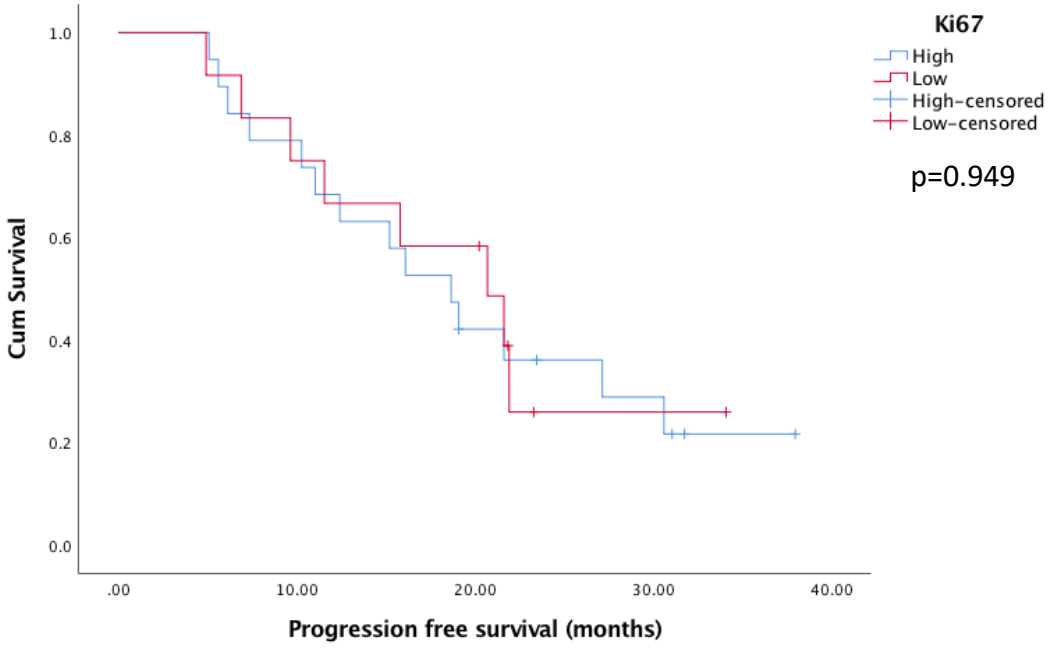


Figure 6.2. Progression free survival according to high and low Ki67 at biopsy



CD31

20 (65%) biopsy samples recorded a high CD 31 count above the mean (5.3 vessels). There was no association between high and low CD31 count and OS (23.2 vs 20.5 months) and PFS (21.2 and 15.2 months)

Figure 6.3. Overall survival according to high and low CD31 at biopsy

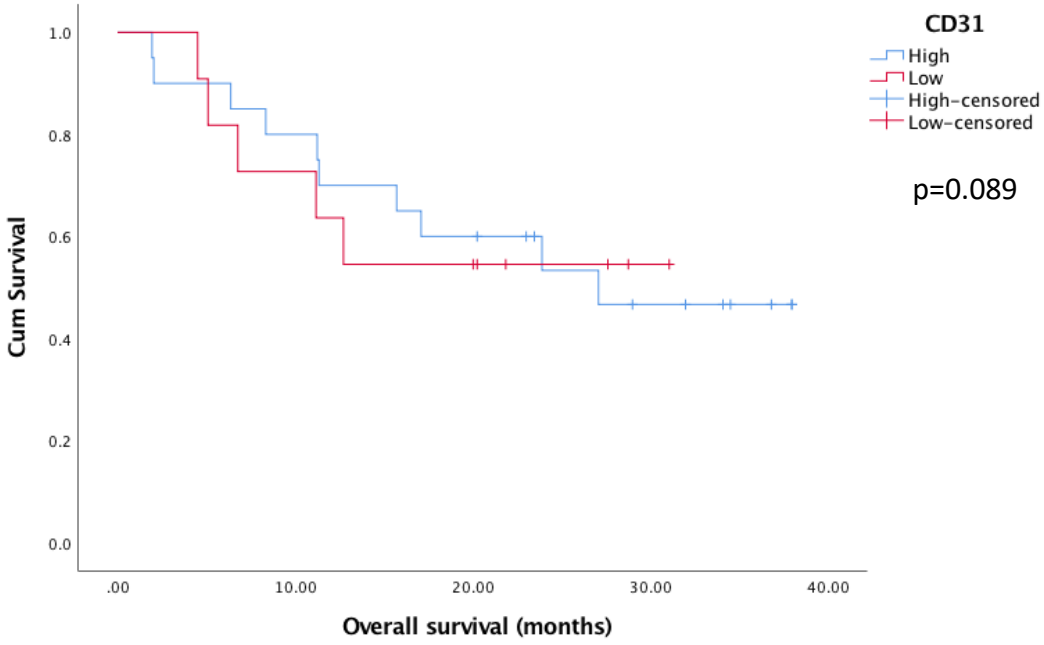
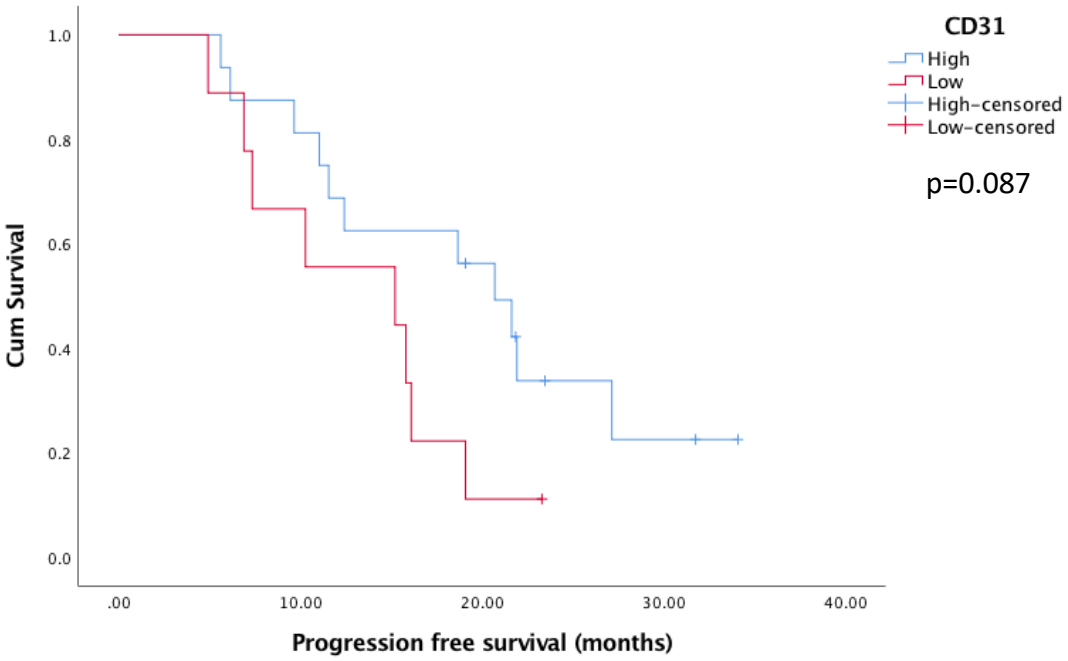


Figure 6.4. Progression free survival according to high and low CD31 at biopsy



PDGFR

11 (35%) biopsy samples recorded a high PDGFR count above the mean (4.3 vessels)

There was no association between high and low PDGFR count and OS (20.0 vs 22.4 months) and PFS (19.1 vs 18.9 months)

Figure 6.5. Overall survival according to high and low PDGFR at biopsy

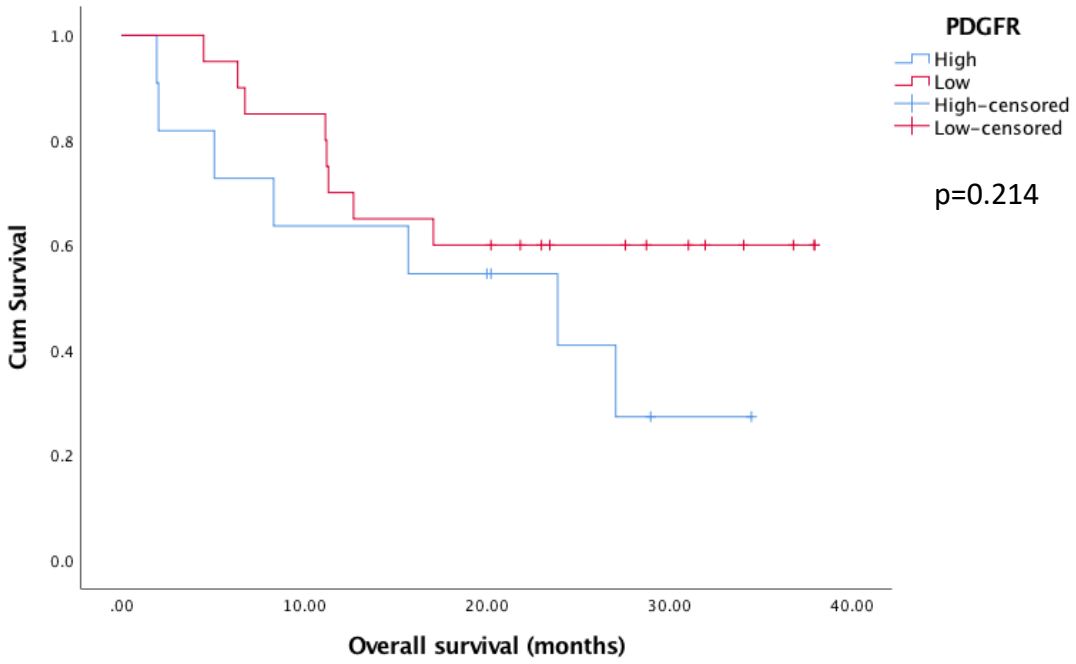
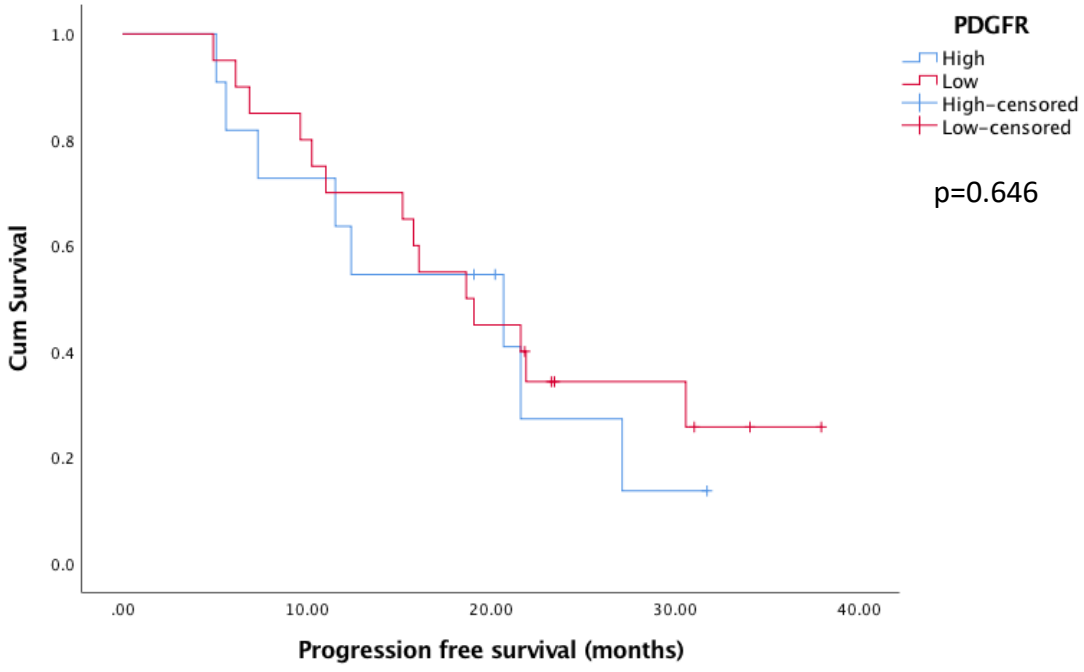


Figure 6.6. Progression free survival according to high and low PDGFR at biopsy



When values of CD31, Ki67 and PDGFR measured in biopsy samples were expressed continuously there was no association with risk of death or recurrence as detailed in table 6.3 and 6.4.

Table 6.3 Univariate analysis of tumour markers at biopsy and overall survival

Tumour markers	**Hazard ratio (CI)**	**P value**
Ki 67	0.989 (0.97-1.01)	0.307
CD 31	1.043 (0.85-1.28)	0.688
PDGFR	1.112 (0.94-1.31)	0.208

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

Table 6.4 Univariate analysis of tumour markers at biopsy and progression free survival

Tumour markers	**Hazard ratio (CI)**	**P value**
Ki 67	1.01 (0.99-1.02)	0.561
CD 31	0.92 (0.73-1.15)	0.447
PDGFR	0.98 (0.82-1.17)	0.832

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

There was also no evidence of association between proliferative markers and markers of tumour vasculature in biopsy samples: Ki 67 and CD 31 ($p = 0.461$) and Ki 67 and PDGFR ($p= 0.781$).

6.4.1.3. Mid treatment CT response

42 women had a mid-treatment CT. 3 women died after the first cycle of chemotherapy. 4 (9%) women had a complete response at mid treatment CT. 2 (5%) had stable disease and the remaining 36 (86%) had a partial response. Examining those 31 women who had a biopsy, tumour markers were dichotomised to assess an association with CT response. There was a strong positive relationship between Ki67 and CT response (τ_b = .563, p = 0.003).

10% (2/12) women with a low Ki67 had stable disease whereas none had a complete response. Conversely 18% (3/17) with a high Ki67 had complete response to adjuvant treatment whereas no women with low Ki67 had a complete response to treatment. Categorised CD 31 did not have any correlation with tumour response as measured by CT imaging (Table 6.5).

There was a significant association between complete response on CT scan and complete resection at surgery (p=<0.0001).

Table 6.5. CT response and levels of tumour markers

	Ki67		**CD31**		**PDGFR**	
	Low Ki67	High Ki67	Low CD31	High CD31	Low PDGFR	High PDGFR
Complete response	0	3	1	2	2	1
Partial response	10	14	9	15	16	8
Stable disease	2	0	1	1	2	0
Coefficient	0.563 P=0.003		0.342 P=0.073		0.424 P=0.021	

*2 women who had biopsy died before they had a CT

6.4.1.4. Surgical resection

When I compared baseline tumour markers with surgical resection outcomes, there was no significant difference in tumour makers between those who had suboptimal or optimal surgery (Table 6.6)

Table 6.6 Comparison of tumour markers in those who had optimal and suboptimal surgery

	Optimal	**Suboptimal**	**P value**
Tumour markers			
Ki 67	22.3 (16.1)	26.4 (29.3)	0.77
CD 31	4.1 (2.4)	3.1 (2.5)	0.43
PDGFR	2.7 (2.0)	2.1 (2.4)	0.40

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

6.4.2. Tumour vasculature and proliferation markers at debulking surgery

6.4.2.1 Recruitment

41 (82%) women underwent elective debulking surgery. 9 (18%) women never had surgery as they were too unfit or died. Of the 41 women who had debulking surgery, 33 (80%) cases had tissue available from surgery for analysis. 3 women had an open and close procedure where the disease was felt not to be resectable by the surgeon and no tissue was taken for histology in these cases. Those 5 (10%) women who had primary surgery were also excluded as I wanted to compare a more homogenous group of patients and this group had no treatment prior to surgical resection.

In order to assess the change in markers 21 women had tissue available from both biopsy and debulking surgery. The remaining 10 who had a biopsy did not have tissue available from surgery for the following reasons (3 died after one cycle of chemotherapy, 3 had open close procedures and 4 were unfit or had too extensive disease so did not have surgery).

6.4.2.2 Surgical outcomes

18 (44%) women had a post-operative complication. Table 6.6 describes details of the post-operative complications. 1 woman had 4 complications (wound urine, chest infection, ileus). 1 had a late complication of deep vein thrombosis and cerebral vascular event at 35 days post operatively. 3 (7%) women had chemotherapy delayed due to post-operative complications (1 due to thrombosis, 2 due to prolonged recovery from surgery). 7 (17%) women had a prolonged hospital stay and 7 (17%) required intensive care admission.

Table 6.7. Description of complications at debulking surgery

Complication (n=18)	**N (%)**
Haemorrhage	7 (17)
Intestinal perforation	2 (5)
Ileus	3 (7)
Cardiopulmonary	1 (2)
Infection	
Wound	5 (12)
Urine	1 (2)
Chest	2 (5)

10 (24%) women had extended procedures. 3 women had 2 extended procedure. Table 6.8 describes the details of extended procedures.

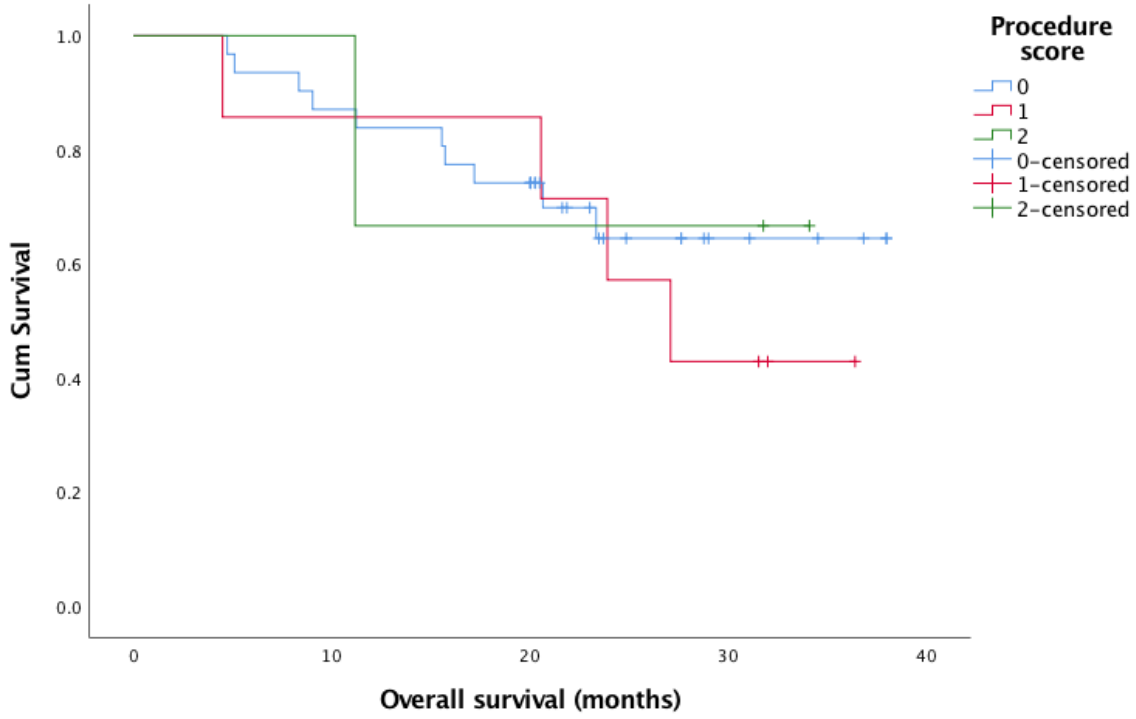
Table 6.8. Details of extended procedures

Extended procedure	**N (%)**
Splenectomy	3 (7)
Para aortic LN resection	1 (2)
Bowel resection/stoma	6 (15)
Diaphragm resection	3 (7)

2 (5%) women died in the post-operative period. Both were within 30 days and post mortem examination detailed cause of death as pneumonia. 1 woman who died had a score of 1 as required a bowel resection.

There was no difference in overall survival according to the surgical procedure score as demonstrated in figure 6.7 below (p=0.78).

Figure 6.7. Overall survival according to procedure score



I used logistic regression to ascertain association between markers at biopsy and complications at surgery. For every increase in PDGFR by 1 there was a reduction in risk of post-operative complications (p=0.037) but no significance was found. This model was adjusted for age, procedure score and treatment.

Table 6.9. Logistic regression of tumour markers and post operative complications.

Clinical variables	Post-op complications (Yes/ No)		ITU admissions (Yes/ No)		Extended hospital stay (Yes/ No)	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
CD31	1.13 (0.75, 1.71)	0.560	0.83 (0.51, 1.36)	0.456	1.03 (0.68, 1.58)	0.886
Ki67	1.01 (0.96, 1.06)	0.739	1.03 (0.97, 1.10)	0.361	1.03 (0.97, 1.08)	0.330
PDGFR	0.42 (0.19, 0.95)	0.037	1.23 (0.66, 2.27)	0.510	0.67 (0.35, 1.29)	0.227

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

6.4.3 Longitudinal change in tumour proliferative and vascular markers after treatment

Table 6.10 describes the mean and SD of tumour markers at biopsy and debulking surgery. There was a significant reduction of 20.7 % in Ki67 after treatment with chemotherapy and bevacizumab. PDGFR was significantly reduced by 1.6. CD 31 also decreased but the change was not significant.

Table 6.10 Tumour markers at biopsy and surgery.

Tumour markers	Biopsy Mean (SD)	Debulking surgery Mean (SD)	Difference Mean (SD)	P value
Ki 67	47.4 (22.2)	26.7 (18.4)	-20.7 (25.7)	0.0008
CD 31	4.8 (2.1)	3.9 (2.5)	-0.8 (3.2)	0.19
PDGFR	4.0 (2.8)	2.4 (1.9)	-1.6 (3.1)	0.04

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

When looking at the changes in those who had chemotherapy only compared with those who had chemotherapy and bevacizumab (ICON 8b), there was a greater fall in CD 31 count in those women who had Bevacizumab with chemotherapy (p= 0.009). The fall in PDGFR and Ki67 was not significantly different between the groups (Table 6.11).

Table 6.11 Comparison of the difference in mean tumour markers according to treatment

Tumour markers	Difference Neoadj. chemotherapy (Mean SD)	Difference ICON8b Mean (SD)	P value
Ki 67	-18.3 (25.1)	-26.7 (28.6)	0.50
CD 31	0.27 (2.8)	-3.5 (2.4)	0.009
PDGFR	-1.1 (3.2)	-2.8 (2.6)	0.47

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

I explored the association between surgical outcome and the difference from baseline to visit 2 for the tumour markers (Table 6.12). There were no significant associations found. However, a rise in ki67 decreased the odd of having an optimal resection by 9% and this approached significance.

Table 6.12. Logistic regression for debulking surgery outcome in relation to change in tumour factors after treatment

Tumour markers	**OR (95% CI)**	**P value**
Ki 67	0.91 (0.82-1.01)	0.057
CD 31	1.1 (0.78-1.6)	0.56
PDGFR	0.84 (0.58-1.2)	0.34

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

6.4.3.1.Survival outcomes

I also used logistic regression to investigate whether the change in baseline to visit 2 was able to predict the odds of progression free survival or overall survival (Table 6.13). Although a reduction in Ki67 was associated with worse OS was close to significance, no other associations were found.

Table 6.13. Logistic regression for odds of OS and PFS in relation to change in tumour

	OS		PFS	
Tumour markers	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
Ki 67	1.1 (1.01-1.14)	0.057	0.97 (0.93-1.01)	0.071
CD 31	1.3 (0.81-2.04)	0.29	1.27 (0.88-1.84)	0.21
PDGFR	0.77 (0.54-1.01)	0.14	0.84 (0.61-1.17)	0.30

*Ki67 is recorded as a percentage figure. PDGFR and CD31 is recorded as number of vessels counted

6.4.3.2. Correlation between change in markers

I used a linear regression model to explore whether there was association between proliferative makers and the vasculature in the tumour (Table 6.14). There is evidence of significant association between percentage change in CD31 and Ki67 with or without adjusting for treatment. For every unit change (i.e. 1%) in Ki67 the percentage change of CD31 increases by 0.68. There was no association with change in PDGFR and Ki67.

Table 6.14 Estimates of the association of %change in Ki67 with PDGFR/CD31.

	Estimate (SD)	**p-value**
Outcome: %change in PDGFR		
%change in Ki67	0.004 (0.214)	0.986
	-0.070 (0.228)*	0.7646*
Outcome: %change in CD31		
	0.682 (0.137)	<0.001
%change in Ki67	0.652 (0.138)*	<0.001*

*Model estimates after adjusting for treatment.

6.4.4 Chemotherapy response score

Chemotherapy response score was recorded in 36 patients. Poor was defined as chemotherapy response score as 1 and a good score 2 and 3 combined. 25 (69%) patients had a good score and 11 (31%) a poor score. Figures 6.8 and 6.9 display CRS and OS and PFS. Overall and progression free survival was worse in women who had a poor chemotherapy response score (18.8 vs 25.1 and 12.7 vs 21.2 months respectively)

Figure 6.8 Progression free survival according to good or poor chemotherapy response score (CRS)

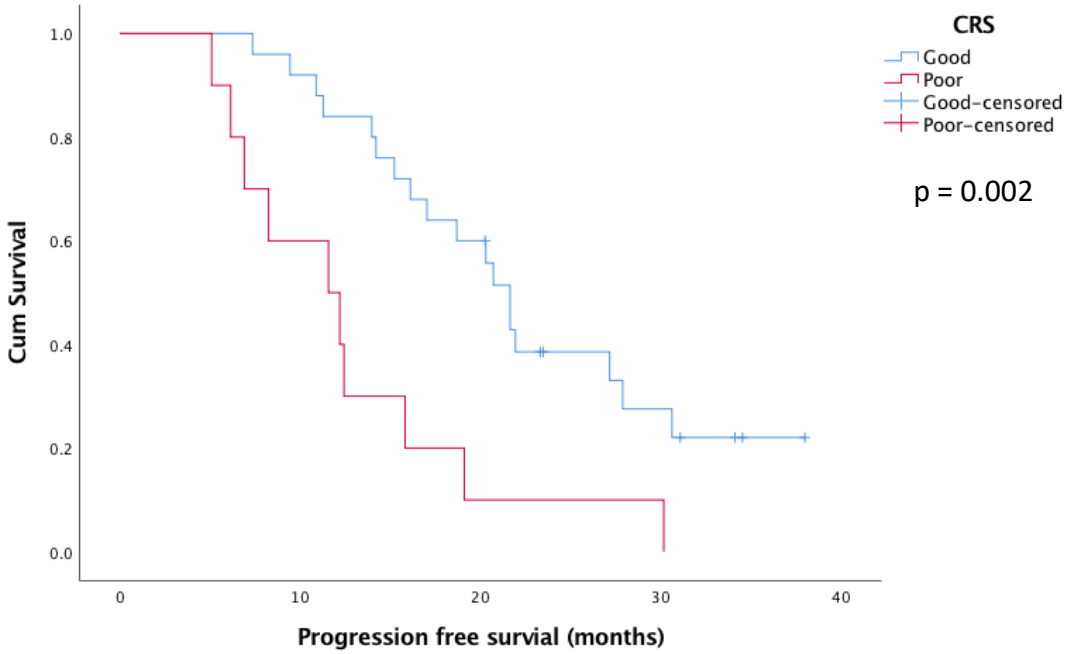
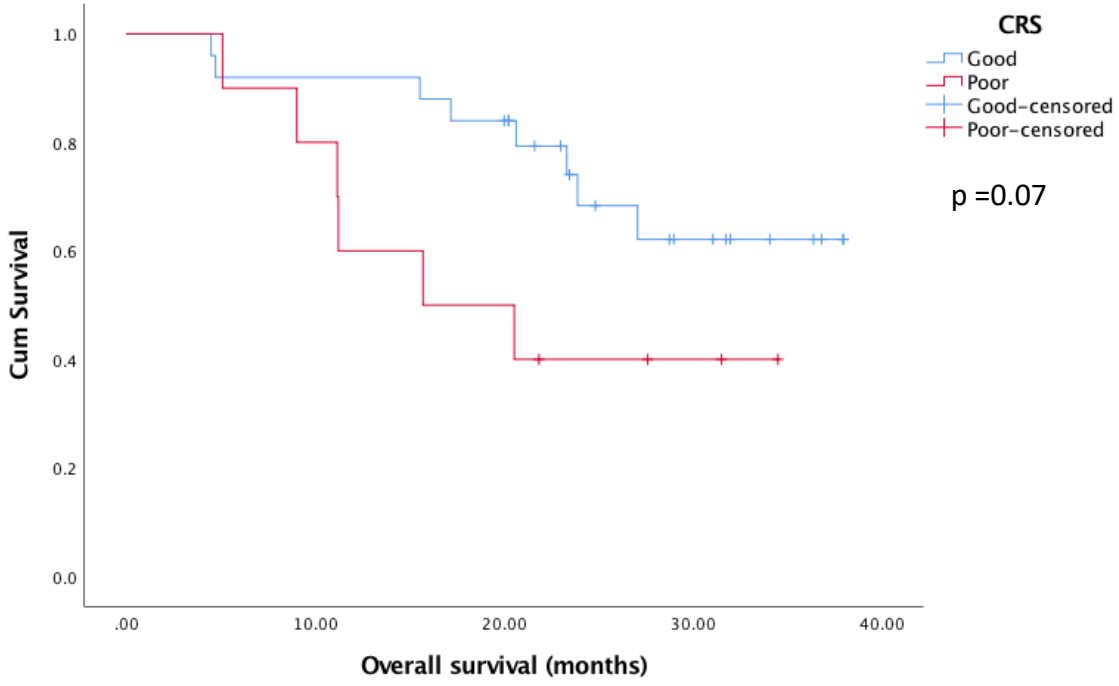


Figure 6.9 Overall survival according to good or poor chemotherapy response score (CRS)



There was a significant association with CRS and surgical outcome. Those patients with a 'good' CRS were associated with having a complete or optimal debulking outcome at interval surgery (p=0.04).

The change in tumour vasculature markers from baseline to surgery did not predict chemotherapy response score although a rise in Ki 67 was associated with reduced odds of a good score (p=0.045) (Table 6.15)

Table 6.15. Logistic regression for good or poor CRS in relation to change in tumour factors after treatment

Tumour markers	**OR (95% CI)**	**P value**
Ki 67	0.955 (0.913-.99)	0.045
CD 31	0.8 (0.5-1.6)	0.24
PDGFR	1.2 (0.9-1.7)	0.29

6.4.5.Testing the association of SCD at baseline with CD31 at biopsy

In order to ascertain whether there was a link between vascular marker in the tumour (CD31) and peripheral vasculature (SCD) I used linear regression (Table 6.16). CD31 measured at biopsy was the main outcome. For 1-unit increase in either maximal basal or mean capillary density, CD31 is expected to decrease by 0.06 and 0.07 respectively however this was not significant.

I also tested the association between Ki67 and PDGFR to skin capillary density and no correlation was found.

Table 6.16. Estimates of the association between CD31 and SCD density.

Variable	Estimate (SD)	p-value
Maximal SCD at Baseline	-0.068 (0.036)	0.157
Mean basal SCD at Baseline	-0.078 (0.046)	0.129

I also investigated the association between the % change in tumour marker with the angiogenic markers and skin capillary density measurements recorded at baseline after adjusting for treatment and age. Increasing the value of VEGF by 100 units at baseline was associated with a fall in CD31 from biopsy to surgery (p= 0.0343) (Table 6.17).

Table 6.17. Estimates of the association of %change in PDGFR and CD31 with each angiogenic marker and SCD measure.

	Estimate (SD)	p-value
Outcome: % change in PDGFR		
VEGF at baseline	0.02226 (0.02357)	0.3653
Max SCD at baseline	-0.01623 (0.01975)	0.4274
Mean basal SCD at baseline	-0.01757 (0.01978)	0.3920
Outcome: % change in CD31		
VEGF at baseline	-0.04925 (0.02039)	0.0343
Max SCD at baseline	0.01062 (0.02042)	0.6123
Mean basal SCD at baseline	0.01090 (0.02053)	0.6051

6.5. Discussion

In this cohort there was a trend of worse OS in those women with low Ki67 at biopsy although this did not reach significance. There was no difference in OS or PFS according to CD31 levels. There was a positive correlation between high Ki67 and CT response after 3 cycles of treatment. This does correlate with aforementioned evidence in the literature that high Ki67 levels at biopsy are associated with improved response to chemotherapy.[197, 198] Response to chemotherapy radiologically is key to informing the MDT decision making regarding plans to proceed with surgery. Histological assessment of response to chemotherapy as calculated by the CRS is based on the presence of viable tumour alongside regression associated inflammatory changes.[255] A rise in Ki67 after treatment was associated with reduced likelihood of a good CRS. Although this was close to significance it does go further to strengthen the association between cell proliferation and chemotherapy efficacy.

The data does also validate the prognostic significance of CRS in regard to OS and PFS. I was able to demonstrate a strong correlation of a good CRS and complete surgical resection which may be an explanation for the former association as complete surgical resection is accepted to be a significant factor in improved OS and PFS.

This is the first study to investigate the longitudinal changes in tumour vasculature and proliferative markers after chemotherapy in ovarian cancer. I have demonstrated a significant reduction in Ki67 and PDGFR after 3 cycles of chemotherapy. CD31 did reduce by a greater amount in those who had Bevacizumab alongside chemotherapy compared to those who had chemotherapy alone. I was not able to demonstrate an impact on OS, PFS in relation

to the degree of these changes. This may be due to the lack of mature data and small numbers within this cohort who had matched tissue from both biopsy and debulking surgery. Nonetheless it is a promising finding that requires validation.

I was unable to demonstrate any association between CD 31 or PDGFR and skin capillary density. Previously I have demonstrated a significant association between skin capillary density and surgical resection. This was not demonstrable in relation to tumour vasculature.

6.6 Conclusion

This is the first study to demonstrate a fall in Ki67 and PDGFR after chemotherapy for ovarian cancer. High levels of Ki67 at diagnosis correlate to radiological tumour response and complete surgical resection is associated with a good chemotherapy response score.

Chapter 7. The effect of anti-angiogenic therapy on skin capillary density

7.1 Introduction

Bevacizumab is a recombinant humanised monoclonal IgG antibody that targets VEGF and an anti-angiogenic therapy used in women with ovarian cancer. Bevacizumab has been shown to improve progression free survival in women with ovarian cancer and those with a higher risk of disease progression have the greatest benefit.[152, 153] Locally Bevacizumab is currently given as part of a trial or in first line treatment for women with stage 4 disease but despite extensive research there is no established biomarker for Bevacizumab that helps to target or monitor treatment.

There are known side effects as a result of the use of Bevacizumab, the most common being hypertension. Not only can this place women at increased risk of morbidity but can delay treatment causing clinical and psychological distress. The mechanism of hypertension secondary to anti angiogenic treatment is multifactorial and endothelial dysfunction, thrombosis and capillary regression are all likely to play a role.[153]

An association between blood pressure and skin capillary density has been investigated in healthy patients and a common feature in essential hypertension is that of microvascular rarefaction characterised by reduced capillary density.[242-244] A small study of patients with renal cell cancer has linked a reduction in skin capillary density with treatment induced hypertension[229] thus it is a reasonable hypothesise that SCD may be an independent clinical biomarker of response to treatment with antiangiogenic inhibitors in ovarian cancer.

7.2 Aims

- To assess the change in SCD in a specific population of patients on anti-angiogenic therapy
- To assess the effect of anti-angiogenic therapy on blood pressure and association with skin capillary density
- To assess the prognostic role of SCD in patients on Bevacizumab treatment

7.3 Methods

Women who received Bevacizumab alongside standard treatment for high grade serous ovarian cancer were included in this subgroup. Pre -treatment measurements were those at baseline prior to treatment. End of treatment measurements were after standard treatment was completed including surgery. All the patients included in this cohort underwent interval debulking surgery. At each visit skin capillary density was measured and blood pressure recorded. Further details of skin capillary measurement are described in the main methods section (section 2.7 page 57). Hypertension as defined as systolic blood pressure >140mmHg and diastolic blood pressure >90mmHg. Non responders were defined as those who had a recurrence of cancer.

7.3.1. Statistics

When investigating the change skin capillary density and blood pressure, the difference between baseline and end of treatment was calculated. Wilcoxon test was used to compare blood pressure and SCD before and after treatment. Dichotomised variables were tabulated as high and low values above the mean. The associations between the baseline and calculated difference in values and PFS and OS were assessed with Kaplan Meier plots stratified by the dichotomised variables. Log rank test was used to ascertain significance in survival data. Linear regression was used to test association between blood pressure and skin capillary density. Where relevant, statistical significance was described when $p = <0.05$.

7.4 Results

7.4.1. Recruitment

15 (30%) women had Bevacizumab treatment. 6 (40%) of these had Bevacizumab treatment alongside chemotherapy from the start of treatment as part of the ICON 8b trial. The remaining 9 (60%) women had Bevacizumab added after surgery. This was due to stage 4 disease or suboptimal debulking surgical outcome. There were three occasions when treatment was delayed due to uncontrolled hypertension.

Table 7.1 shows the demographics of the patients enrolled in this subgroup.

Table 7.1 Demographics of patients

	N= 15
Characteristic	
Age (years) at diagnosis	66.5 (9.2)
<65	5(27%)
65-70	5 (27%)
>70	10 (66%)
BMI (kg/m^2)	27.1 (4.56)
History of hypertension (yes)	0 (0%)
Ca-125	4298 (73, 40000)
Performance status at diagnosis	
0	2 (13%)
1	13 (87%)
2	0(0%)
Tumour stage	
3c	5 (34%)
4	10(66%)
Treatment	
Neoadjuvant chemotherapy	9 (60%)
Icon8b	6 (40%)
Type of surgery	
Complete/optimal	11 (73%)
Suboptimal	4(27%)

Chemotherapy response score	
1	7 (47%)
2	6 (40%)
3	2 (13%)
Death (Yes)	6 (40%)
Recurrence (Yes)	12 (80%)

7.4.2. Longitudinal change in skin capillary density during treatment

Table 7.2 demonstrates the mean skin capillary density in patients before and after treatment. The mean basal skin capillary density reduced by 12.8 (SD5.9) capillaries and the maximal by 13.6 (SD 4.8) capillaries.

Table 7.2: Mean (SD) of skin capillary density (vessels/mm^2) before and after treatment with Bevacizumab.

	Mean basal SCD Mean (SD)		**P value**	**Maximal (SCD) Mean (SD)**		**P value**
	Pre	Post		Pre	Post	
Skin capillary density	64.0 (9.1)	51.2 (10.9)	0.0019	68.0 (9.7)	54.4 (10.2)	0.0013

Figures 7.1 and 7.2 demonstrate the individual plots of SCD at start and end of treatment and indicate a uniform reduction amongst all patients.

Figure 7.1: Individual plots of maximal SCD (vessels/mm^2) pre and post treatment with Bevacizumab

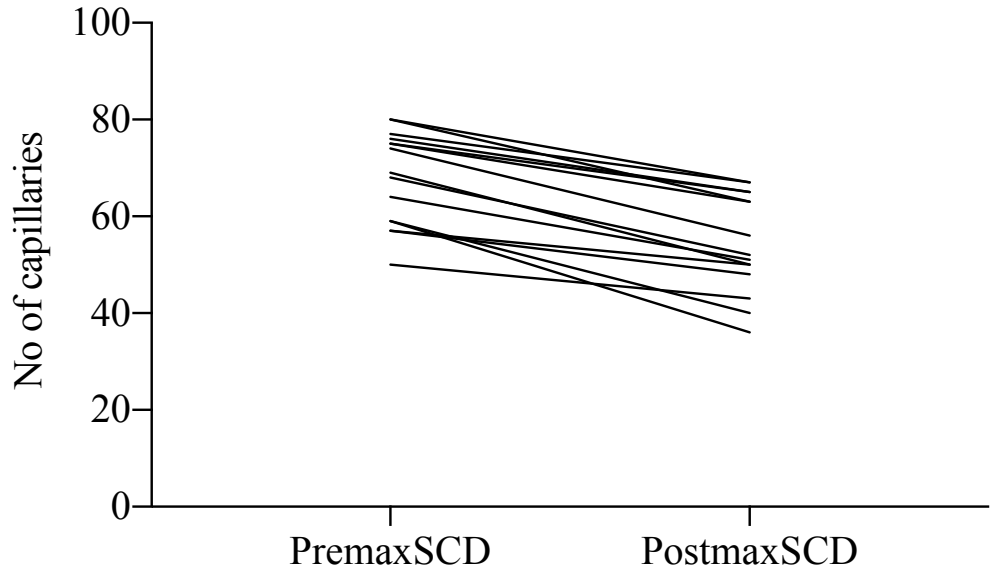
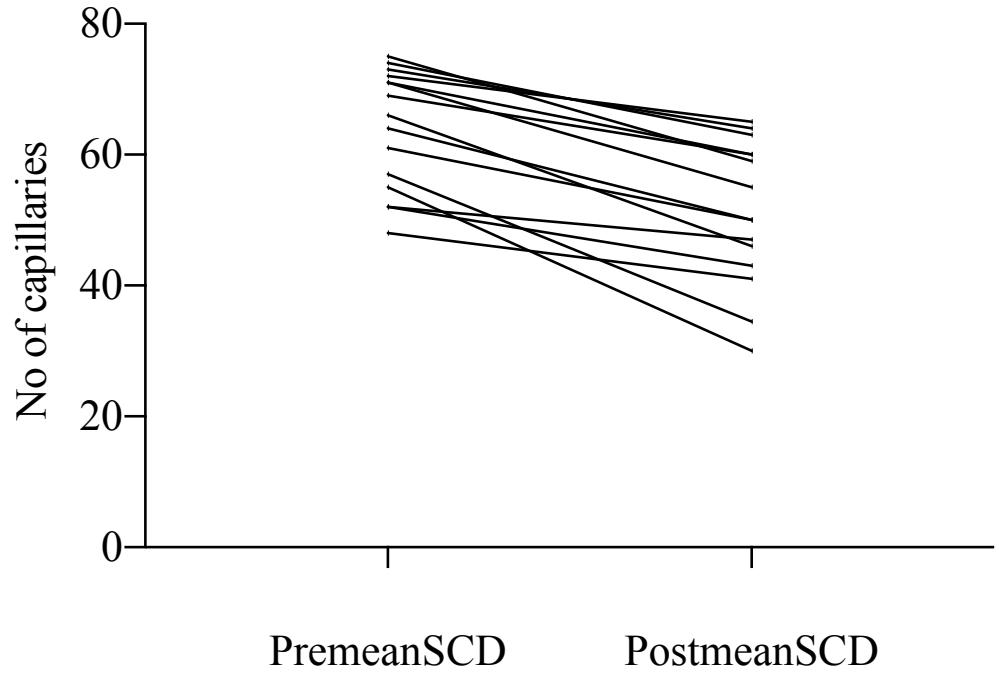


Figure 7.2: Individual plots of mean basal SCD (vessels/mm^2) pre and post treatment with Bevacizumab



Capillary rarefaction in both mean basal and maximal SCD occurred after treatment with bevacizumab (p= 0.0019 and p= 0.0013 respectively). Figures 7.3 and 7.4 demonstrate this change.

Figure 7.3: Box plot demonstrating fall in mean basal SCD (vessels/mm^2) in patients after treatment with Bevacizumab

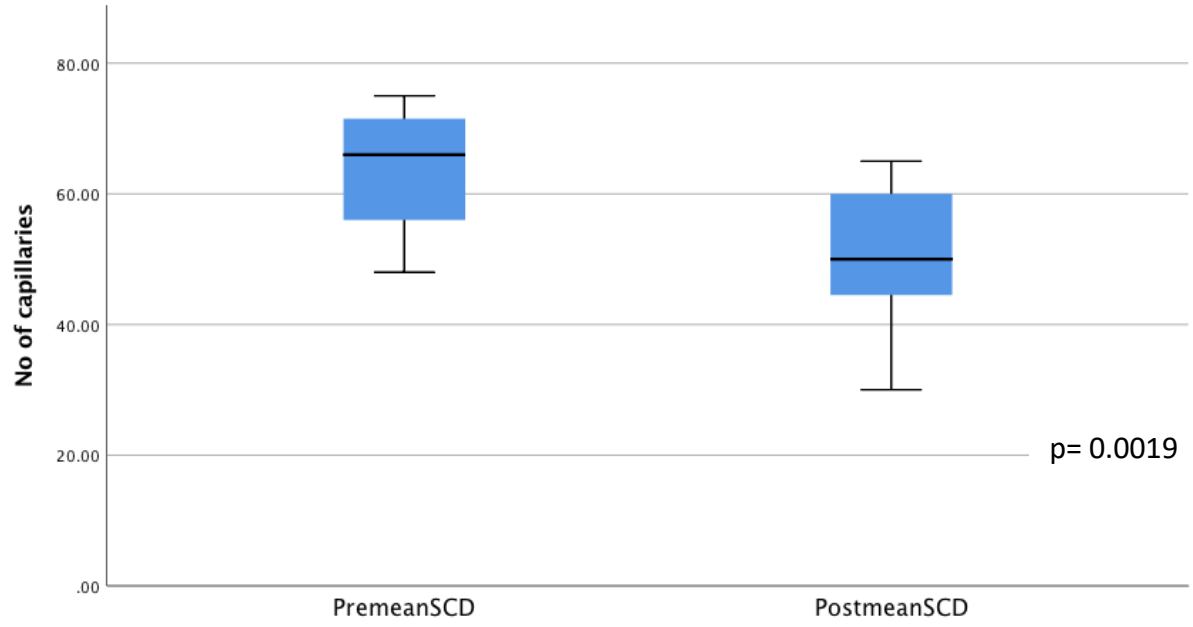
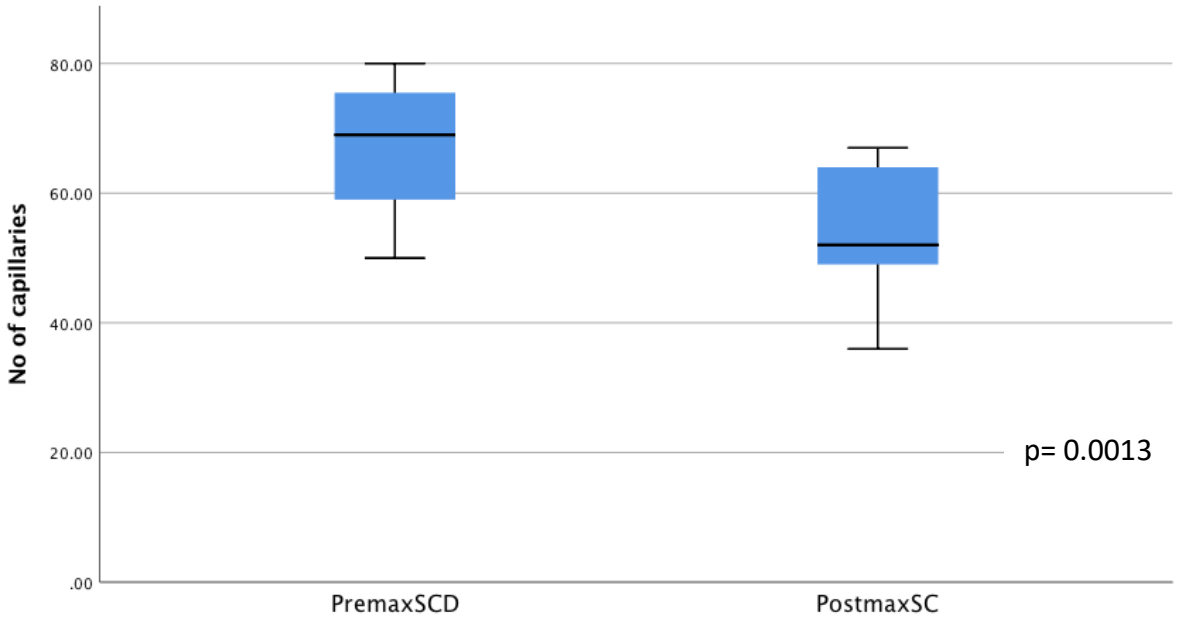


Figure 7.4: Box plot demonstrating fall in maximal SCD (vessels/mm^2) in patients after treatment with Bevacizumab



I then investigated whether the drop in skin capillary density was different according to the patients who responded compared to those who did not. Non responders were classified as those who had a recurrence of their disease or progression on the end of treatment CT scan according to RECIST.

Figures 7.5 and 7.6 demonstrate the fall in SCD stratified according to response to treatment. SCD rarefaction occurred in both groups but the difference was not significant.

Figure 7.5: Box plot demonstrating fall in mean basal SCD (vessels/mm^2) in patients after treatment with Bevacizumab according to response to treatment.

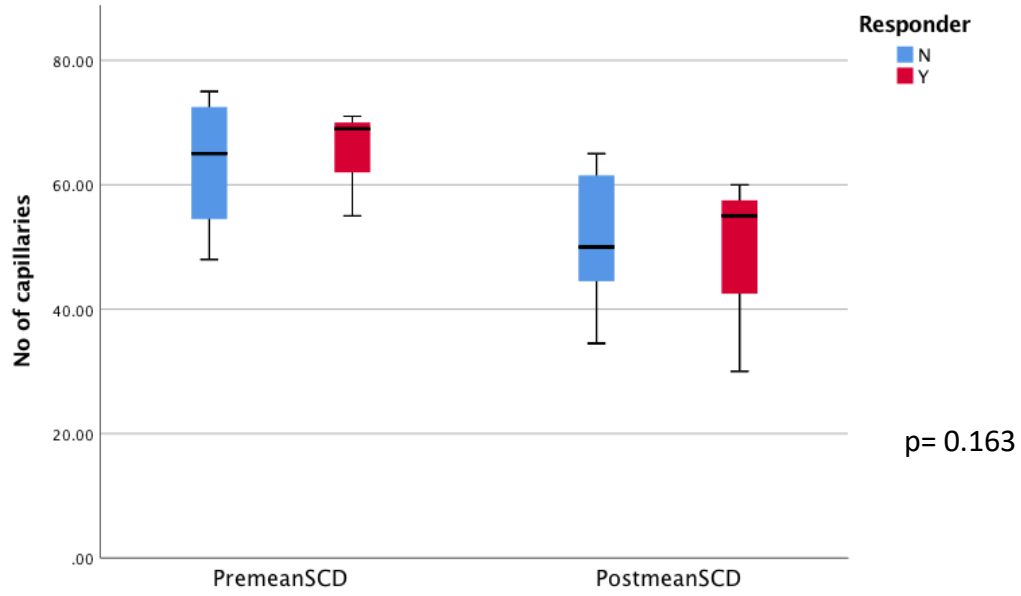
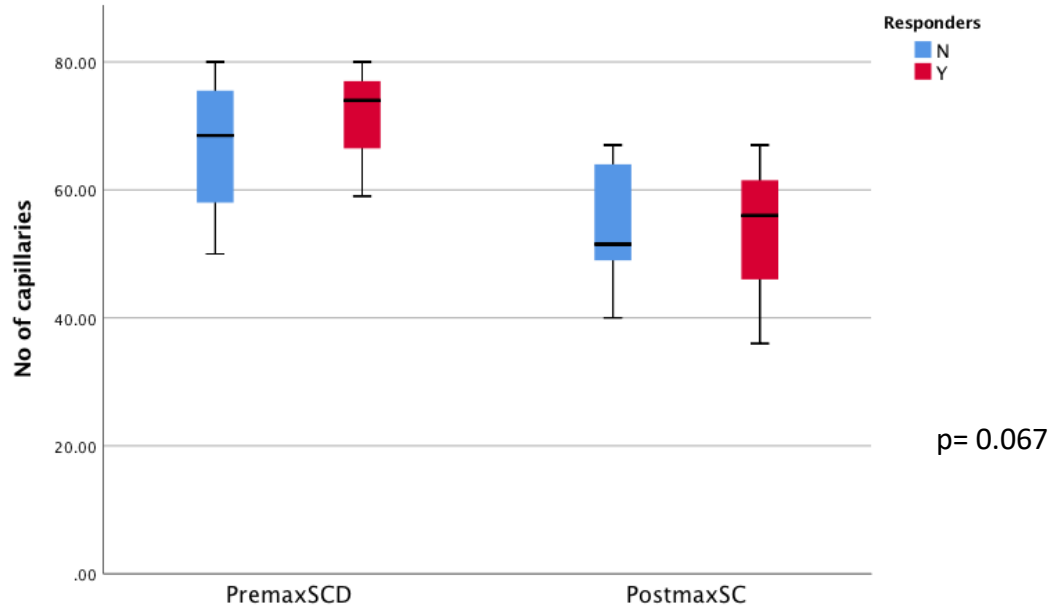


Figure 7.6: Box plot demonstrating fall in maximal SCD (vessels/mm^2) in patients after treatment with Bevacizumab according to response to treatment.



7.4.3 Blood pressure changes and skin capillary rarefaction

There was also a rise in the diastolic and systolic blood pressure after treatment with Bevacizumab. I used a linear regression model to investigate the association between the change in mean basal and maximal skin capillary density to the change in blood pressure. I demonstrated a linear inverse trend between fall in skin capillary density and rise in systolic and diastolic blood pressure (Figure 7.7).

Figure 7.7: A. Difference maximal SCD compared to systolic blood pressure. B. Difference mean basal SCD compared to diastolic blood pressure. C. Difference mean SCD compared to systolic blood pressure. D. Difference maximal SCD compared to diastolic blood pressure. All SCD measurements were in vessels/mm^2.

Figure 7.7 A.

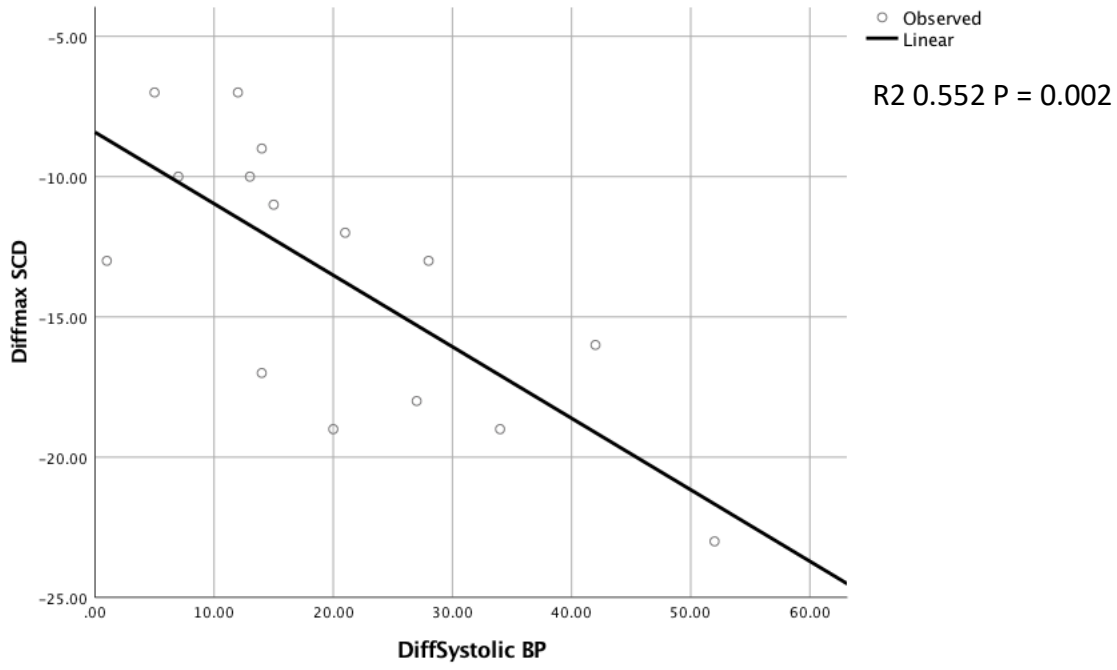
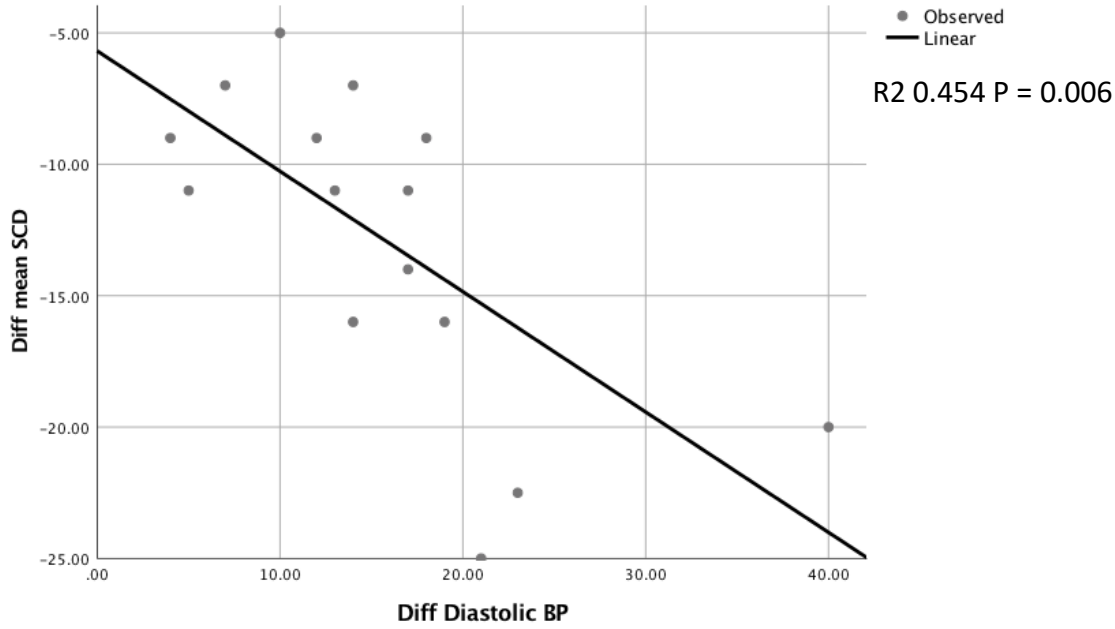
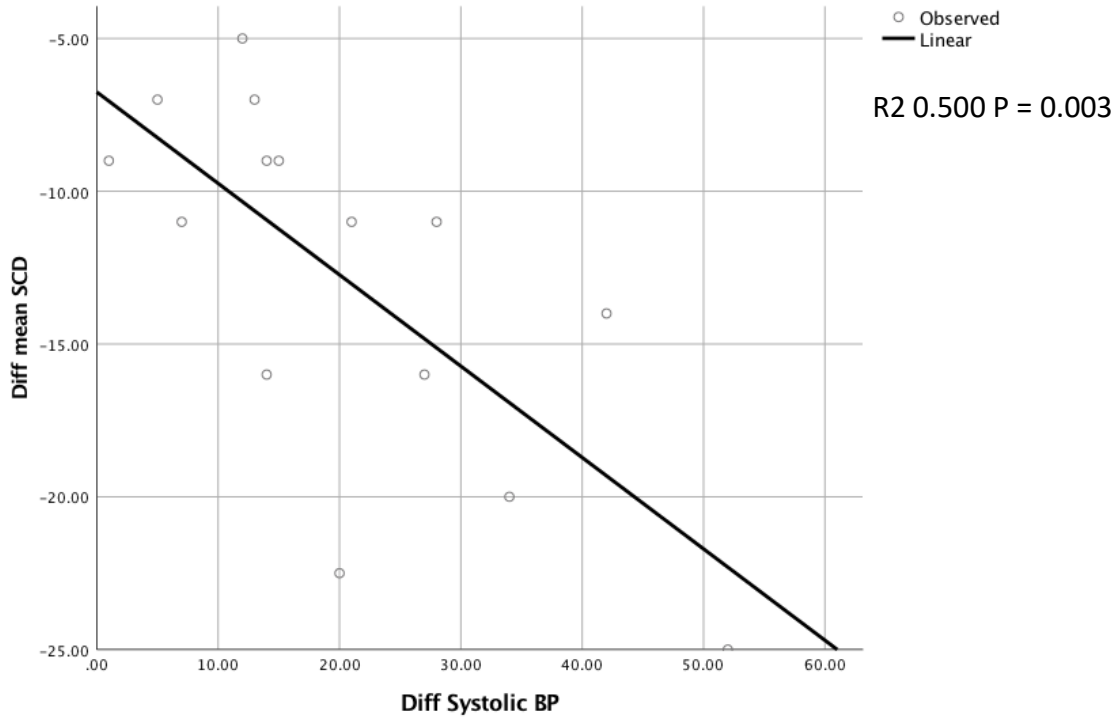


Figure 7.7 B



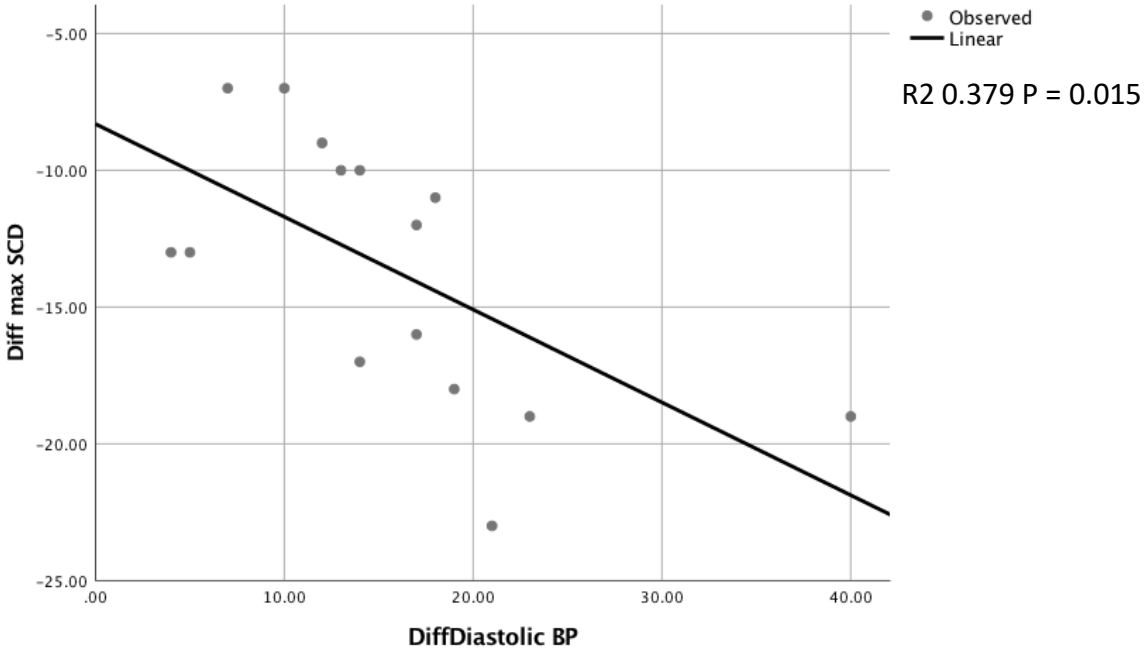
R2 0.454 P = 0.006

Figure 7.7 C



R2 0.500 P = 0.003

Figure 7.7 D



7.4.4 Survival outcomes

In these patients treated with Bevacizumab I carried out survival analyses to see whether the vascular changes were related to clinical outcomes.

The changes in maximal and mean basal skin capillary density did not have a significant predictive value for PFS (21 vs 19 months p= 0.568 and 22 vs 20 months p=0.657). (Figures 7.8A, B). Those patients with a change in both mean basal and maximal skin capillary density above the mean hence a smaller reduction in the number of capillaries showed a trend to worse OS but this was not significant (24 vs 32 months p= 0.761 and 24 vs 31 months p=0.594)

Figure 7.8: A. Progression free survival according to values above and below the mean of the difference in mean basal SCD. B. Progression free survival according to values above and below the mean of the difference in maximal SCD. C. Overall survival according to values above and below the mean of the difference in maximal SCD. D. Overall survival according to values above and below the mean of the difference in maximal SCD. All SCD measurements were in vessels/mm^2.

Figure 7.8 A

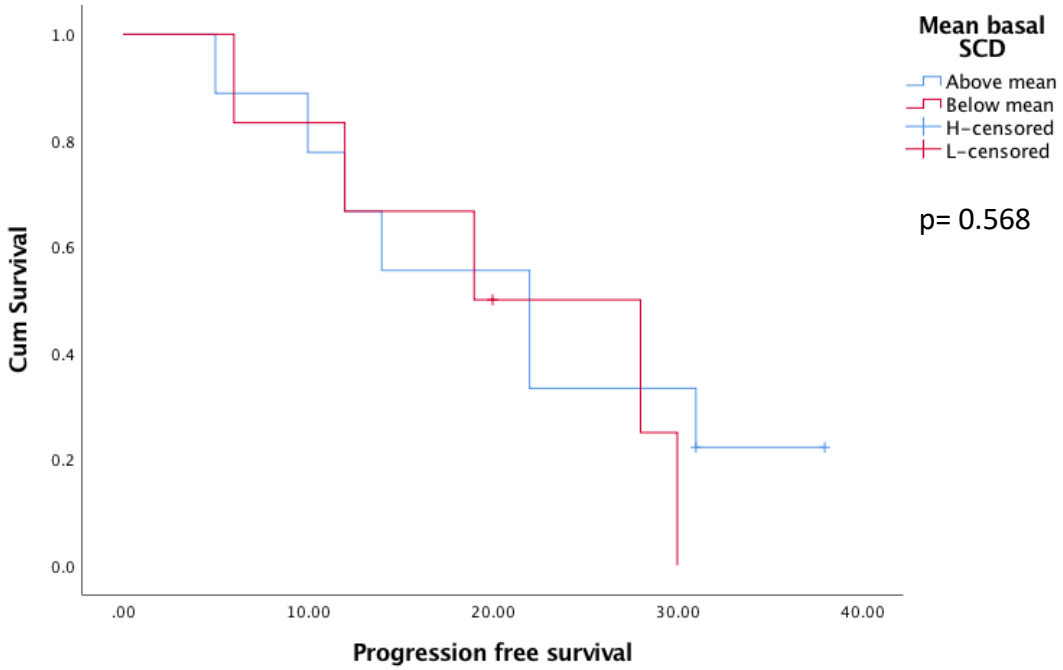


Figure 7.8 B

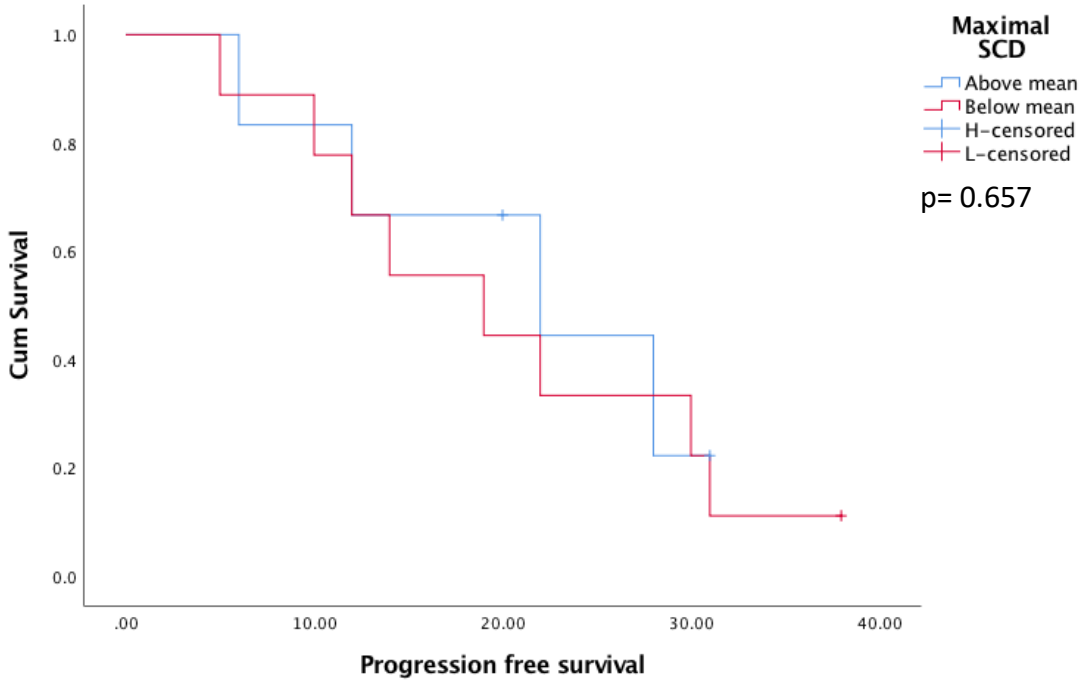


Figure 7.8 C

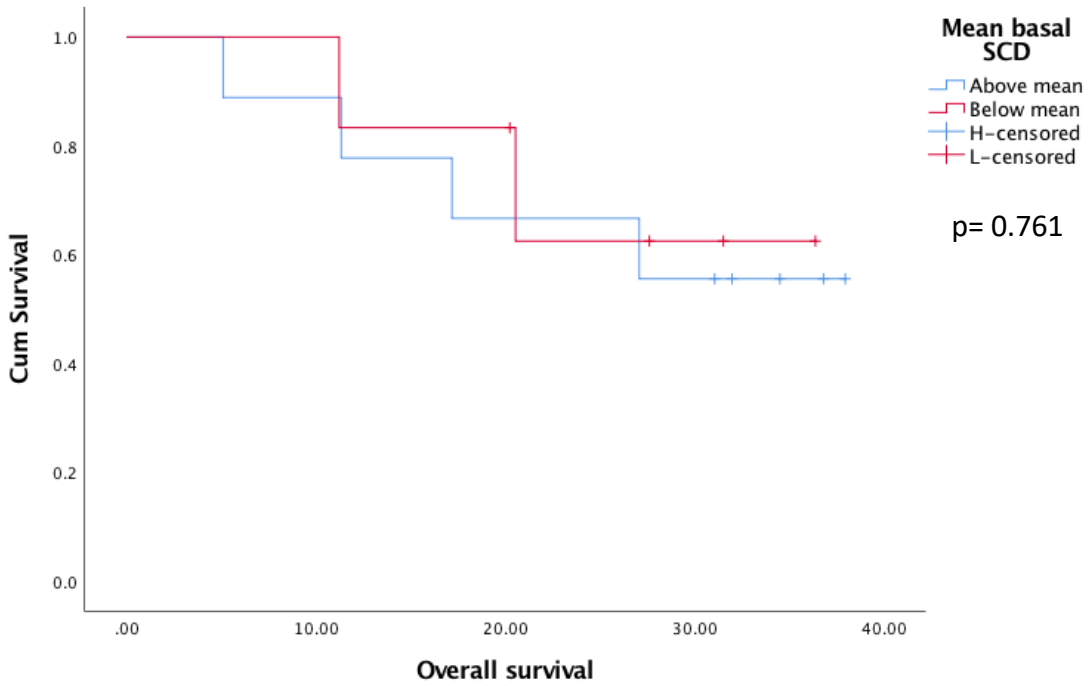
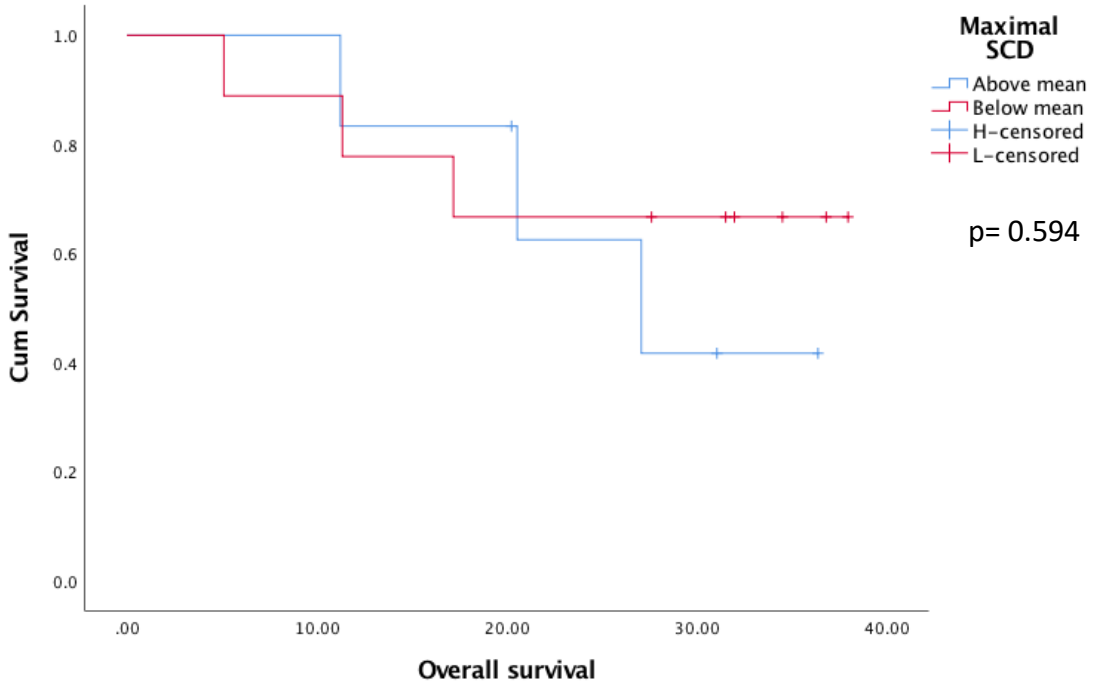


Figure 7.8 D



I also looked at mean basal and maximal SCD at baseline in this subgroup of women who received Bevacizumab treatment. Those women with higher SCD at baseline showed a trend to worse OS but this was not significant (27 vs 29 months p=0.200) (Figure 7.9 A, B). In both these analyses when the means were dichotomised the same results were seen hence both mean basal and maximal data was combined on one figure.

Figure 7.9: A. Overall survival according to values above and below the mean in baseline mean basal and maximal SCD. B. Progression survival according to values above and below the mean in baseline mean basal and maximal SCD. All SCD measurements were in vessels/mm^2.

Figure 7.9 A

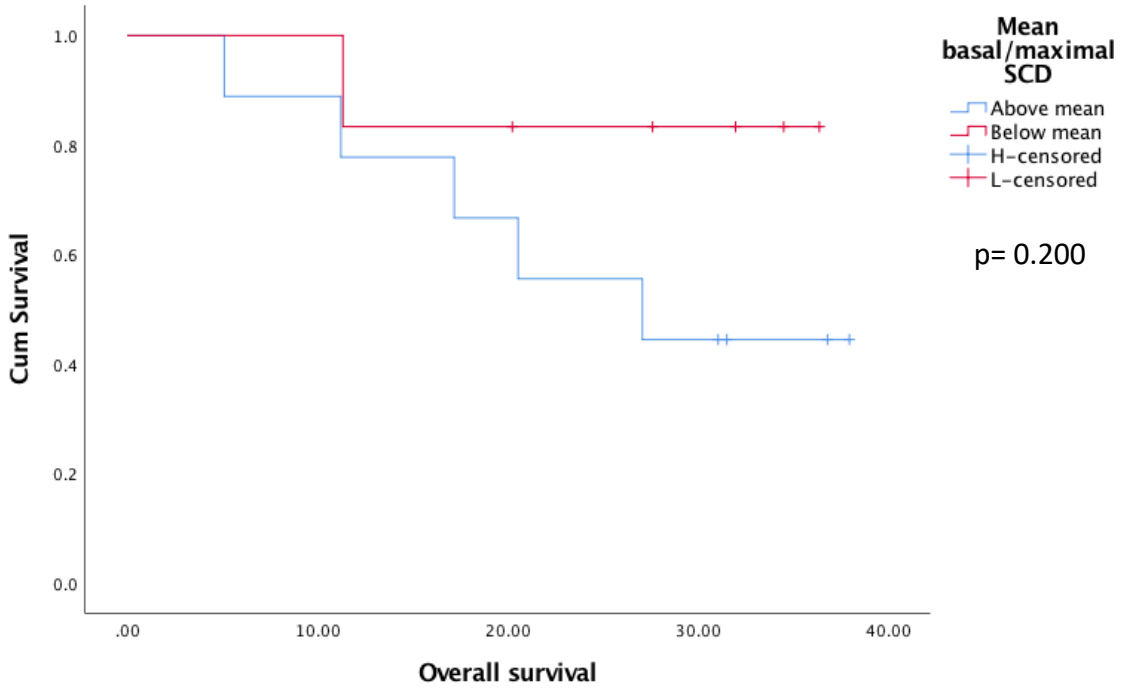
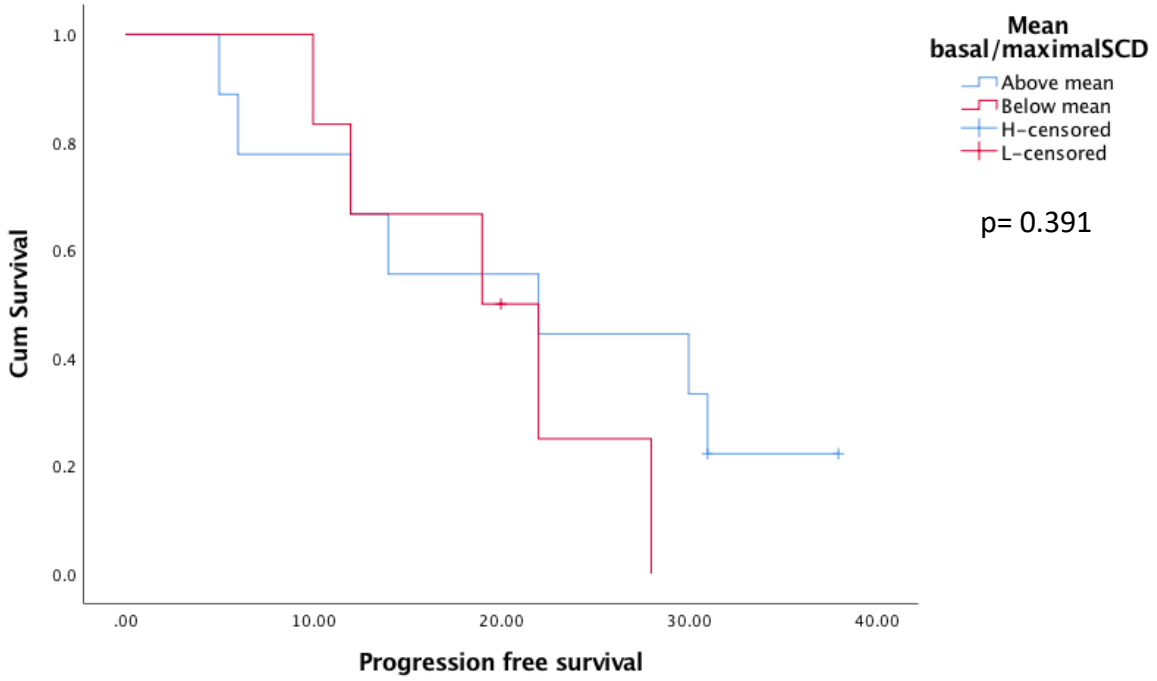


Figure 7.9 B


Mean
basal/maximalSCD
Above mean
Below mean
H-censored
L-censored
p= 0.391
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
.00
10.00
20.00
30.00
40.00
Progression free survival

7.5 Discussion

In this chapter I have investigated a small subgroup of the study population to assess the effect of Bevacizumab on SCD and ascertain whether these changes are related to clinical outcomes. I have been able to demonstrate capillary rarefaction with Bevacizumab treatment and a direct association with the development of hypertension. I found no difference in the capillary rarefaction according to response to Bevacizumab treatment. There was a trend for improved overall survival in those patients who had a greater drop in SCD but this was not significant.

In this subgroup, both functional and structural rarefaction occurred which corresponds with a reduction in the number of capillaries as well as non-perfusion of the capillary networks. This effect on skin capillary density may be explained by VEGF inhibition. Reduced endothelial cell survival secondary to dysfunction, thrombosis and apoptosis with subsequent destruction of micro vessels have been postulated as mechanisms.[265, 266] Additionally, capillary non perfusion may be due to reduced vasodilation due to impaired nitric oxide synthesis as a result of VEGF blockade.[251] These processes are likely to cause increased vascular tone and impaired vasodilation which could lead to hypertension.[252, 265]

A common feature in essential hypertension is that of microvascular rarefaction characterised by reduced capillary density.[242] Similar to that described in this data, this structural decline in capillary density is seen in the skin and contributes to increased systemic vascular resistance and hypertension.[242-244] I did not demonstrate a significant association with blood pressure and capillary rarefaction in those treated with cytotoxic therapy alone which may

suggest that the development of hypertension may also be a result of capillary rarefaction in these patients.

Hypertension is a recognised side effect of Bevacizumab treatment and the prevalence of grade 2-3 hypertension has been reported to be as high as 22%.[153, 249] In some cancer sites such as breast and colorectal cancer, hypertension may represent a biomarker of tumour response to anti angiogenic therapy[265] and has been shown to be linked to improved overall survival.[232, 233]

I have not been able to demonstrate SCD rarefaction in Bevacizumab treatment with improved OS or PFS. However, the association between reduction in SCD and rise in blood pressure means we may be able to predict and manage hypertension in a timely fashion by measuring SCD in clinical practice to prevent treatment delays.

7.6. Conclusion

Both functional and structural capillary rarefaction occurred in patients with Bevacizumab treatment and this appears to be of a greater magnitude than that seen in patients on cytotoxic chemotherapy alone. There is a strong association with capillary rarefaction and hypertension in patients treated with Bevacizumab, the mechanism of which is likely to be multifactorial. Although there is no significant association of capillary rarefaction in these patients and cancer outcomes, this preliminary data shows a promising trends that require further validation.

Chapter 8: General discussion

I conducted a longitudinal cohort study to investigate skin capillary density in ovarian cancer. In this study I recruited 50 women with high grade serous ovarian cancer and studied them at 5 time points starting at diagnosis. I studied the longitudinal effects of cancer treatment on skin capillary density and correlations with serum and tumour angiogenic factors. I have explored the association of skin capillary with cancer outcomes and surgical resection and morbidity in ovarian cancer patients.

I made a number of novel findings and in this chapter, I aim to reflect on the main findings and discuss them in the context of the current literature and my original hypotheses. I will consider the limitations of my study and the future implications and further study in skin capillary density in ovarian cancer which is a novel and promising area of research.

8.1 Summary of main findings and implications in research

8.1.1 Capillary rarefaction occurred during treatment for ovarian cancer and VEGF level reduced during treatment

Capillary rarefaction occurred in all women irrespective of treatment group. The largest drop in SCD was between baseline and visit 2, after 3 cycles of chemotherapy with or without Bevacizumab. VEGF and Ang1 also demonstrated a reduction during treatment.

The overall trend of decrease in VEGF seen in my study is supported by other studies in other cancer sites in the literature where serum VEGF fell after resection of tumours and completion of chemotherapy.[214, 261, 262] VEGF levels did rise transiently after surgery in the immediate post-operative period. This may be reflection of increased angiogenesis for healing and hypoxia mediated stimulation of VEGF.[259]

Ang 1 also fell in patients during treatment. Raised Ang 1 levels are found in ovarian cancer patients compared to controls.[97, 139] Pre-treatment high Ang 1 have been shown to be associated with improved PFS for patients treated with Bevacizumab.[217, 267] Additional attempts to assess molecular biomarkers for response to tyrosine kinase inhibitor Sunitinib, decreasing levels of Ang 1 were associated with improved PFS.[140] Although I was not able to demonstrate an increased magnitude of fall in levels in patients on Bevacizumab this is the first study to demonstrate the dynamic change of levels during treatment which may be a result of reduced tumour bulk and angiogenesis.

A new addition to the literature from this study is the parallel fall in skin capillary density that occurred alongside a reduction in VEGF.

Skin capillary density is a measure of microvascular networks and the dynamic change in my cohort is a promising finding that can provide an indication of angiogenic activity. Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis and directly induces endothelial cell proliferation.[66] It is logical therefore that a fall in VEGF and Ang 1 corresponds with a fall in SCD as they act synergistically to stimulate angiogenesis and Ang 1 along with VEGF have been shown to increase capillary density in animal studies.[268]

It is likely that there may also be a direct impact of cytotoxic chemotherapy on angiogenesis and skin capillary density as well as the effect of the cancer itself. This is supported by my finding that capillary rarefaction occurred during treatment with chemotherapy only.

To my knowledge, capillary rarefaction in patients on cytotoxic chemotherapy has not been demonstrated before in the literature. The capillary rarefaction in all patients was both structural and functional.

Taxanes target microtubules disrupting mitosis and normal cell division leading to apoptosis.[269] Vascular dysfunction from cytotoxic drugs is multifactorial and chemotherapy agents as well as the cancer itself are likely to have an impact. Decreased endothelial nitric oxide bioavailability after cytotoxic treatment causes reduced vasodilation and perfusion. Oxidative stress and pro inflammatory cytokines can lead to platelet activation, thrombosis and capillary regression.[263] This may suggest that capillary density is impacted directly by taxane chemotherapy.

8.1.2 Capillary rarefaction occurred during anti angiogenic treatment and was correlated with a rise in blood pressure

Those women who had bevacizumab had a greater fall in skin capillary density from start to end of treatment compared to those on chemotherapy alone. Both functional and structural rarefaction occurred which corresponds with a reduction in the number of capillaries as well as non-perfusion of the capillary networks.

This effect on skin capillary density can be explained by reduction in VEGF due to reduced tumour activity as alluded to above. However further reduction in VEGF by the direct inhibition of Bevacizumab may have additive effect on capillary rarefaction.

The effects on capillaries secondary to anti angiogenic treatments may be secondary to the neutralisation of VGEF effects on the vessel wall and endothelial cells. Reduced endothelial cell survival secondary to dysfunction, thrombosis and apoptosis with subsequent destruction of micro vessels have been hypothesised as mechanisms of reduction in skin capillary density secondary to VEGF blockade.[265, 266] Additionally, capillary non perfusion may be due to reduced vasodilation due to impaired nitric oxide synthesis.[252, 270]

These processes are likely to cause increased vascular tone and impaired vasodilation which may explain to correlation between Bevacizumab and hypertension.[252, 265]

There is supporting evidence of reduction in SCD due to VEGF blockade which has also been demonstrated in patients who received sunitinib for metastatic renal cell cancer.[229, 234] Hypertension was correlated with this, and regrowth of the microcapillary network occurred after treatment was discontinued.[234] It is likely given this evidence that capillary rarefaction does play a role in VEGF inhibitor-induced hypertension. However, it may not be the sole factor.

The mechanism for Bevacizumab induced hypertension is debated. Veronese *et al*. refuted the theory it was related to adrenergic or renovascular cause and found no difference in humoral factors during treatment with anti-angiogenic therapy despite a rise in blood

pressure being commonplace.[270] Mourad *et al*. reported a decrease in endothelium dependant vasodilation due to impaired nitric oxide synthesis as a result of VEGF blockade in Bevacizumab therapy[251] which resulted in a reduction in functional and structural capillary density.

A common feature in essential hypertension is that of microvascular rarefaction characterised by reduced capillary density.[242] A structural decline in capillary density is seen in skin beds in patients with essential hypertension which contributes to increased systemic vascular resistance. This rarefaction appears to precede the development of hypertension.[243, 244]

Vascular rarefaction as a consequence of VEGF inhibition is likely to be multifactorial. Angiogenic factors such as VEGF stimulate the growth of new capillaries which is expected to reduce vascular resistance.[251] In mouse models vessel narrowing and cessation of blood flow was an initial response to anti VGEF treatment followed by regression mediated by endothelial cell apoptosis.[257]

The concurrent rise in blood pressure and capillary rarefaction seen in patients on Bevacizumab has not been demonstrated in ovarian cancer before. This association was not seen in those who had a capillary rarefaction during cytotoxic treatment alone. This interesting paradox may hint that capillary rarefaction in those patients on anti-angiogenic therapy is a result of hypertension rather than the cause. However, the magnitude of rarefaction in those on Bevacizumab was considerably greater which may be the explanation for hypertension in this group alone if it is indeed mediated by increased vascular resistance.

Platinum based chemotherapy such as cisplatin is associated with hypertension and endothelial cell dysfunction, platelet activation and reduced NO availability are mechanisms that are implicated in the aetiology.[263] Paclitaxel halts endothelial cell proliferation by reducing smooth muscle migration.[271, 272] When Taxanes are combined with angiogenic inhibitors at other cancer sites (breast and lung) the hypertensive effects seem to be heightened.[238, 273]

8.1.3 Skin capillary density at baseline is strongly associated with outcome of debulking surgery

I have found a significant association between skin capillary density at baseline and surgical debulking outcome. For every unit increase in mean basal and maximal SCD the chance of optimal debulking increased by 19 and 22% respectively. Maximal SCD of over 66 vessels/mm^2 and mean basal over 61 vessels/mm^2 at baseline predicted the probability of achieving optimal surgery in more than 80% of patients. Although in my cohort 78% of those who underwent debulking surgery had optimal resection, reports in the literature are variable ranging between 50-80%. The higher number reported are in studies of ultra-radical surgery where morbidity is higher.[253, 274-278]

Various models have been constructed to aid accurate prediction for surgical resection using a combination of imaging techniques, clinical features such as albumin and presence of ascites, laparoscopy, and biological markers.[279] However, no reliable and reproducible method has yet been established. Radiological assessment is heavily relied upon to aid decision making and many patients have suboptimal outcomes. It is clear that suboptimal

surgery does not improve survival but increases morbidity therefore appropriate patient selection for surgery is paramount.

I hypothesise that high skin capillary density at baseline may result in increased tissue perfusion and thus increase delivery and efficacy of chemotherapy and bevacizumab treatment. This is turn is likely to have a favourable outcome of reducing tumour bulk thus increasing the likelihood of complete resection at surgery.

Skin capillary density in isolation or in combination with other factors may predict the success of surgical resection. I have demonstrated that skin capillary density can predict surgical resection with high sensitivity and specificity. This would not only guide appropriate counselling for patients, but may prevent excess morbidity by avoiding suboptimal outcomes.

8.1.4. Skin capillary density at baseline does not significantly predict progression free or overall survival

I found no association between baseline SCD and cancer survival outcomes. As regards change in capillary density before and after treatment, those patients who had a greater reduction in maximal (structural) capillary density had an improved progression free survival. There was a trend to reduced overall survival but this was not significant. For those patients who received Bevacizumab I was unable to demonstrate any association to survival outcomes.

We have discussed earlier that hypertension may represent a clinical biomarker of tumour response[231] and studies in patients with renal cell carcinoma that have demonstrated a link between treatment induced hypertension and improved PFS after treatment with antiangiogenic inhibitors including Bevacizumab and Sunitinib.[226-230] If capillary rarefaction precedes hypertension then it is logical that a greater reduction in SCD may predict improved outcomes.

Demonstration of a significant association between SCD and recurrence gives promise of SCD as a biomarker in ovarian cancer treatment. The relationship between structural capillary rarefaction and reduced recurrence is likely to be multifactorial as previously discussed. However, if this is a reflection of reduced angiogenesis as a result of effective treatment, it opens the possibility that SCD may be an easily measured surrogate of this.

8.1.5. Skin capillary density is higher in patients with cancer compared to healthy controls

Although there is variation in values of skin capillary density in the normal population, I have demonstrated a clear increase in both maximal and mean basal SCD in those with ovarian cancer compared to healthy controls.

Currently a combination of factors such as CA 125, radiological and clinical assessment are used to determine the presence of ovarian cancer.[31, 32] There is no reliable screening tool for ovarian cancer[33] and the biological indolent nature means most cases present with advanced disease. Serum VEGF levels are increased in those with malignant compared to benign ovarian tumours[95-97] secondary to increased angiogenesis and tumour derived VEGF. We

have previously discussed the association between VEGF and SCD which may also explain the finding in this group.

8.1.6. Capillary rarefaction does not correlate with tumour vasculature and proliferative markers. Proliferative marker Ki67 is associated with chemotherapy response

This is the first study to investigate the longitudinal changes in tumour vasculature and proliferative markers after chemotherapy in ovarian cancer. I have found a reduction if Ki67 and PDGFR after treatment with chemotherapy. High Ki67 at diagnosis was associated with an improved radiological response to chemotherapy and there was a trend of rise in Ki67 after treatment and reduced likelihood of a good CRS.

This does correlate with evidence in the literature that high Ki67 levels at biopsy are associated with improved response to chemotherapy.[198] Histological assessment of response to chemotherapy as calculated by the chemotherapy response score (CRS) is based on the presence of viable tumour alongside regression associated inflammatory changes. A rise in Ki67 after treatment was associated with reduced likelihood of a good CRS. Although this was close to significance it does go further to strengthen the association between cell proliferation and chemotherapy efficacy.

CD31 did reduce in those who had Bevacizumab alongside chemotherapy compared to those who had chemotherapy alone. I was not able to demonstrate any impact on OS or PFS or any correlation with tumour vascular markers and SCD.

CD31 has been associated with worse OS in ovarian cancer patients[185] however there is heterogeneity in methods for calculating microvessel density (MVD) which means results from multiple studies are difficult to correlate. MVD in cancer models has been shown to decrease after anti angiogenic therapy due to apoptosis of tumour endothelial cells[69] and CD31 did fall more significantly in the subgroup treated with Bevacizumab in this study compared to those treated with chemotherapy alone.

PDGFR also fell during treatment. PDGFR is a marker of pericytes which are required for stabilisation of new vessels and are a maker of active angiogenesis.[192, 193] The dynamic change in PDGR may be more representative of the impact of treatment on angiogenic activity in the tumour.

I was not able to demonstrate a correlation between SCD and CD31 or PDGFR. CD31 reflects the microvascular environment in the tumour whereas SCD is more representative of the wider capillary network under influence of other factors. In comparison to the values of CD31 in other studies, my values are low. This may be because of the method used to measure CD31. Nevertheless, I have demonstrated that SCD is dynamic and changes over short time periods. Inconsistencies with the values of MVD in studies may be that it is not a measure of active tumour angiogenesis.

8.1.7. Skin capillary density as a surrogate marker of angiogenesis

It can thus be argued quite confidently that SCD is a marker of active angiogenesis. Bevacizumab works by inhibiting VEGF and thus reducing angiogenesis. As we have seen SCD

falls as a result of bevacizumab's effect on microvessels.[251] Skin capillary density as a marker of angiogenesis is further supported by the concept of capillary rarefaction with age when angiogenesis is reduced.[280] As discussed cytotoxic chemotherapy causes vascular dysfunction which leads to hypoperfusion and capillary regression[263] and I have demonstrated capillary rarefaction in patients undergoing this treatment also. Capillaroscopy allows access to capillary function and angiogenesis and may allow clinicians to monitor the effects of cancer treatment by assessing vascular reactivity.

8.2 Strengths and limitations

8.2.1. Skin capillary density technique and confounding factors

I made attempts to validate my values by measuring SCD in a group of age matched controls and those with benign disease. I also developed a learning curve to improve my skill in obtaining a good image. Initial images were not as good quality as I was learning the technique, so I ensured I practised on colleagues before recruiting patients.

Additionally, I allowed time for each visit with patients to ensure the environment and conditions were suitable for reliable and reproducible measurements. There is however a degree of intra observer and interobserver variability, but the above measures were taken to reduce this as much as possible. I recorded videos to allow time to count capillaries and did this after each visit. I did have access to an automated counter but found this was not reliable.

A limitation is this method of skin capillary estimation is the difficulty in highly pigmented skin. Melanin absorbs light strongly in the visible spectrum which makes assessing SCD in this population difficult. This has limitations for applicability to the general population and recruitment of patients to studies. Fluorescence video microscopy could be an alternative method for these patients.

I was unable to demonstrate that SCD was predictive of response to Bevacizumab or survival. This may be due to the small numbers and lack of power in the study. Additionally, as the data matures this may be more apparent. PFS and OS in my cohort was shorter than those reported in recent trials with the addition of Bevacizumab.[35, 153] This may be because women had advanced stage 3c or 4 disease but may have had an impact on my findings.
Nevertheless, this study has established the potential of recruiting patients alongside larger multicentre trials as capillaroscopy is non-invasive and inexpensive it is likely to be welcomed by patients.

Ageing is a known factor in capillary rarefaction due to loss of dermal volume.[281] The age range of this cohort was narrow and the majority of patients were over 65. Age was a factor adjusted for in statistical modelling. Additionally, capillary rarefaction is described in hypertension. No patients in the cohort had uncontrolled hypertension. Evidence suggest that those with controlled hypertension have no difference in capillary density compared to normotensive controls.

8.2.2. Study cohort and sample size

My cohort was of patients with advanced ovarian cancer with the majority having stage 3c and 4 disease. These women have poorer outcome and pose a challenge to treatment thus were of most interest. Additionally, the vast majority of patients with ovarian cancer present with advanced disease so it can be argued that my cohort is representative of clinical reality.

A limiting factor was sample size. This was influenced by the time constraints of the recruitment period due to the length of my research post and working alone to recruit patients and collect data. Furthermore, the number of women diagnosed with ovarian cancer varied on a monthly basis. The data collection (n=256 visits) was also time consuming as patient interactions lasted around 60 minutes at each visit. Calculating SCD from videos required additional time. Counting vessels in tissue also required around 15 minutes per slide by two people and ELISA experiments took several weeks to complete. The only way to increase the sample size would have been to prolong my research period or engage additional staff from other sites to recruit and collect data. The sample size may have contributed to the lack of associations drawn between changes in SCD and markers and outcomes. Additionally, allowing the data to mature over time and then further reanalysis may reveal more information.

I was only able to recruit 6 women who were enrolled in the ICON8b trial which limited the amount of data available in women treated with Bevacizumab. However, during the study period, a total of 7 women were enrolled into the trial by the department. The remaining patient that was not included in my study was not approached for recruitment due to

significant emotional distress and I felt it unethical to consent. As the visits are carefully timed with treatment, I was unable to re discuss at a later date. The low number in this subgroup is a reflection of the cancer centre recruitment and suitability of patients for the trial.

I did not miss a visit for either blood collection or SCD measurement. This required scrupulous organisation and flexibility to ensure there were no missing data points. I was unable to follow women up after they stopped Bevacizumab as treatment was continued as maintenance for 15 months by which point I had completed my research time.

8.2.3. VEGF 165b and serum samples

The inability to produce data for VEGF165b was disappointing. Multiple attempts were made to improve the technique and despite this, accurate curves were only produced for some plates. Even in those cases the values were erratic or undetectable. Nonetheless this is an interesting finding. Since I carried out the ELISAs for this research a recent study in breast cancer patients found similar results in that 34% of patients had unmeasurable levels of VEGF165b. Levels did rise after treatment with chemotherapy, but levels were heterogenous with large interindividual variability.[214]

VEGF165b has been found to be down regulated in cancer tissue in favour of the switch to the proangiogenic isoform which is vital for tumour development.[214, 282, 283] To my knowledge there have only been two studies measuring VEGF165b in blood samples in cancer.[208, 214] The second study found the ratio of VEGFxxxb to VEGF could be helpful in selecting patients for

Bevacizumab due to a correlation between PFS in those with low levels of anti-angiogenic isoform.[208]

The fact that levels are undetectable is in keeping with down regulation of VEGF165b at tissue level and are consequently too so low to be detectable by this particular ELISA kit.

Another reason that may explain the lack of measurable values is the use of serum. The aforementioned study found levels were higher and more often detectable in plasma samples in healthy controls. All my samples were serum as is much of the data in VEGF and cancer. I recognise that levels may be elevated due to the effects of thrombocyte VEGF production. However much of my work has been investigating trends over treatment and the consistent use of serum as well as standardised preparation of samples means that data is still interpretable and meaningful.

I stored serum in 500 micro litre aliquots. On reflection the ELISAs required smaller volumes which meant time was spent aliquoting samples at a later date. My limited funding and number of samples meant I was only able to measure a small number of cytokines. Luminex micro array assays would have allowed a wider range of cytokines to be measured using smaller volumes of sample.

8.3. Future work and implications

The potential for further research in this area is limitless but I will focus on suggestions that I would wish to pursue.

8.3.1. Skin capillary density

I have demonstrated in this proof of concept study that skin capillary density shows promise as a marker of angiogenesis in ovarian cancer and potential biomarker. In order to develop a biomarker, it first has to demonstrate dynamic change in response to an intervention and I have been able to demonstrate this with SCD and cytotoxic treatment and anti-angiogenic therapy.

Skin capillary density now needs to be studied on a wider scale and measured alongside interventions either in current trials or in a new study. In the first instance accurate sample size calculation to inform a repeated larger scale longitudinal study to replicate my work. The study could also be conducted as a cross sectional evaluation to provide greater numbers and require multi centre collaboration.

A longitudinal study mapping skin capillary changes during maintenance treatment with Bevacizumab and after completion of treatment would provide additional information regarding capillary rarefaction and anti-angiogenic treatment.

I did not find any difference in SCD between those with benign tumours and cancer but showed a significant difference between healthy controls and cancer. An informative study would be one that measures SCD in benign disease on a larger scale as the number in my cohort were small.

The development of a validated automated capillary counter would improve reliability and efficiency for capillaroscopy. This is important is capillaroscopy is to be considered on a wider scale as part of a large trial or integrated into clinical practice.

8.3.2. Angiogenic markers

VEGF165b in cancer is a promising area for future research. Collaboration is required among research groups to validate a reliable method and ELISA technique for detection in a larger cohort to determine whether VEGF165b has a role in stratifying patients for anti-angiogenic therapy.

I was unable to demonstrate any change in soluble endoglin with treatment. Soluble endoglin is implicated in vascular resistance and hypertension but I was not able to demonstrate any correlation with serum levels and capillary rarefaction. More recently however endoglin has been thought to be more reliable in identifying vessels involved in tumour angiogenesis compared to CD31.[258] Some studies have demonstrated an association between increased MVD assessed by CD105 and worse outcome, advanced stage and suboptimal primary cytoreductive surgery in ovarian cancer.[175, 179, 180, 284] An additional area of research would be to measure endoglin in ovarian cancer tissue and correlate with SCD.

Serum biomarkers of Bevacizumab are still allusive. Proteomic analysis of paired serum samples from women in my study who received chemotherapy or chemotherapy and Bevacizumab would allow identification of potential pathways of further interest which may allude to potential new biomarkers of bevacizumab. I have begun work on this and conducted proteomics on matched samples and am embarking on data analysis.

8.3.3. Clinical applications

I have seen that Bevacizumab treatment was withheld in some cases which caused a delay in treatment and undoubted angst for the patient. The link between hypertension and capillary rarefaction is clear and I have demonstrated this in patients taking Bevacizumab. It could be feasible that SCD is used as a biomarker of hypertension secondary to Bevacizumab and predict the onset of hypertension to allow timely instigation of treatment thus ensuring patients do not suffer both the physical and mental effects of hypertension and treatment delay. Capillaroscopy could be easily incorporated into chemotherapy pre assessment clinics prior to delivery of treatment.

8.4 General conclusion

Skin capillary density has not been measured in patients with ovarian cancer before. Neither have serum or tissue markers been monitored during cytotoxic treatment. I have demonstrated a dynamic change in SCD during treatment. The concept of capillary rarefaction in the presence of anti-angiogenic therapies is plausible. However, reduction in SCD with cytotoxic treatments such as chemotherapy is less explored and has not been demonstrated before. Additionally, skin capillary density is able to predict surgical resection outcomes in women undergoing interval debulking surgery for advanced disease.

Although this data requires validation in larger studies, it can be postulated that skin capillary density could be useful as a biomarker of response to treatment and cancer outcomes and act as a surrogate marker of angiogenesis in cancer. It is a reproducible, cheap and non-invasive investigation that is acceptable to patients and shows promise in helping to guide treatment and prognostic information in the era of personalised medicine.

I believe I have demonstrated proof of the need for ongoing work to enable skin capillary measurement to be applicable clinically to improve the care of women with ovarian cancer.

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Appendix 1. Grant approval

Above +Beyond
For Patients. For Health. For Bristol.

RESEARCH FUNDING COMMITTEE

Research and Innovation, Level 3
UH Bristol Education Centre, Upper Maudlin Street, Bristol BS2 8AE
Chair: Professor David Wynick, Tel: 0117 342 0233 email: d.wynick@bristol.ac.uk

8 March 2018

Re: Project no. 2017-Aut-02: Ovarian cancer and capillary density of the skin (OCCLUDES study).

Dear Dr Cass,

I am pleased to confirm the following minor revisions to your Above and Beyond grant:

Awarded	**£14,415 (breakdown below)**
Start and end dates:	**1 April 2018 – 31 March 2019**
Start deadline:	**14/06/2018**
Cost centre:	**58802G2194**

Instructions for accessing funds and conditions of award are found below.

Yours sincerely

[redacted]

Professor David Wynick
Chair, Research Funding Committee

cc: Ankaret Fillipich, Above and Beyond Charities
Kirsty Cepek, Assistant Director of Finance, Financial Management
Dan Hancock, University of Bristol Finance Office
Professor A Lopez Bernal, Bristol Medical School

Appendix 2. Ethics and Research and Development (R&D) Approval

NHS
Health Research Authority

East Midlands - Nottingham 2 Research Ethics Committee
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839525

26 October 2015

Mr. Vivek Vasudev Nama
Consultant Gynaecological Oncology
University of Bristol
St. Michael's Hospital, Southwell Street
Bristol
BS2 8EG

Dear Mr. Nama

Study title:	**The assessment of skin capillary density before, during and after treatment for epithelial ovarian cancer and its correlation to treatment outcome.**
REC reference:	**15/EM/0489**
Protocol number:	**Protocol v.2**
IRAS project ID:	**60542**

The Proportionate Review Sub-committee of the East Midlands - Nottingham 2 Research Ethics Committee reviewed the above application on 26 October 2015.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Assistant, Joanne Unsworth, NRESCommittee.EastMidlands-Nottingham2@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

1. Use the IRAS short title for the Participant Information Sheet, Consent Form and GP letter.
2. Add the names of the research team to the Participant Information Sheet.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Summary of discussion at the meeting

The PR Sub-Committee confirmed the study raised no material ethical issues under the following headings: Social or scientific value; scientific design and conduct of the study, recruitment arrangements and access to health information, and fair participant selection, favourable risk benefit ratio; anticipated benefit/risks for research participants (present and future), care and protection of research participants; respect for potential and enrolled participants' welfare and dignity, suitability of the applicant and supporting staff, independent review, other general comments and suitability of research summary.

Ethical issues raised, noted and resolved in discussion:

- **Informed consent process and the adequacy and completeness of participant information**

  The Sub-Committee agreed the short title from IRAS should be used in the Participant Information Sheet and Consent Form.

  They also stated the research team should be named on the Participant Information Sheet.

- **Suitability of supporting information**

  The Sub-Committee agreed the short title from IRAS should be used for the GP letter.

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Poster]	1.2	12 October 2015
Covering letter on headed paper [Cover Letter]		12 October 2015
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance letter]		12 October 2015
GP/consultant information sheets or letters [GP letter]	1.1	12 October 2015
IRAS Checklist XML [Checklist_16102015]		16 October 2015
Letter from funder [David Telling letter]		12 October 2015
Letter from sponsor [Insurance letter]		12 October 2015
Letters of invitation to participant [Info letter]	1.2	12 October 2015
Participant consent form [Consent form]	1.3	12 October 2015
Participant information sheet (PIS) [Info sheet]	1.6	12 October 2015
REC Application Form [REC_Form_16102015]		16 October 2015
Research protocol or project proposal [Protocol]	3	12 October 2015
Summary CV for Chief Investigator (CI) [CV - Nama]		12 October 2015
Summary CV for student [CV - Platt]		12 October 2015

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at
http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

15/EM/0489	**Please quote this number on all correspondence**

Yours sincerely

pp. [redacted]

Dr Ian Ross
Chair

Email: NRESCommittee.EastMidlands-Nottingham2@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Dr Birgit Whitman*
Dr Diana Benton, University Hospital Bristol NHS Trust

NHS
Health Research Authority

East Midlands - Nottingham 2 Research Ethics Committee
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0207 1048154

15 February 2016

Mr. Vivek Vasudev Nama
Consultant Gynaecological Oncology
University of Bristol
St. Michael's Hospital, Southwell Street
Bristol
BS2 8EG

Dear Mr. Nama

Study title:	**The assessment of skin capillary density before, during and after treatment for epithelial ovarian cancer and its correlation to treatment outcome.**
REC reference:	**15/EM/0489**
Protocol number:	**Protocol v.2**
IRAS project ID:	**60542**

Thank you for your response of 8th February 2016. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 26 October 2015

Documents received

The documents received were as follows:

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP letter]	1.3	08 February 2016
Participant consent form [Consent form]	1.4	03 February 2016
Participant information sheet (PIS) [Info sheet]	1.7	03 February 2016

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Poster]	1.2	12 October 2015

Covering letter on headed paper [Cover Letter]		12 October 2015
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Insurance letter]		12 October 2015
GP/consultant information sheets or letters [GP letter]	1.1	12 October 2015
GP/consultant information sheets or letters [GP letter]	1.3	08 February 2016
IRAS Checklist XML [Checklist_16102015]		16 October 2015
Letter from funder [David Telling letter]		12 October 2015
Letter from sponsor [Insurance letter]		12 October 2015
Letters of invitation to participant [Info letter]	1.2	12 October 2015
Participant consent form [Consent form]	1.3	12 October 2015
Participant consent form [Consent form]	1.4	03 February 2016
Participant information sheet (PIS) [Info sheet]	1.6	12 October 2015
Participant information sheet (PIS) [Info sheet]	1.7	03 February 2016
REC Application Form [REC_Form_16102015]		16 October 2015
Research protocol or project proposal [Protocol]	3	12 October 2015
Summary CV for Chief Investigator (CI) [CV - Nama]		12 October 2015
Summary CV for student [CV - Platt]		12 October 2015

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

15/EM/0489 **Please quote this number on all correspondence**

Yours sincerely

[redacted]

Carolyn Halliwell
REC Manager

E-mail: NRESCommittee.EastMidlands-Nottingham2@nhs.net

Copy to: *Dr Birgit Whitman*
Dr Diana Benton, University Hospital Bristol NHS Trust

NHS
Health Research Authority

East Midlands - Nottingham 2 Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

07 August 2017

Mr. Vivek Vasudev Nama
Consultant Gynaecological Oncology
University of Bristol
St. Michael's Hospital, Southwell Street
Bristol
BS2 8EG

Dear Mr. Nama

Study title:	**The assessment of skin capillary density before, during and after treatment for epithelial ovarian cancer and its correlation to treatment outcome.**
REC reference:	**15/EM/0489**
Protocol number:	**Protocol v.2**
Amendment number:	**Modified Amendment 1**
Amendment date:	**22 May 2017**
IRAS project ID:	**60542**

Thank you for submitting the above amendment, which was received on 15 June 2017. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 12 May 2017 refers).

The modified amendment has been considered on behalf of the Committee by the Alternate Vice-Chair.

Ethical opinion

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved are:

Document	*Version*	*Date*
Covering letter on headed paper		10 July 2017
GP/consultant information sheets or letters	1.4	19 December 2016
Notice of Modified Amendment	Modified Amendment 1	22 May 2017
Participant consent form	1.5	19 December 2016
Participant consent form	1.6	19 December 2016
Participant information sheet (PIS)	1.8	19 December 2016

NHS
Health Research Authority

Participant information sheet (PIS)	2.0	19 December 2016
Research protocol or project proposal	4	24 May 2017

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

15/EM/0489:	**Please quote this number on all correspondence**

Yours sincerely

PP [redacted]

Mr Jon Merrills
Chair

E-mail: NRESCommittee.EastMidlands-Nottingham2@nhs.net

Copy to: *Diana Benton, University Hospitals Bristol NHS Foundation Trust*
Dr Birgit Whitman
Gemma Cass

RED Research & Enterprise Development

University of BRISTOL

Mr Vivek Nama
University of Bristol
St. Michael's Hospital
Southwell Street
Bristol BS2 8EG

23rd September 2016

Dear Vivek

AGREEMENT BY THE UNIVERSITY OF BRISTOL TO ACT AS RESEARCH SPONSOR.

Project Title: The assessment of skin capillary density before, during and after treatment for ovarian cancer and its correlation to treatment outcome (our ref 2340)
Chief Investigator: Mr Vivek Nama

Study duration	15.02.16 – 14.02.22
Ethics	15/EM/0489; 15.02.16
IRAS ID	60542
Study documentation	Versions of study documents as detailed on NHS REC favourable opinion 15.02.16
Insurance reference	CT1844
Contract reference	n/a
Funder	David Telling
Study sites	UH Bristol

The University of Bristol hereby confirms that it agrees to act as the research sponsor for the above referenced project with you as Chief Investigator.

We would like to remind you of your obligation to comply with all relevant regulatory guidance and legislation.

If you intend to make any changes to the study, including an extension to the end date, you must inform us as Sponsor prior to submission to the Regulatory Authority. Annual Progress Reports are due on the anniversary of when NHS REC favourable opinion was granted, please send us a copy.

The University currently monitors approximately 10% of active studies via a Service Level Agreement with UH Bristol. If your study is selected for monitoring you will be contacted by members of UH Bristol.

May we take this opportunity to wish you every success with your research.

Yours sincerely

[redacted]

Birgit Whitman
Head of Research Governance

OCCLUDES STUDY CONSENT FORM

Ovarian Cancer and CapiLlary DEnsity of Skin: Role of skin capillary density in Epithelial Ovarian Cancer

Please sign initials in box below:

1. I confirm that I have read and understand the information sheet **(version 1.8) dated 19.12.2016** for the above study. I have had the opportunity to ask questions about the study and I understand why the research is being carried out.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. If I withdraw consent, I understand that samples already taken will be kept as part of the study but no further tests will be taken.	☐
3. I agree to allow data to be retained in anonymised form for five years after the completion of this study in line with NHS regulations.	☐
4. I consent to the removal, storage and use of my tissue and blood samples for the above study in line with the Human Tissue Act 2004.	☐
5. I agree that the samples collected during this study can be stored and used for future studies, with ethical approval	☐
6. I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.	☐
7. I give permission to be contacted either by phone, email or by post for research purposes	☐
8. I consent to my GP being informed of my participation in this study	☐
9. I agree to take part in the above study	☐

Name of Patient (Capitals)	**Date**	**Signature**
Researcher	**Date**	**Signature**

OCCLUDES STUDY INFORMATION SHEET

Ovarian Cancer and CapiLlary DEnsity of Skin

Role of skin capillary density in Epithelial Ovarian Cancer

Invitation:

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP to decide whether you would like to take part. Ask us if there is anything that is not clear or if you would like more information. Thank you for reading this.

What is the Purpose of this Study:

Currently ovarian cancer is treated by a mixture of surgery and chemotherapy. The chemotherapy drugs given are the same for all patients. We do not have a way of working out who will benefit from chemotherapy or surgery first, or who may need a different type of chemotherapy. This study may help us to work this out by using some simple new tests.

Why have I been Chosen?

You have been chosen as we you have been diagnosed with ovarian cancer. Please let the doctor know if you suffer from any blood circulation problems, as you might not be suitable for the study.

Do I have to take part?

Taking part in this research is entirely voluntary. It is up to you whether or not to take part. If you do decide to take part you will be asked to sign a consent form. You are free to change your mind and withdraw from the study at any time. If you do withdraw you do not have to give your reasons for doing so and it will not affect the standard of care you receive.

What will happen to me if I take part?

You will be seen on 5 occasions at St. Michael's Hospital when you are attending for routine visits. You will not have to make any extra visits.

The first visit will be before you start treatment either with an operation or chemotherapy. At each visit you will be asked to sit in a chair whilst your forearm and hand will be placed on an armrest. A video will be recorded of the blood vessels on the skin using a microscope and a camera; a painless technique. You will be able to view your own blood vessels on the computer screen if you wish. A baby blood pressure cuff will be wrapped around the bottom

of a finger and then inflated to increase the number of blood vessels that we can see. The video will continue recording for two minutes whilst the cuff is blown up.

This test will be done after the operation, 9 weeks later and at the end of treatment.

At each visit, a blood sample (about 2 tablespoons) will be taken at the same time as your routine blood tests. This blood test will be used only for research , and would not provide information that can be used for your health care.

If you have surgery, the tissue removed will be sent to the pathologist for routine testing. Once the routine lab tests are finished we will request a very small amount of the tissue (that would otherwise have been disposed of) for research.

What do I have to do?

You do not need to make any changes to your lifestyle except to abstain from coffee and smoking for at least 2 hours before you attend for your capillary study.

What will happen to the samples?

After analysis for the study with your consent tissue samples will be stored anonymously in a laboratory and **kept for potential future studies with ethical approval.**

What are the possible Disadvantages and Risks of taking part?

Capillary microscopy is a painless test. You may feel slight tingling in your finger when the blood pressure cuff is blown up and for the 2 minutes that it remains inflated, but this disappears as soon as the cuff is released.

What are the possible Benefits of taking part?

This study is of no direct benefit to you but it may help us understand how different ovarian cancers respond to more specialised chemotherapy. Your medical care would not be adversely affected by not taking part in this study.

What if something goes wrong?

We do not envisage any harm coming to you as a consequence of taking part. If you have any concerns or other questions about this study, or the way it has been carried out, you should contact the Patient Support & Complaints Team, Trust Headquarters, University Hospitals Bristol, Marlborough Street, Bristol, BS1 3NU. *Tel No:* 0117 342 3604 *email:* pals@uhbristol.nhs.uk

Will my participation in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential.

What will happen to the results of the Research Study?

It is anticipated that the results from this research study will be published in a medical journal several months after its completion. The exact journal is not known at this stage, although we will be able to give this information and the actual results at a later date. No participants of this research study will be identified in any report/publication.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people called a Research Ethics committee to protect your safety, rights, well-being and dignity. This study has been reviewed and given a favourable opinion by the NRES Committee.

CONTACT FOR FURTHER INFORMATION:

If you require further information regarding any part of this research study, please feel free to contact **Dr Gemma Cass (Gynaeoncology Research Fellow)** or Dr. Sarah Platt (Subspecialty Registrar) or Carol Shahin, Research coordinator on 0117 3425756

Research team:

Dr Gemma Cass, Gynaeoncology Reseach Fellow

Dr Vivek Nama, Consultant Gynaecologist

Dr Sarah Platt, Subspecialty Fellow, Gynae-Oncology

Mr Amit Patel, Consultant Gynaecologist

Dr Axel Walther, Consultant Oncologist

Dr Jo Bailey, Consultant Gynaecologist

Mr John Murdoch, Consultant Gynaecologist

Many thanks for your interest in the study and taking the time to read this information.

You will be given a copy of this Patient Information Sheet and a signed Consent Form to keep.

University of BRISTOL

University Hospitals Bristol NHS
NHS Foundation Trust

If you have recently been diagnosed with Ovarian Cancer, you may be eligible to help us in the OCCLUDES clinical research study.

OCCLUDES STUDY

Ovarian **C**ancer and **C**api**L**lary **DE**nsity of **S**kin

The aim of the study is to assess skin capillary density before, during and after treatment for ovarian cancer. We can hopefully then identify which patients will benefit most from different types of chemotherapy.

We are recruiting all women who have recently been diagnosed with Ovarian Cancer.

If you are interested, please discuss this with your health care professional in the gynaecology clinic, or ask the receptionist to contact:

Dr Gemma Cass (Clinical research Fellow)

Gemma.Cass@UHBristol.nhs.uk

Appendix 4. Table S4.1. Variability in SCD measurements

Intraobsever					Interobserver				
1	2	Ab diff	SD	% Ab diff	1	2	Ab diff	SD	% Ab diff
56	56	0	0	0	45	50	5	4	11
54	57	3	2	6	65	63	2	1	3
66	68	2	1	3	65	60	5	4	8
50	48	2	1	4	61	66	5	4	8
53	55	2	1	4	62	63	1	1	2
53	52	1	1	2	55	58	3	2	5
69	71	2	1	3	68	70	2	1	3
46	49	3	2	7	67	63	4	3	6
80	82	2	1	3	49	48	1	1	2
67	69	2	1	3	63	60	3	2	5
54	53	1	1	2	59	60	1	1	2
57	60	3	2	5	63	60	3	2	5
54	55	1	1	2	55	56	1	1	2
72	72	0	0	0	78	74	4	3	5
70	75	5	4	7	82	79	3	2	4
65	66	1	1	2	55	50	5	4	9
78	80	2	1	3	59	57	2	1	3
58	58	0	0	0	66	68	2	1	3
75	71	4	3	5	55	60	5	4	9
71	71	0	0	0	48	49	1	1	2
				3					5
				2.2					2.9