VEGF Levels in Women with Gynaecological Cancers

Jeffrey Lee

A thesis submitted for the degree of

Bachelor of Medical Science (Honours)

at the University of Otago, Christchurch,
New Zealand.

November 2010

Abstract

Background: High blood levels of Vascular Endothelial Growth Factor (VEGF) have been reported in patients with gynaecological cancer and have been correlated with advanced disease and poor outcome. However, results are few and varied and most studies used single measurements. There is also debate as to whether serum or plasma measurement reflects circulating VEGF.

Aims: To describe and compare the properties of serum and plasma VEGF in women with ovarian and endometrial cancer. To clarify their relationships with platelets and investigate their correlation with disease activity. In addition we wished to investigate the effect of green tea (which has known angiogenic activities) consumption on blood VEGF.

Methods: VEGF was measured by ELISA method in serum and plasma in women with gynaecological cancers. Serial measurements were taken in women with endometrial or ovarian cancer, and in women without cancer. Blood samples were also obtained in women before and after surgery for endometrial or ovarian cancer. The association of platelets and CRP to blood VEGF was determined using combined data. In addition, the effect of green tea on serum and plasma VEGF was evaluated in women with persistent cancer who were treated with 6 days oral green tea extracts equivalent of 900mg EGCG daily.

Results: The median week to week coefficient of variance (CV) was approximately 10% for serum and 30% for plasma VEGF. There was significant overlap in VEGF concentrations between women with disease and control. Serum VEGF but not plasma correlated with platelets and CRP and there appeared to be a multiplicative relationship

between plasma and serum VEGF. There was no significant difference between blood VEGF before and after surgery. Short term green tea extracts were safe but did not lead to significant changes in blood VEGF.

Conclusion: Blood VEGF measurements are elevated in some women with advanced gynaecological cancers, single measurements appear a reliable indication of the levels in different patients but levels did not appear to be a good indicator of change in tumour load within individual patients. Serum VEGF varied with platelet count and CRP and may be reflection of the systemic response to advanced malignancy. It is not clear to what extent plasma VEGF reflects circulating VEGF. We did not see a reduction in blood VEGF after consumption of green tea. Future studies are needed to assess to what extent plasma VEGF reflect circulating VEGF, and the effect of green tea extracts.

Acknowledgement

First of all, I would like to thank my supervisors Peter Sykes and John Evans for their guidance and patience; it has been a rewarding and satisfying experience. Huge thanks to Dianne Harker, whose vast experience in research and ongoing support have been invaluable in streamlining various parts of the project. Isabela Banea, who is no longer in the department, thank you for all the administrative and moral support. Helen Morrin and the Angiogenesis Research Group, I am grateful to you for advice on specimen processing and the valuable fridge space. The clinical team has been excellent, especially Yvonne Shennan, for teaching me the art of phlebotomy; Bryony Simcock, Mike Laney; Bernie Fitzharris, Michelle Vaughan, and David Gibbs and all the staff at Christchurch Women's Outpatients Department and the Oncology Department for helping me with recruitment. The members of LCPR, James Dann, Gloria Evans, and Kenny Chitcholtan, you guys have been great in providing general advice and excellent nutritional contributions. Barbra Pullar, Frances Okey, and anyone I have missed out from O&G, thank you for making the department such an amazing place to work in. I would also like to acknowledge Judith McKenzie and the Haematology Research Group for our collaboration and Jenny Smith from Bio Concepts for supplying the Green Tea Extracts. Last but not least, I cannot thank my parents enough for their support in my education since day one.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABN	Australian Biospecimen Network
ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AST	Aspartate Transaminase
BIL	Bilirubin
BMI	Body Mass Index
CA125	Cancer Antigen 125
CDHB	Canterbury District Health Board
CI	Confidence Intervals
CTAD	Citrate-Theophylline-Dipyridamole-Adenosine
CRP	C-Reactive Protein
CV	Coefficient of Variation
EC	Epicatechin
ECG	Epicatechin Gallate
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGC	Epigallocatechin
EGCG	Epigallocatechingallate
ELISA	Enzyme-Linked Immunosorbent Assay
ET-1	Endothelin-1
FDA	Food and Drug Administration

FIGO	International Federation of Gynaecologists and Obstetricians
g	9.8 Newtons per kilogram
GGT	Gamma-Glutamyl Transpeptidase
GTE	Green Tea Extracts
HPV	Human Papillomavirus
OR	Odds Ratio
PF4	Platelet Factor 4
PlGF	Placenta Growth Factor
PSA	Prostate-Specific Antigen
r	Correlation Coefficient
RR	Relative Risk
SD	Standard Deviation
SEM	Standard Error of the Mean
TP	Total Protein
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPF	Vascular Permeability Factor

1 Introduction

1.1 Ovarian Cancer

Ovarian cancer accounts for 5% of all female cancers and is the 4th leading cause of cancer mortality in women in New Zealand. In 2005, there were 301 new cases of this disease and 190 deaths [1]. Eighty to 90% of ovarian cancers are epithelial in origin and they occur predominantly in perimenopausal and postmenopausal women [2], with a peak incidence at age 60. Unfortunately, this disease is difficult to detect in early stages due to the absence of symptoms [3] and 70% of women present with cancer that has spread to the peritoneal surfaces, with poor prognosis [3]. Much effort to fight this "silent killer" has been focused on developing population screening. However, there is no evidence that screening is effective in reducing mortality [4]. Treatment consists of cytoreductive surgery followed by combination chemotherapy. While improvement in both modalities over the last two decades saw an increase of five-year-survival from 30% to 50%, long term survival for patients with advanced disease remains only 20-25% [5].

1.2 Endometrial Cancer

Endometrial cancer is the commonest gynaecological malignancy in developed countries [6], including New Zealand. In New Zealand during 2005, 383 women were diagnosed and there were 80 deaths [2]. It is predominantly a cancer of postmenopausal women, with 91% of cases worldwide occurring in women aged 50 and older [6]. Endometrial carcinomas can be classified into two types on the basis of biological and histopathological features. Type I endometrial cancers account for 80% of cases, are usually well-differentiated and endometrioid in histology. This type is associated with long-term unopposed oestrogen stimulation, or other hyperoestrogenic risk factors, such as obesity [7]. The hormonal alteration causes endometrial cell proliferation, inhibits apoptosis, and promotes angiogenesis [8]. Patients with this type of cancer typically exhibit disease at an early stage and have a favourable prognosis [9]. Type II tumours, in contrast are often poorly differentiated, non-endometrioid, not associated with hyperoestrogenic risk factors and are more likely to metastasise and recur [9]. The cornerstone of curative treatment for all endometrial carcinoma is surgery. External pelvic radiotherapy and/or vaginal brachytherapy are used postoperatively for patients with high risk of recurrence and a poor prognosis. In women with advanced disease, chemotherapy is indicated. However, 5-year survival rates for advanced endometrial cancer remain low at 10-40%, despite current therapies [10].

1.3 Angiogenesis in Cancer

Angiogenesis is the formation of new blood vessels from pre-existing vasculature. The close association between tumour growth and increased vascularity was reported more than a century ago by several investigators, including the German pathologist Rudolf Virchow [11]. In the 1920s, a significant advance in angiogenesis research came with the development of a transparent chamber, which allowed microscopic observation of vessel growth in tumours in real time [11, 12]. In a seminal study published in 1945, Algire and colleagues [13] used transparent chambers to quantitatively assess vessel proliferation in a variety of normal and malignant tissues transplanted onto mice, a technique still used today. It was observed that transplanted tumours, in contrast with normal tissues, caused a striking increase in vascularity. Moreover, this vascular growth preceded the phase of rapid tumour growth. This led the authors to conclude that "the rapid growth of tumour explants is dependent on the development of a rich vascular supply" [13]. In 1971, Judah Folkman proposed the innovative idea of targeting angiogenesis as a strategy to treat human cancer [14]. These concepts have led to intense investigations in the field of angiogenesis, which led to important discoveries of underlying molecular mechanisms, key angiogenic cytokines, as well as the development of novel anti-angiogenic therapies. It has been shown that tumours cannot grow beyond $2mm^3$ without establishing a vascular supply [15], limited by the diffusion of nutrients and wastes. Furthermore, angiogenesis can facilitate tumour metastasis by providing a route for tumour cells to be shed into the circulation, and increased vascularity of primary tumours has been correlated with increased likelihood of mestatasis and decreased survival (reviewed in [16]). The angiogenic process is a highly complex, dynamic process and is regulated by a number of pro- and anti-angiogenic molecules. A current model of angiogenesis in tumours suggests that this process involves recruitment of sprouting vessels from pre-existing

vasculature and incorporation of endothelial progenitor cells [17]. One of the major pathways mediating this process involves the vascular endothelial growth factor (VEGF) family of protein and receptors.

1.4 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor is a homodimeric, 34-45 kDA glycoprotein and the VEGF family comprises of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) [17]. VEGF-A (commonly referred to as VEGF) has isoforms of 121, 165, 189, and 206 amino acids, due to alternative splicing of the *VEGF-A* gene [18]. $VEGF_{165}$ is the predominant isoform, exists in both soluble and ECM-bound, and is commonly overexpressed in a variety of human tumours [17]. The receptors of VEGF are tyrosine kinase receptors, located primarily on endothelial cells and include VEGFR-1 and VEGFR-2. More recently neuropilin-1 and -2 have been identified as non-tyrosine kinase co-receptors [18]. VEGF is a pleiotrophic growth factor, results in endothelial cell survival, mitogenesis, migration and differentiation, as well as mobilization of endothelial progenitor cells from the bone marrow to the peripheral circulation [17]. VEGF also increases microvascular permeability (therefore it is also known as vascular permeability factor, or VPF), results in leakage of plasma proteins and transforms the stroma into an pro-angiogenic environment [19]. Furthermore, VEGF has been associated with malignant pleural and ascetic effusions [19]. As mentioned previously, with a very few exceptions, VEGF overexpression has been strongly associated with advanced tumour stage and poor survival, in breast, lung,

colorectal, gastric, pancreatic, hepatocellular, prostate, bladder, ovarian, head and neck, and even in other malignancies such as osteosarcoma, melanoma and leukaemia [20]. VEGF expression in the tumour has also been shown to be a significant prognostic factor of recurrence or survival, independent of other conventional clinicopathologic prognosticators (reviewed in [20]).

1.4.1 Circulating VEGF

Kondo and colleagues first reported that VEGF was detectable in serum of tumour-bearing mice and cancer patients [21]. The results indicated that VEGF was secreted from *in situ* tumours and released into circulating blood. Moreover, patients with endometrial, ovarian, cervical and lung cancer had higher levels of VEGF than individuals with no sign of disease and it was proposed that serum VEGF had potential as a serum diagnostic marker. In agreement and in addition to these findings, higher serum VEGF levels were found in patients with disseminated cancer than those with local disease, and patients undergoing cancer therapy had lower values than those without [22]. This study by Salven suggests that serum VEGF may reflect tumour status as angiogenesis is essential in tumour progression.

In ovarian cancer, pre-operative serum VEGF levels have been repeatedly shown to be elevated compared to healthy controls [23, 24] or in patients with benign and low malignant potential tumours [24-28]. Levels have been reported to decrease after successful removal of tumours [23-25, 29]. Elevated VEGF concentrations have also correlated significantly with poorer disease-free survival, overall survival [30, 31] and

more ascites [24]. In addition, using multivariate analysis, some studies have shown serum VEGF to be a strong prognostic indicator of survival, independent of established prognostic parameters such as stage [27, 28, 30, 32]. Along with association between VEGF expression and poor prognosis [33], the findings provide relatively consistent evidence that higher VEGF levels are indicative of more aggressive clinical behaviour in ovarian carcinoma.

In endometrial cancer, the data are more limited; increased serum VEGF in endometrial cancer patients compared to healthy controls was first reported by Kondo et al [21]. This is supported by a Chinese study which has shown increased serum VEGF in patients with ovarian, endometrial but not cervical cancer [23]. In addition, elevated serum VEGF concentrations that were observed in endometrial hyperplasia and endometrial cancer stage I and II, decreased one month after treatment [34]. Elevated pre-treatment plasma VEGF concentrations were associated with increased risk of progression and death in a phase II trial of thalidomide (an anti-angiogenic drug with unknown mechanism) in patients with refractory or recurrent endometrial cancer [35]. However, these studies were hampered by a relatively small number of samples (up to 72 [34]) and more extensive investigations are required to confirm these findings.

One direction cancer research has taken is the quantification of tumour angiogenesis for diagnosis and prognosis [36]. In assessing the angiogenic potential of tumours, several techniques have been used. Intratumoral microvessel density is most commonly used and is assessed by immunohistochemical staining with specific endothelial cell markers such as Factor VIII related antigen or CD31 [37]. Measurement of circulating angiogenic proteins, such as VEGF, that are secreted from

tumour tissue is more indirect but has several potential advantages. First, it can be performed without the need of a tumour specimen and is therefore theoretically applicable to all cancer patients. Second, it is technically simpler, less expensive and time-consuming. Third, repeated/serial measurements can be made in the same patient before and after anticancer treatment which may be useful for therapeutic considerations, such as neoadjuvant therapy. Fourth, measurement of circulating angiogenic factor levels by quantitative immunoassay can be more precise compared with semiquantitative techniques such as immunostaining [20]. The majority of studies using this approach have employed an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of circulating angiogenic factors and it appears to be a reliable and reproducible technique [20].

1.4.2 Assumptions and Limitations

Since VEGF is soluble, elevated circulating VEGF levels is interpreted as "overspill" of the excess local VEGF production by tumours, either through simple diffusion or exocytosis. This is supported by positive correlation between serum VEGF level and tumour VEGF expression [38, 39] and the decrease of serum VEGF level after surgical resection [23-25, 29]. Furthermore, increased VEGF levels in mesenteric blood draining from the tumour compared with peripheral blood in colorectal cancer patients has been documented, although other studies did not find such a relationship [40, 41].

However, there are certain “complications” in relating circulating VEGF to tumour production - other adult organs are known to express VEGF, such as lung, kidney, adrenal gland, heart, stomach mucosa, leucocytes and platelets [42, 43] and may contribute to blood VEGF. An important observation was made by Banks and colleagues in 1996 that VEGF in serum samples is higher than in matched plasma samples [44]. Serum is the liquid part of blood after *in vitro* coagulation involving platelet activation. Subsequent investigations revealed serum VEGF correlated highly with platelet counts in breast cancer patients [45]. Significantly higher VEGF concentrations were found in serum samples than in plasma in cancer patients [45] as well as healthy volunteers [44, 46] and activation of platelets increased VEGF [44-46]. The presence of VEGF in platelets was confirmed ultrastructurally to be in the α-granules [47]. Therefore it has been argued that plasma VEGF is more suitable in measuring free, basal circulating VEGF as serum samples reflect blood platelet counts rather than VEGF production by peripheral tissues [44, 48]. Higher plasma VEGF concentrations are observed in malignant disease and levels are increased further on the development of metastasis [48, 49]. However, higher VEGF per platelet has been reported in cancer patients using lysed platelets [50]. This is confirmed by subsequent investigations which showed raised calculated VEGF per platelet in cancer patients compared with healthy controls [51] (determined by dividing the difference of serum and plasma VEGF by platelet count), and higher VEGF per platelet in colorectal cancer patients than in benign disease patients [52]. Therefore, although serum VEGF levels are affected by platelets, platelet-derived VEGF also reflects biology of the cancer cells and therefore serum is more useful for measurement of circulating VEGF [51]. Indeed, the clinical relevance of serum VEGF is demonstrated by the fact that the majority of studies of circulating VEGF employed serum VEGF and they did show a positive

correlation between serum VEGF and clinical parameters such as patient survival [39]. In fact, both serum and plasma VEGF have been shown be indicative of prognosis in most studies [39], although this remains to be confirmed in studies of ovarian and endometrial cancer, since most studies used serum only, as discussed earlier although interestingly McMeekin and colleagues reported plasma VEGF and not serum was associated with progression free survival and overall survival in refractory or recurrent endometrial cancer [35].

It has been proposed that platelets are "scavengers" of VEGF in the circulation, preventing induction of angiogenesis except where coagulation takes place, such as in wound healing [53]. Excess VEGF in the plasma, presumably tumour-derived, may be scavenged by platelets [52], which may partly explain the discrepancy between high angiogenic activity of cancer patient's serum and the lack of angiogenesis outside the tumour [53]. In a recent study, it was shown that platelets selectively sequester angiogenic proteins (including VEGF) in mice implanted with VEGF-enriched Matrigel or tumour xenografts, which apparently prevents a corresponding increase in plasma VEGF [54]. This new discovery in platelet biology supports the theory that plasma and platelet VEGF may be in a state of dynamic equilibrium. In a study by Kusumanto and colleagues, up to 69% of total VEGF in whole blood was found in granulocytes, particularly neutrophils [55] and although leucocyte count did not correlate with serum and plasma VEGF [56], its contribution to plasma and serum measurements remains to be elucidated. In summary, while there is evidence to support the use of both serum and plasma, there is still controversy as to which provide the best prognostic information or reflect the tumour status.

Another major setback in measuring circulating VEGF is the lack of an internationally standardised protocol, and important inconsistencies in the pre-analytical phases exists. These differences may explain in part the widely scattered range of VEGF concentrations in healthy subjects, such as 0 to 1750ρg/ml for serum VEGF [57]. Plasma VEGF values, in general have been reported to be low and lie close to the lower detection limit of the assays [52]. Factors that have been shown to affect VEGF levels are time delay from blood collection to processing; anticoagulant type (for plasma); the temperature at which the samples are held and centrifugal force [57-59]. Plasma is commonly prepared from tubes with EDTA, citrate or heparin as anticoagulant. Banks and colleagues reported EDTA plasma samples to be higher than matched citrated samples, presumably due to more platelet activation by EDTA, and it was suggested that VEGF increases with increasing time delay, although wide interindividual differences were seen and the sample size was small [44]. The authors recommended that an optimal specimen of citrated plasma should be processed within one hour [44]. This is consistent with a study of patients with rheumatoid arthritis in which significantly lower VEGF concentrations were found in citrated plasma in comparison to EDTA or heparin plasma [59]. Measuring CD62 expression as a marker of platelet activation alongside VEGF, Zimmermann and colleagues found significant in vitro platelet activation for EDTA samples, which correlated with VEGF concentration. Citrated plasma produced less platelet activation, and CTAD tubes (a combination four anticoagulants of citrate, theophylline, dipyridamole and adenosine) was the most effective in preventing platelet activation, as well as having the lowest VEGF level [60]. In agreement, another study using platelet-specific platelet factor 4 (PF4), found CTAD to be more effective in inhibiting platelet activation than citrate, producing low or nearly undetectable values of PF4 [57]. Therefore it was concluded CTAD plasma should be adopted, although most

of the VEGF levels were undetectable [57].

Hormbrey and colleagues' investigation showed a reduction of plasma VEGF with increasing relative centrifugal force, which was mirrored by a reduction in platelet count in the supernatant. They therefore recommended that samples should be spun at high centrifugal force to minimise contamination from platelet-derived VEGF [58]. This is consistent with reports of lower plasma VEGF with step-wise increase in centrifugal force [59].

Delay between venipuncture and processing, or the "clotting time" is shown to be more important for serum than for plasma. Serum VEGF increases markedly after collection and has been reported to reach a plateau at one hour [61] or two hours [57]. However, mixed results have been reported with regard to effect of time delay for plasma. Plasma VEGF was reported to be stable for three hours [58] to up to eight hours [61], while it had increased by 80% in 2 hours in Hetland's study [59]. Interestingly, this increase was reduced markedly when samples are stored at 4°C until processing [59]. In contrast, others have found cooling tends to raise VEGF level in plasma [58], although lower platelet activation at 4°C compared to room temperature was observed in citrated plasma, which is consistent with Hetland's results [57]. For serum, while Hormbrey et al. reported significantly lower VEGF levels in samples that are cooled (19% of room temperature equivalent) [58], Hetland's study did not specify such effect and found less increase in VEGF over time when serum is stored at 4°C compared to room temperature [59].

Reference intervals for both serum VEGF and plasma VEGF in healthy controls have been determined. However, there are discrepancies across the literature with regard to the effect of age and sex. While no correlation was observed between age or sex and VEGF levels [59, 62], it had been reported that plasma VEGF concentrations were higher in individuals over 30 years of age [49] where as negative correlation was observed between serum VEGF and age [63]. Another study found a negative correlation with age in children (4-17 years), and a positive correlation with age in adults [64]. Serum VEGF concentrations in men were reported to be higher [65, 66]. However, it is interesting to note that the difference between the sexes disappeared when only non-smokers were compared [65], and therefore smoking may be a potential inducer of circulating VEGF. Other studies observed increased VEGF levels in women [67, 68]. Studies investigating the effect of the menstrual cycle on circulating VEGF have produced inconsistent results. For premenopausal females, cyclical variation, namely lower VEGF levels in the luteal phase compared to the follicular phase had been reported, which was absent in controls consisting of postmenopausal women or woman on oral contraceptives [69]. In contrast, another study of six women found serum VEGF to be lowest in the secretory phase [70]. Larger, more recent studies found no difference during the menstrual cycle[68, 71].

Single measurements are typically used in studies to determine baseline VEGF levels in healthy volunteers as well as in studies which reported higher circulating VEGF in women with ovarian cancer [26-28, 30-32]. Kraft and colleagues assessed serum VEGF concentrations weekly for six weeks in 7 healthy volunteers and reported no increases over the 95^{th} percentile level of VEGF values obtained from 145 healthy controls [62]. In 16 healthy controls and 21 rheumatoid arthritis patients, plasma

samples was taken at seven time points over 24 hours, and no systematic diurnal variation was noted, except that plasma VEGF at 7AM was significantly lower than at 1PM and 4PM [59]. Five plasma samples collected over three weeks in 32 participants, which were repeated twice to investigate long-term variability indicated that plasma VEGF levels were different at each of these rounds and the authors suspected both pre-analytical and biological variation contributed to this [59]. The variation of VEGF levels over time in women with established ovarian or endometrial cancer has not been reported previously, and it has not been confirmed that VEGF levels increase continuously alongside progression of tumour, such as Cancer Antigen 125 (CA125) which is used routinely to monitor disease progression in ovarian cancer. (Although in a study of women endometrial cancer, blood sampling was carried out every three months after surgery, variation was not assessed [34].)

Although circulating VEGF has not been validated as a tumour marker, there have been a few reports which suggest it may be a useful predictive marker of efficacy of chemotherapy. In patients with metastatic non-small cell lung cancer and metastatic colorectal cancer, VEGF normalization after chemotherapy was asscociated with tumour response and stable disease and one year survival rates was also higher than in patients with persistently high VEGF levels [72]. In another trial, basal VEGF levels in metastatic colorectal patients decreased significantly after cetuximab plus irinotecan treatment and for ones with at least 50% reduction, a higher response rate, longer disease free survival as well as overall survival was observed [73].

Other diseases and physiological states are known to affect circulating VEGF levels. Diabetes mellitus [74], smoking, obesity and metabolic syndrome have been

positively associated with VEGF levels [68]. It also appears the inflammatory response elevates VEGF levels. Higher VEGF levels have been reported in patients with rheumatoid arthritis [59, 75], inflammatory bowel diseases [76], polyarteritis nodosa [77] and other rare systemic vasculitis such as Wegener's granulomatosis and Churg-Strauss Syndrome [78]. Serum VEGF in patients with acute viral or bacterial meningitis are higher than in healthy blood donors [62]. Wound healing is initiated by platelet degranulation of a number of growth factors, including VEGF, and later many other cell types also contribute VEGF for the maturing wound [79]. Inflammation and wound healing are inevitable consequences of surgical trauma, and serum VEGF has been reported to increase post-operatively, reaching a maximum 14 days after surgery [80]. Moreover, this increase was twice as much in the major abdominal surgery group (transthoracic oesophagectomy) than in the group undergoing a less invasive laparoscopic procedure (cholecystectomy). Post-operative serum VEGF levels were also significantly higher in patients with inflammatory lung complications [80]. In another study, plasma VEGF concentrations were significantly elevated after minimally invasive colorectal resection, for as long as one month (four weeks) [81].

1.5 Targeted Therapy on Angiogenesis

Targeted therapy refers to the use of biological agents that interfere with molecular and biochemical pathways that cause the malignant phenotype, including proliferation, angiogenesis, invasion, metastasis and decreased apoptosis [82]. Such targeted therapy exploits differential expression of specific targets in cancer cells when

compared with normal cells [82]. One of the most promising strategies under investigation is antiangiogenic therapy. As VEGF plays a central role in the angiogenesis of different types of carcinoma, much effort has been focused on its potential role as a therapeutic target. Bevacizumab (Avastin, Genentech) was the first antiangiogenic drug approved by the US Food and Drug Administration (FDA), based on significant improvement in both progression-free and overall survival in patients with metastatic colorectal cancer, when it is combined with 5-fluorouracil-based chemotherapy [83]. It is a recombinant humanized monoclonal IgG1 antibody directed against human VEGF [84]. The role of antiangiogenic therapies are being investigated in ovarian and endometrial cancer.

1.5.1 Ovarian cancer

Bevacizumab is the antiangiogenic drug with the widest clinical development as well as published information in the treatment of ovarian cancer [85]. A number of Phase II studies, usually in patients with recurrent, heavily pre-treated and platinum-resistant ovarian cancer, have obtained favourable results, showing antitumour activities both as single agent and in combination with chemotherapy (reviewed in [85]). Leading on from these results are Phase II studies evaluating the role of bevacizumab as a first-line treatment in ovarian cancer – which also showed encouraging response rates and acceptable toxicities to chemotherapy alone [86] and in fact, the highest response rates (up to 80%) was observed in this setting [87]. Although Bevacizumab is generally well-tolerated with mild frequent toxicities such as hypertension, proteinuria and bleeding, the drug has been associated with other uncommon, yet potentially life-threatening side effects such as arterial thromboembolism, gastrointestinal

perforation and wound healing complications, causing a number of trials to end prematurely [85]. Two large phase III multicentric studies are currently underway to clarify the efficacy (improvement in disease-free survival and overall survival) and safety of bevacizumab as first-line therapy for advanced ovarian cancer.

Other antiangiogenic therapies consist of VEGF Trap – soluble fusion protein that binds to the A and B isoforms of VEGF, small-molecule tyrosine kinase inhibitors that target VEGFRs, such as sorafenib, sunitinib and pazopanib. They are currently under investigation in Phase II studies, with promising results [85].

1.5.2 Endometrial cancer

In a murine model Kamat and colleagues injected endometrial cancer cell lines Ishikawa and Hec-1A into the uterine horn then treated the mice with bevacizumab and/or docetaxel. The combination compared with each agent alone obtained significantly more tumour growth inhibition, tumour vascularity and resulted in less extentive metastasis [88]. The use of bevacizumab for patients with recurrent or persistent endometrial cancer is currently being evaluated in a Phase II study [10]. There is also interest in steroid receptors as steroid hormones such as oestrogen and progesterone regulate angiogenesis and VEGF expression in the uterus [10].

1.5.3 Integrative therapy

The concept of maintenance therapy in cancer – continuing chemotherapy beyond the standard 4 to 6 cycles of treatment in an attempt to prevent recurrence of disease – is not new and is of particular interest in ovarian cancer since most patients, despite receiving current standard treatment of surgery plus chemotherapy, will eventually relapse, with a median progression free survival of 18 months [86]. Prolonged treatment with paclitaxel, an anti-neoplastic agent as well having anti-angiogenic properties, has been shown to improve progression free survival in ovarian cancer, however, any benefit must be balanced with the increased toxicities associated [82]. One of the phase III trials of bevacizumab is also investigating its role as maintenance therapy [86].

Natural anti-angiogenic compounds present in food may be potential candidates that can be integrated into conventional treatment with cytotoxic drugs and radiotherapy. They have the advantage of interacting with multiple angiogenic pathways, as well as displaying pleiotropic modes of activities such as cell signaling, the apoptotic pathway and the interaction of cancer with the immune system [89]. Thus there is a lower likelihood of the development of chemoresistance [90]. These natural products may be most effective in impeding cancer recurrence after cytotoxic therapy and have potential to increase overall survival and quality of life [89]. In addition, as discussed earlier, surgery, such as colorectal resection has been associated with postoperative increase in plasma VEGF. Although the clinical effect of this is unclear, it has been proposed that this may create a proangiogenic environment that stimulates angiogenesis of microscopic residual tumour [91]. Natural anti-angiogenic compounds may also be useful in shifting the balance, as long as wound healing is not compromised. Using food compounds is also likely to more economical, less toxic and with high patient

compliance since complementary and alternative medicine use in cancer patients is high (prevelance up to 64% [92]). One such compound is a polyphenol, epigallocatechingallate, from green tea.

1.6 Green Tea

Tea is the second most consumed beverage in the world, after water [93]. Green tea, like black and Oolong teas, is made from leaves and buds of *Camellia sinensis*, a species of the Theaceae family. It differs from the other tea types by the processing method. Green tea is produced by drying and heating the fresh leaves and thereby inactivating oxidative enzymes such as polyphenol oxidase. Oolong and black tea go through partial and complete fermentation, respectively, before the dry heat treatment, resulting in the oxidation of simple polyphenols to a more complex, condensed polyphenol, which gives rise to the dark colour of black tea. Therefore green tea contains the most abundant simple polyphenols, of the flavonoid subgroup known as catechins – which constitute approximately 30% of the dry leaf weight [94]. The four main polyphenolic compounds of green tea are (−)-epigallocatechingallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epicatechin (EC). Green tea, and its main catechin EGCG have been attributed to a wide range of health benefits including antioxidant activity, prevention and/or control of cancer, atherosclerosis, hypertension, obesity, diabetes, dental caries, as well as having antibacterial, antiviral, antifungal and neuroprotective properties (reviewed in [95-97]). In clinical investigations of green tea and EGCG, although tea beverage had been used,

the doses of the polyphenol were often unknown [98]. This arises from the fact that the content of the polyphenols differs largely depending on the tea "vintage" (such as "Sencha" from Japan), as determined by the species of plant, the soil for tea cultivation, age of the tea leaves, the method of processing tea leaves and season of harvest. The percentage weight of EGCG can vary between 6 to less than 1 percent [99] across different vintages. Moreover, the length of infusion produces further variation (majority of EGCG was not extracted until after 6 to 8 minutes of infusion [99]). Changing one's dietary habit is not easy and consuming 20 to 25 cups of green tea daily is impractical [100], as high doses are often required. Therefore, to avoid these issues and improve compliance, standardised oral supplements of concentrated green tea extract in the form of capsules and tablets can be administered [100] and have indeed been used extensively in clinical trials [98].

1.6.1 Anticancer and Antiangiogenic Properties of Green Tea

Although numerous epidemiological studies have shown an inverse association between drinking tea and the risk of developing human cancer, overall the evidence is inconclusive [101]. Of the 127 case-control studies and 90 cohort studies reviewed on the relationship between tea consumption and cancer risk (including colon, lung, breast, prostate, ovary and others), only 51 and 19 showed an inverse relationship respectively, while the rest showed no association [101]. A recent Cochrane review assessing the association between green tea consumption and the risk of cancer incidence also concluded that the evidence is insufficient and conflicting [102]. Meta-analyses evaluating the effect of tea in general and the risk of developing the two gynaecological

cancers of interest show some evidence that green tea in particular is protective for endometrial cancer [103]. While this was not reproduced in the meta-analysis for ovarian cancer, there appeared to be a trend for studies done in Asia to report a reduction in cancer risk [104]. In Asian countries green tea is the most widely consumed tea. Another study in China also reported that green tea consumption enhances survival in women with ovarian cancer. Therefore, green tea may still be protective for ovarian cancer but in a meta-analysis the effect was masked by studies that predominantly measured black tea consumption. Laboratory studies of green tea and EGCG have indicated a number of mechanisms in explaining how catechin-containing teas could help prevent cancer. EGCG has been shown to inhibit tumour growth as well as down-regulating angiogenesis and VEGF in various animal models of human cancer cell lines. A small number of clinical trials have been published to date, and they showed that green tea extract was able to reduce or slow down progression of several pre-cancerous lesions, as well as producing a clinical response in chronic lymphocytic leukaemia.

.

1.6.1.1 Epidemiological studies

1.6.1.1.1 Endometrial cancer

A recent meta-analysis of tea consumption and risk of endometrial cancer supported the protective effect of tea on endometrial cancer [103]. Two cohort and five case-control studies were included and the combined relative risk was 0.85 for ever drinkers versus non/lowest drinker. In addition, when categories of tea consumption were placed on a common scale, an increase in tea drinking of two cups a day was associated with a 25% decreased risk of developing endometrial cancer. Subgroup

analysis revealed that only green tea was statistically associated with the decreased risk, however, only two studies measured black tea drinking [103]. It was also found that the relationship between tea and risk of endometrial cancer was only apparent in studies done in Asia and not in the USA, and therefore the authors stated that care must be taken before extrapolating these results to other populations. This discrepancy may be partly attributed to the variation in the types of tea consumed, as green tea represented more than 90% tea consumption in China and Japan, whereas black tea would be the most common in the USA [103].

1.6.1.1.2 Ovarian cancer

A meta-analysis of tea consumption and ovarian cancer risk, which evaluated 2 cohort studies and 7 case-control studies did not show an association (RR = 0.85; 95% CI 0.66-1.07) [104]. Of the reviewed articles, a study from China which reported a considerable risk reduction was shown to be significantly different from the other studies, which were in Western countries [105]. This implied that there might be some important difference between the study in China and in Western countries and again it was highlighted that more than 90% of tea drinkers in China consumed green tea, while in Western countries people mainly drink black tea [104].

Another meta-analysis, which included studies reviewed by Zhou and colleagues [104] as well as four more recent studies (one case-control and three cohorts) found no significant association between ovarian cancer and tea consumption (pooled OR = 0.85; 95% CI 0.71-1.01), however, in evaluating cohort studies alone an inverse relationship was found which was statistically significant [106].

A case-control study assessing caffeinated drinks and ovarian cancer risk found that among teas, neither black tea nor herbal tea were associated with risk but women who drank one or more cups of green tea a day had a 54% reduction in ovarian cancer risk. Moreover, the findings were similar when Asian women cases and controls were removed from the analysis [107].

Interestingly, increased tea consumption was shown to increase survival in epithelial ovarian cancer patients in a cohort study in China [108]. 254 women in Zhejiang province who had histopathologically confirmed epithelial ovarian cancer were followed up for survival time and frequency and quantity of tea consumption post diagnosis. 92% of women drank green tea only. At three years post diagnosis, the survival was different between tea drinkers and non-drinkers. 77.9% of tea drinkers survived to the time of the interview compared to only 47.9% still alive among the non-drinkers. The adjusted hazard ratios, compared to non-drinkers were 0.55 (95% CI = 0.34-0.90) for tea drinkers. Using other measures of tea consumption, such as the number of cups drunk, frequency of dried tea batches brewed and quantity of dried tea leaves consumed, higher tea intake was consistently associated with risk reduction (0.38-0.44) and the dose-response relationship was also statistically significant ($P < 0.05$).

The aforementioned Cochrane review [102], which concluded that there were "insufficient and conflicting" evidence regarding green tea consumption for cancer prevent, only referenced two case-control studies for ovarian cancer and none for

endometrial cancer. Even under the list of studies that had been excluded for methodological reasons, no studies for endometrial cancer could be found. It appeared that a number of studies of evaluating effect to green tea on cancer had been overlooked, and this might have influenced the conclusion.

1.6.1.2 Preclinical studies

In contrast to epidemiological studies, green tea and its active components have been demonstrated in many animal models to inhibit many molecular pathways in carcinogenesis (reviewed in [109]) as well as showing an antiangiogenic effect such as decreasing VEGF.

EGCG, the main catechin of green tea has shown similar effects – namely inhibition of growth and VEGF secretion for a number of cancers such as colon [110], breast [111], skin [112], prostate [113]and pancreas [114]. Together, these studies suggested that green tea may have anti-cancer effect, partly through down-regulating angiogenesis and VEGF. Laboratory studies of green tea/EGCG on gynaecological cancers are discussed below.

1.6.1.2.1 Cervical cancer

In a study by Zhang et al ([115]), EGCG and green tea extract were shown to suppress hypoxia-induced VEGF protein production and mRNA expression in human cervical carcinoma cells (HeLa), in a dose dependent manner. Similarly, hypoxia-induced HIF-1α protein, a transcriptional factor crucial to VEGF synthesis, was also inhibited by EGCG. Results suggest that inhibition of VEGF and HIF-1α by EGCG is via blocking PI3k/Akt and ERK1/2 signal transduction pathways, as well as

promoting the degradation of HIF-1α protein accumulation via the proteasome degradation pathway.

Further evidence has been reported more recently, in which green tea extracts and EGCG were shown to inhibit the HPV-16 oncoproteins-induced expression of HIF-1α protein, and VEGF protein and mRNA in human cervical carcinoma cells C-33A [116].

1.6.1.2.2 Ovarian cancer

Spinella and colleagues reported EGCG reduced the growth in ovarian carcinoma cells HEY and OVCA 433 dose-dependently and the effect was associated with apoptotic changes [117]. It was shown that this is mediated through inhibiting endothelin-1 (ET-1) and its receptor ET_AR (ET-1/ET_AR), which play a key role in development and progression of ovarian carcinoma. Furthermore, oral administration of green tea to nude mice bearing HEY ovarian carcinoma xenographs, resulted in reduced tumour growth, as well as reduction in ET-1, ET_AR and VEGF, at both mRNA and protein levels.

1.6.1.2.3 Endometrial Cancer

Recently, our laboratory had shown that EGCG significantly reduced VEGF secretion from primary endometrial cells and also in cells treated with Cobalt Chloride, a hypoxic-mimicking agent which increases VEGF by stabilising HIF-1α [118].

1.6.1.3 Clinical studies

A number of clinical trials have shown that green tea decreased progression of

pre-malignant lesions in oral, colon, cervical and prostate cancers [119-123]. In a phase one trial in patients with chronic lymphocytic leukemia, green tea extract supplementation produced clinical response for the majority of patients [124]. In addition, one recent study reported that green tea polyphenols reduced serum levels of Prostate Specific Antigen (PSA) and various growth factors, including VEGF in prostate cancer patients [125, 126].

A double blind intervention trial showed that in comparison to placebo, oral and topical treatment of mixed tea compounds in patients with oral leukoplakia, a premalignant lesion to oral cancers, produced higher rates of reduction in lesion size, lower rate of increase in lesion size, and also reduction in markers of DNA damage and cell proliferation [121]. Similarly, a 12-week trial of green tea extract, in higher doses, improved clinical response rates in patients with high risk oral premalignant lesions and in those responded, biopsies showed decreased downregulation in stromal VEGF and cyclin D1 expression [123].

In 71 patients who previously had colorectal adenomas removed, but were currently polyp-free, daily supplementation with 1.5 grams of green tea extract for 12 months reduced the relapse of metachronous adenomas (15% compared to 31% in control group) at a repeat colonoscopy. The size of the adenomas was also smaller in the green tea extract group [122].

Although no clinical studies evaluated the effect of green tea extract in ovarian or endometrial cancer, one study investigated its clinical efficacy in human papilloma virus

infected cervical lesions, ranging from chronic cervicitis, mild dysplasia, moderate dysplasia to severe dysplasia. In the treatment group a green tea extract ointment was applied twice weekly and/or received daily green tea extract capsule for twelve weeks, and showed a total response rate of 69%, compared to 10% in the controls – suggesting that green tea extracts may be potential therapy for these lesions [119].

In a trial of men with high-grade prostate intraepithelial neoplasia, daily oral green tea extracts or placebo were administered in 12 months. In the treatment group, 1 man (3%) progressed to having prostate cancer, where as this was the case for nine men (30%) in the control group. Moreover, lower urinary tract symptoms and quality of life scores improved in men from the treatment group who also had coexistent benign prostate hyperplasia [120]. Interestingly, the difference in cancer prevalence between the two groups was sustained two years after the administration of green tea extracts was suspended which suggested that protective effect of green tea extract were long-lasting [125]. In a pilot study of which the serum marker of interest VEGF was measured, a brief treatment of green tea extract in 26 patients with prostate cancer diagnosed by biopsy showed a significant reduction in serum PSA, hepatocyte growth factor and VEGF [126].

In a Phase I trial of green tea extracts in patients with previously untreated early stage chronic lymphocytic leukemia, one patient achieved remission, 33% had a decrease in lymphocyte count, and 92% of patients with palpable adenopathy saw a 50% or greater reduction in the sum of the nodal areas. It suggested that green tea may be used as a potential disease stabilising agent in chronic lymphocytic leukemia, which is currently incurable [124].

1.6.2 Safety of Green Tea Extract

In general, standardised green tea extracts (GTE) have been well tolerated in human clinical trials; 100mg of EGCG is equivalent to 1-2 cups of green tea [127]. In a Phase I study of oral GTE in adult cancer patients, sides effects were gastrointestinal (abdominal bloating, dyspepsia, flatulence, nausea, and vomiting) and neurological (agitation, dizziness, insomnia, tremors, and restlessness), and likely to be caffeine-related [100]. Single oral doses ranging from 50mg to 1600mg administered to healthy male volunteers were reported to be safe [128]. Also, 800mg of EGCG once a day or in divided doses given for four weeks were well-tolerated [127]. However, recently there has been a number of case reports of liver toxicity associated with GTE, EGCG-containing weight-loss products and in some cases even green tea infusions [129]. In particular, Exolise, a weight-loss product containing a hydroalcoholic extract of green tea was suspended by French and Spanish authorities in April 2003 because the product was suspected of causing elevated liver enzymes (and in one case, progression to liver failure requiring liver transplantation [130]. The risk of toxicity has been estimated to be low (1 case out of 83812 treatments, cited in [129]), and it is difficult to establish a causal relationship, as the patients were taking other drugs or natural remedies simultaneously. Nevertheless, in some cases a rechallenge of the green tea formulation was positive (elicited the same reaction such as raised liver enzymes), and therefore it is reasonable to assume a certain toxic potential of GTEs (reviewed in [129]). The mechanism of hepatotoxicity has not been established.

1.6.2.1 Bioavailability and Food

EGCG has low bioavailability in humans, and is mostly present in the plasma in the free form [127, 131]. Chow et al reported >92 % free form, based on the ratio free versus total (free and conjugated) EGCG [127]. In an animal toxicology study involving dogs, no adverse effects were noted when 500mg EGCG / kg body weight / day was administered to pre-fed dogs in divided doses. However, this dose caused morbidity when administered to fasted dogs in a single bolus [132]. A similar effect of food has also been shown in human studies where significantly higher free plasma EGCG levels was observed when the GTE was taken following an overnight fast, in comparison with those taken with food [133].

A recent systematic review on the safety of GTE by the US Pharmacopeia, citing the adverse events reports, pharmacokinetic and toxicological evidence, has proposed that all GTE to be taken with food [134].

1.7 Aims of the study

- To compare plasma and serum VEGF in women with active endometrial/ovarian cancer and women with similar demographics but currently without active cancer.
- To establish reference ranges of blood VEGF levels in the above groups.
- To correlate blood VEGF levels with cancer type, stage, and histological subtype.
- To compare and contrast VEGF measurements in plasma and serum components

of blood.

- To describe the variability of blood VEGF measurements in weekly serial samples and hence determine the reliability of individual VEGF samples.
- To determine whether surgical removal of disease is associated with reduction of VEGF levels.
- To determine whether VEGF levels are proportionate to disease activity and are therefore a tumour marker.
- To determine whether green tea suppresses VEGF levels in women with persistent endometrial/ovarian cancer.

2 Methods

2.1 Plasma and Serum Vascular Endothelial Growth Factor Levels in Women with Gynaecological Cancer

2.1.1 Ethical Approval

Ethical approval for this prospective cohort study was granted by the **Upper South A Regional Ethics Committee**, ethics Ref URA/09/01/007.

2.1.2 Participants

2.1.2.1 Recruitment

All potential participants were recruited from the Christchurch Gynaecological Oncology Service. Three cohorts of women with different tumour status were recruited. Inclusion criteria were: Cohort 1 – women who had received curative treatments for endometrial and/or ovarian cancer and were without evidence of disease; Cohort 2 – women with clinical evidence of endometrial and/or ovarian cancer and were

not currently undergoing treatment; Cohort 3 – women who were to undergo definitive surgical treatment for endometrial and/or ovarian cancer. Potential woman who fitted the inclusion criteria were identified by their treating physician then individually approached for informed consent. .

2.1.2.2 Cohort 1

Participants were recruited from the Outpatients Department. They were women who were being followed after receiving curative treatments for primary endometrial and/or ovarian cancer, and were currently without evidence of disease. Eighteen women ages 31-79 were recruited, of which ten and eight were treated for endometrial or ovarian cancer, respectively. One woman was Asian, one Maori and the rest were New Zealand Europeans.

2.1.2.3 Cohort 2

Women with clinical evidence of endometrial or ovarian cancer, as assessed by their treating clinician and were not currently undergoing active treatment for their cancer (active treatment included palliative chemotherapy/radiotherapy).

Unfortunately, due to the rarity of advanced endometrial cases, we were unable to recruit any women with advanced endometrial cancer. Seven women, aged 59-84 with advanced ovarian cancer who were not receiving active treatment were recruited.

2.1.2.4 Cohort 3

Women who were to undergo primary cytoreductive surgery for endometrial cancer and/or ovarian cancer were included. These included women with known cancer or women with adnexal mass(es). Postoperative pathology results of the tumour/mass provided verification of the tumour types.

2.1.2.5 Exclusion Criteria

There were no exclusion criteria for Cohort 1, 2 and 3, other than inability to give informed consent, or those deemed inappropriate to participate (such as anxious/emotional patients) by the treating clinician. However, one woman in Cohort 1 was withdrawn from the study after informed consent because of difficulty accessing veins.

2.1.3 Samples

2.1.3.1 Collection

Cohort 1 and 2 women had 20ml blood specimens collected at Outpatients Clinic weekly for four weeks and monthly for two months (six specimens in total).

Cohort 3 women had one 20ml blood specimen collected before and one after surgery. These collection times coincided with their routine preadmission appointment

and their post-operative follow-up appointment, usually six weeks after surgery. The post-operative blood was taken before chemotherapy if the woman was to receive post-operative chemotherapy (four weeks after surgery).

20ml of blood was collected in Vacutainer tubes (BD, Plymouth, United Kingdom) and each blood collection comprised of:

- 8.0ml in two tubes containing potassium EDTA (purple top, Ref 367839)
- 4.5ml in a tube containing Sodium Citrate (blue top, Ref 367691)
- 4.5ml in a tube containing Lithium Heparin (green top, Ref 367375)
- 4.0ml in a tube containing Clot Activator (red top, Ref 368975)

They were placed on ice immediately after venipuncture and were processed within eight hours.

2.1.4 Processing

2.1.4.1 Full Blood Count and C-Reactive Protein

Two 4.5ml tubes containing potassium EDTA and Lithium Heparin were sent to and analysed by Canterbury Health Laboratories (Christchurch, New Zealand) for a Full Blood Count and C-Reactive Protein.

2.1.4.2 Plasma

Platelet-poor plasma was prepared in accordance to the Australian Biospecimen

Network (ABN) Biorepository Protocols [135] with a slight modification. Firstly, the blood collected with potassium EDTA and Sodium Citrate as anticoagulants were centrifuged at 3200g for 12 minutes, at 4℃ by Heraeus Multifuge 1 S-R (Thermo Scientific, Massachusetts, USA). The supernatant was collected, avoiding contamination by the buffy coat, and then centrifuged at 3200g for 10 minutes at room temperature (the ABN Protocols recommended this centrifugation to be done at 4℃) by Centrifuge 5417 (Eppendorf, Hamburg, Germany). Multiple aliquots (250µl) were collected, avoiding the pellet containing cellular debris and platelets, and stored at -80℃ until analysis.

Our protocol differed in the fact that the first centrifugation was done at 4℃ as opposed to room temperature. Prior to this, the blood samples were also kept in ice rather than in room temperature, as mentioned in 2.1.3.1. Hetland and colleagues' investigation into pre-analytical variability showed that for EDTA plasma, delay of centrifugation after venepuncture was associated with an increase of VEGF (80% after two hours) [59]. When the samples were stored at 4℃, there was no statistical significant increase in VEGF levels for up to 24 hours [59]. We were not able to sufficiently control the time between venepuncture and processing of each sample due to the availability of the investigator and the centrifuge, therefore adopted cooling of samples to reduce errors arising from variation in processing time. The second centrifugation of the supernatant could only be done in room temperature as the Centrifuge 5417 (Eppendorf, Hamburg, Germany) did not have temperature settings.

2.1.4.3 Serum

Blood from tubes containing Clot Activators were centrifuged at 1200g for 10 minutes at room temperature [135], and multiple 250µl aliquots of serum obtained and stored at -80℃ until analysis.

2.1.5 Assays

2.1.5.1 Full Blood Count

Full Blood Count, which provided numbers of circulating cells in blood (red cells, white cells and platelets), was analysed by Sysmex XE2100 (Sysmex Corporation, Kobe, Japan).

2.1.5.2 C-Reactive Protein

C-Reactive Protein in plasma was analysed using Abbott c8000 (Abbott Laboratories, Abbott Park, Illinois, USA).

2.1.5.3 VEGF Immunoassay/ELISA

Preliminary experiments with Human VEGF DuoSet (R&D Systems, Minneapolis, USA), revealed this ELISA kit was not sensitive enough for VEGF measurements in plasma and serum.

Another commercially available kit, Human VEGF Quantikine ELISA Kit (R&D

Systems, Minneapolis, USA) which had been validated for cell culture supernates, serum and plasma, was found to be sensitive enough. It employs the quantitative sandwich enzyme immunoassay technique, using a solid-phase monoclonal antibody and an enzyme (horseradish peroxidase)-linked polyclonal antibody raised against recombinant human VEGF. The substrate is chromogen (tetramethylbenzidine) and colour develops in proportion to the amount of VEGF bound to the solid-phase antibody. Recombinant human VEGF standards were used for calibrations.

Optical densities of the final solution in each well were determined using a microplate reader SpectraMax 190 (Molecular Devices, Victoria, Australia) with readings at 540nm subtracted from 450nm for correction. A standard curve is generated with the software SoftMax Pro v5.3 (Molecular Devices, California, USA) using a four parameter logistic (4-PL) curve fit. The sensitivity of the assay for VEGF detection is 9ρg/ml, as quoted from the manual.

2.1.5.3.1 Summary of Assay Procedure

Serum and plasma samples from participants were incubated for 2 hours at room temperature on 96-well microplates coated with a monoclonal antibody specific for VEGF. All analyses and calibrations were carried out in duplicates of 100 μl.

Next, any unbound substances were washed away, and an enzyme-linked polyclonal antibody specific for VEGF was added. This was allowed to incubate for 2 hours and any unbound antibody was washed away. A substrate solution of chromogen (tetramethylbenzidine) was introduced and colour development was stopped in 25 minutes with sulphuric acid, at room temperature. The optical density of the final coloured solution in the wells was read within 30 minutes.

2.1.5.3.2 ELISA Assay Principle

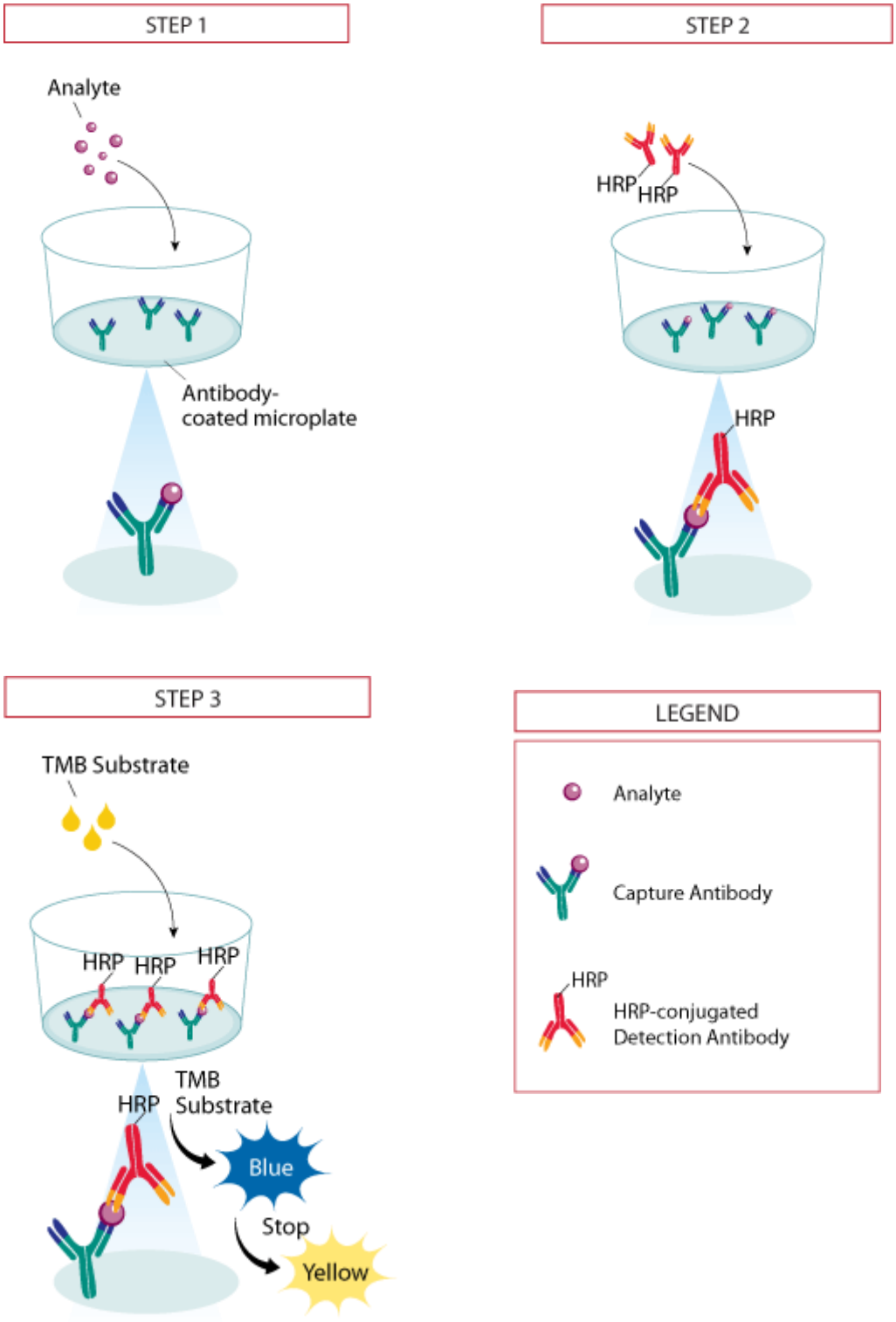


Figure2-1 ELISA Illustrations Image source: [Internet] http://www.rndsystems.com//product_detail_objectname_quantikineelisaassayprinciple.aspx Accessed June 2009

2.1.5.3.3 Quality Control

Multiple aliquots of serum from a healthy, postmenopausal woman as prepared in 2.1.4.3 was obtained and analysed in duplicate on every microplate to act as quality control.

2.1.6 Statistical Methods

Plasma VEGF concentrations as measured by ELISA were adjusted for the dilution effect of the liquid anticoagulant Sodium Citrate (10% tube volume), taking into account the haematocrit, using the following formula: VEGF (ρg) / 10^6 platelets = (VEGF serum – VEGF plasma) × (1 – haematocrit) / platelet number. [136].

2.2 A Pilot Study to Determine the Effect of Green Tea Extract on Circulating Vascular Endothelial Growth Factor in Women with Endometrial and Ovarian Cancer

2.2.1 Ethical approval

Ethical approval for this study was granted by the **Upper South A Regional Ethics Committee**, ethics ref URA/09/07/056.

2.2.2 Participants – (Cohort 4)

All potential participants were recruited from the Christchurch Gynaecological Oncology Service. Potential woman who fitted the inclusion criteria were identified by their treating physician then individually approached. The inclusion criteria were women with active endometrial and/or ovarian cancer who were not undergoing active treatment for at least one week. These women were then screened for any of the exclusion criteria before informed consent.

2.2.2.1 Exclusion criteria

- Known allergy to green tea and/or other ingredients in the Green Tea Extract formulation
- Severe heart disease (eg unstable angina or chronic congestive heart failure)
- Ongoing gastric ulcer
- Concurrent anti-coagulants
- Concurrent antiarryhythmic agents
- Concurrent consumption of natural health products containing green tea compounds
- Ongoing infection or systemic inflammation
- Abnormal liver function test within the last month

In cases when it was debatable whether the woman fall into the exclusion criteria (such as a near-normal liver function test), the treating physician was consulted

2.2.3 Procedure

Women were given 24 capsules of Green Tea Extract (Bio Concepts, Queensland, Australia), each containing dry Camellia sinensis leaf extract equivalent of 337.5mg tea catechins, including 225mg EGCG. The women were required to take two capsules, twice a day, at twelve-hourly intervals, over a period of six days. A first blood test was performed before starting ingesting the Green Tea Extract, and a second was done on the day the capsules were finished. 15ml of blood were taken at each sampling time.

2.2.4 Samples

2.2.4.1 Collection

Blood was collected in Vacutainer tubes (BD, Plymouth, United Kingdom) and each blood specimen comprised of:

- 4.0ml in a tube containing Potassium EDTA (purple top, Ref 367839)
- 4.5ml in a tube containing Lithium Heparin (green top, Ref 367375)
- 4.0ml in a tube containing Clot Activator (red top, Ref 368975)
- 2.7ml in a tube containing Sodium Citrate (blue top, Ref 363095)

They were kept at room temperature and processed one hour after collection.

2.2.4.2 Processing

2.2.4.2.1 Full Blood Count

4.0ml of blood collected in tubes containing potassium EDTA was sent to Canterbury Health Laboratories (Christchurch, New Zealand) for a Full Blood Count.

2.2.4.2.2 Plasma

The 2.7ml Sodium Citrate and 4.0ml Lithium Heparin tubes were prepared for platelet-poor plasma [135], with all processing carried out at room temperature. Firstly, the tubes were centrifuged at 3200g for 12 minutes, at room temperature by Heraeus Multifuge 1 S-R (Thermo Scientific, Massachusetts, USA). The supernatant was pipette off, avoiding contamination by the buffy coat, and then spun at 3200g for 10 minutes at room temperature by Centrifuge 5417 (Eppendorf, Hamburg, Germany). Multiple aliquots of plasma (250μl) were obtained and stored at -80℃ until assaying. 10% ascorbic acid (Sigma, St. Louis, Missouri) equivalent of 5% of the aliquot volume was added to plasma prepared from the heparinised tube, as a preservative.

The processing protocol was a further modification to one used in the previous study, as outlined in 2.1.4.2. Due to the lower number of participants, we were able to control the processing time to one hour ± 5 minutes. Secondly, it appeared that the electronic temperature reading of Heraeus Multifuge 1 S-R (Thermo Scientific, Massachusetts, USA) might be malfunctioning, therefore all processing was kept at room temperature. The 10% ascorbic acid was added as a preservative, with the intention of analysing the green tea catechins using HPLC, using the method described by Unno and colleagues [137] in order to assess compliance and absorption. This analysis was not carried out in this study due to time constraints.

2.2.4.2.3 Serum

4.0ml red tubes containing Clot Activators were spun at 1200g for 10 minutes at room temperature, and multiple aliquots of serum obtained and stored at -80℃ until analysis.

2.2.5 Assays

2.2.5.1 Full Blood Count

Full Blood Count was analysed by Canterbuy Health Laboratories (Christchurch, New Zealand) using Sysmex XE2100 (Sysmex Corporation, Kobe, Japan).

2.2.5.2 VEGF Immunoassay/ELISA

Human VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, USA) was employed and the procedure was as stated in 2.1.5.3 above.

3 Results

3.1 Technical Note

An issue that concerns all the plasma VEGF results is the inherent low levels of plasma VEGF concentration relative to the sensitivity of the ELISA kit used. We used a commercial human VEGF kit, Quantikine (R&D Systems), which is one of the most quoted [58]. Hombrey and colleagues pointed out that although this kit has the sensitivity of 9 ρg/ml (as specified by the manual), only values between 31.25-2000 ρg/ml are "reliable", as defined by the industry standard of coefficient of variance ≤ 10% within the 95% confidence limits between duplicate readings [58]. Using values below 31.25 ρg/ml may introduce bias [58]. In our investigation the majority of plasma samples were indeed below 31.25 ρg/ml and therefore susceptible to unknown bias. "Undetectable" plasma samples, which had absorbance value below our lowest standard of 0 ρg/ml VEGF, were allocated a value 1 ρg/ml, then adjusted for anticoagulant and haematocrit before they were used for further analysis. This adjustment was unlikely to alter the results significantly, as all adjusted values were still below 2 ρg/ml. On the other hand Hormbrey and colleagues recommended allocating all plasma values below 31.25 ρg/ml to 31.25 ρg/ml, provided there was a magnitude of difference when comparing the groups of interest, using values below 31.25 ρg/ml may still offer valid conclusions [58]. From our search we were unable to find another commercial ELISA kit that was considerably more superior in sensitivity (such as one

that was at least one order of magnitude lower). Another commercially available set, QuantiGlo (R&D Systems) offered a sensitivity of 1.61-5.99 ρg/ml (mean 3.30 ρg/ml, compared to 9 ρg/ml) but at a higher cost, therefore we did not choose this kit. A possible advantage of using a kit that has been widely used is that results may be more comparable between studies.

3.2 Study Participants

Key information on participants is summarized below, for more details on individual participants, please refer to the Appendix, page 137.

3.2.1 Cohort 1

Cohort 1 participants consisted of 18 women who had had past history of ovarian and/or endometrial malignancy but were currently without any clinical evidence of disease recurrence. After an outpatient follow-up appointment at the Christchurch Gynaecologic Oncology service in Christchurch Women's Hospital they were referred by an oncology gynaecologist to the principle investigator for informed consent and recruitment for the study. There were 8 women who had had ovarian cancer, and 10 who had had endometrial cancer. All malignancies were epithelial in histological origin except one which was a carcinosarcoma of the uterus. The median age was 57, and ranged between 31 and 79, and only 1 woman was pre-menopausal. Common

co-morbidity for all women was as follows: 2 were overweight or obese (BMI > 25), 5 had hypertension, 1 had coronary artery disease, one had metabolic syndrome with impaired fasting glucose and none had diabetes. 7 women were not on any medication, and for the 11 on medication, 1 was on oral contraceptives, 2 were on hormone replacement therapy, and 3 admitted taking nutritional supplements.

3.2.2 Cohort 2

Cohort 2 participants included 7 women who currently had clinical evidence of ovarian or endometrial cancer, and were not receiving active treatments for cancer. Similar to Cohort 1 they were referred to the principle investigator by their respective gynaecological oncologists. Only one woman had endometrial cancer, while the rest had ovarian cancer and all had had curative treatment in the past but the cancer had recurred. The median age was 65 and ranged between 59 and 84. Common co-morbidity included hypertension (2), cerebrovascular accident (1) and hypercholesterolaemia (1). There were no women with diabetes. Metoprolol was the most common medication for which 2 women were currently on, 1 admitted taking nutritional supplements and 2 were not on any long term medication.

3.2.3 Cohort 3

24 women who were to undergo surgery for suspected ovarian and/or endometrial cancer were recruited for Cohort 3. They were typically recruited after the preadmissions appointment at the Christchurch Gynaecologic Oncology service in

Christchurch Women's Hospital. The median age was 70.5, and ranged from 31 to 85. 6 participants were found to have benign disease, while 9 had endometrial cancer and 9 had ovarian cancer. 8 out of 9 woman of each type had cancer of epithelial origin. Stage I and Stage III disease were the most common stage in endometrial (5 women), and ovarian cancer (4 women), respectively. Common co-morbidity consisted of hypertension (11), hyperlipidaemia (2), Type 2 diabetes (2) and obesity (2). Only 1 woman was not on any medications and the most common medications were bendrofluazide (7), paracetamol (6), aspirin (6), omeprazole (4) and felodipine (4). 3 women admitted taking supplements.

3.2.4 Cohort 4

The 21 participants Cohort 4 could be broken down into two sub-groups – the disease group (15) and the control group (6). They were to participate in a pilot clinical trial of green tea extracts. The characteristics for disease and control groups were similar to Cohort 2 and Cohort 1, respectively. The disease group consisted of women who had active endometrial and/or ovarian cancer and were not on treatment, where as the control group were women who had had one of these cancers and were currently without evidence of disease. In fact, prior to this trial, 3 women in the disease group also participated in Cohort 2, and all women in the control group participated in Cohort 1. In contrast, the majority of women (12/15) had persistent disease, defined as not having a period where the cancer was in remission since diagnosis. The median age was 61 for the disease group (range 31-78) and 55 for the control group (range 48-77). In the disease group, 14 had ovarian cancer, while one had endometrial cancer, and all were epithelial in origin except for one woman who had

epithelioid mesothelioma. In the disease group, 4 women had hypertension and there were no other co-morbidity which more than one participant had. In the control group the most common co-morbidity was hypertension (3), followed by osteoarthritis (2). 3 and 1 women were not taking any medications in the disease and control group, respectively. Omeprazole and fluoxetine were the most common medications, which were both taken by three women, while paracetamol was the most common in the control group taken by two women.

In summary in our investigation into blood VEGF levels in women with endometrial and ovarian cancer, we recruited four cohorts of women, who were mostly post-menopausal. The control groups (Cohort 1 and Cohort 4 controls), with a total of 18 women were without cancer but whom we could assume to have similar characteristics as those with cancer, as the controls had had past history of these cancers. The disease groups consisted of 7 women in Cohort 2, 3 of which also participated in Cohort 4 disease group (18 women in total). They were not on any treatment and allowed us to study changes in VEGF over time in cancer progression, as well as the effect of a short term course of green tea extracts. In addition, Cohort 3 had surgery for suspected cancer and those who had malignant disease would provide insight as to whether VEGF changes after surgical resection of the disease.

3.3 Plasma Anticoagulant Type

To obtain plasma, blood is drawn into tubes containing anticoagulants, as opposed to serum, which is the liquid that remains after clotting. It had been previously shown by others that VEGF values measured by ELISA varied according the anticoagulant preparation in the blood collection tube. In order to determine the most appropriate anticoagulant for the measurement of plasma VEGF, for the first 37 blood samples of this study (across 8 participants) plasma were taken in two tubes in one venepuncture procedure; tubes containing EDTA, and tubes containing sodium citrate. The plasma VEGF concentrations of these two matched samples were analysed by ELISA, of which concentrations measured in sodium citrate tubes were adjusted for liquid anticoagulant volume and haematocrit. This modification was not necessary for VEGF measured in EDTA tubes as EDTA was present as a solid, and raw ELISA values were used. Of the eight participants, half were currently without evidence of cancer, while the other half had active cancer. As blood VEGF levels had been shown to be higher in patients with cancer, this would allow us to explore how (if any) the choice of anticoagulant influenced a wide range of VEGF concentrations.

Plasma samples were divided into two groups according to disease status. The Control group consisted of 19 paired samples from 4 participants in Cohort 1. The Disease group had a total of 18 samples – 3 pre-surgery samples from 3 participants in Cohort 3 and 15 serial weekly samples for 3 participants in Cohort 2. All three participants from Cohort 3 had cancer, which was either established before the surgery (one woman, for palliative surgery) or during surgery, which involved confirmation of the incised tissues/organs (for example the uterus) by a pathologist.

The scatter plots of the individual pairs are shown below for Control group

(Figure 3-1) and Disease group (Figure 3-2)

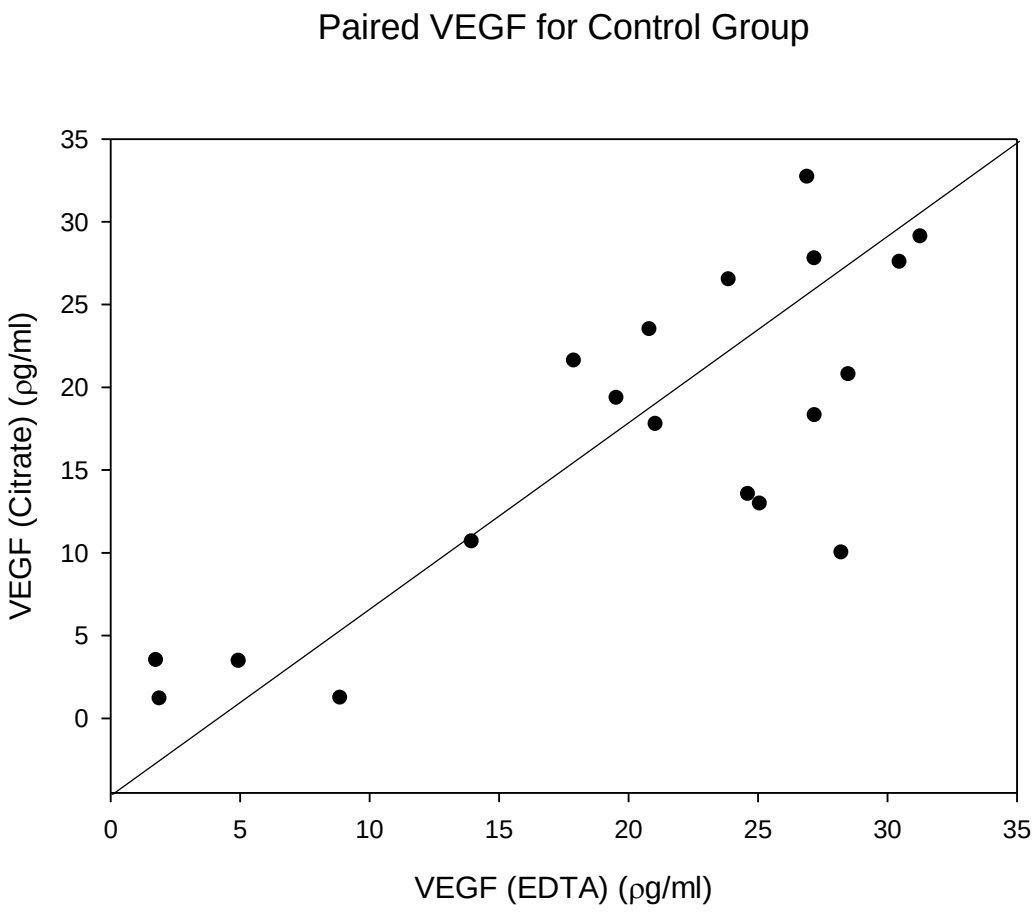


Figure 3-1 Control group VEGF in plasma from EDTA versus VEGF in plasma from citrate tubes. Plasma VEGF processed from EDTA tubes was plotted against matched sample from sodium citrate tubes in Control group - women currently without cancer. Line represents y = x

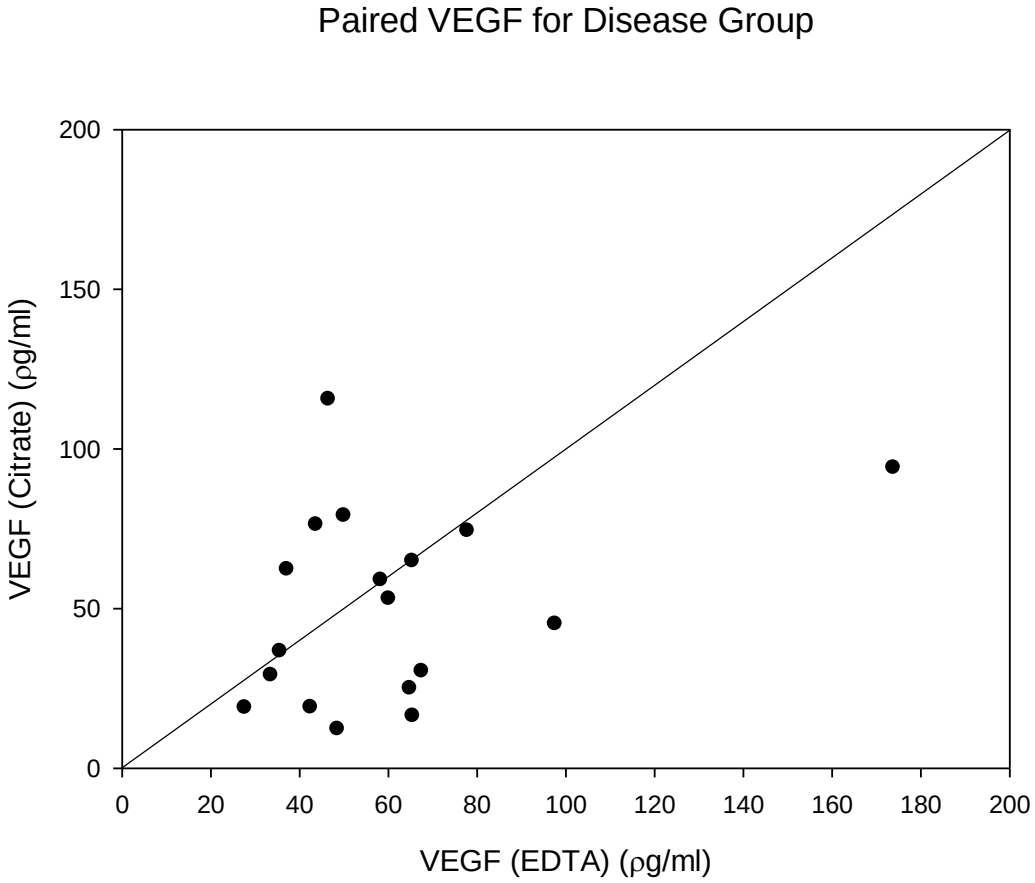


Figure 3-2 Disease group VEGF in plasma from EDTA versus VEGF in plasma from citrate tubes. Plasma VEGF processed from EDTA tubes was plotted against matched sample from sodium citrate tubes in disease group - women with cancer, Cohorts 2 and 3. Line represents y = x

On first observation, the values do not fall on the line of equivalence (y = x), with a higher proportion below the line (10/19 for control group, 12/18 for disease group), indicating that VEGF levels in samples with EDTA as anticoagulant are higher than with citrate, without taking into the account the magnitude of the differences.

EDTA and citrate plasmas had statistically significant different levels of VEGF concentrations (Figure 3-3) as analysed by the paired t-test. VEGF in EDTA plasma (mean, 20 ρg/ml; SD 10 ρg/ml) samples are higher than in matched citrated plasma (mean, 17 ρg/ml; SD 10 ρg/ml), in control (mean difference EDTA vs Citrate, 3 ρg/ml ; 95% CI, 0.3-6 ρg/ml, $P < 0.031$). The trend was similar in the disease group, but this difference was not significant (mean difference EDTA vs Citrate, 10 ρg/ml; 95% CI, -28-8 ρg/ml, $P < 0.24$)

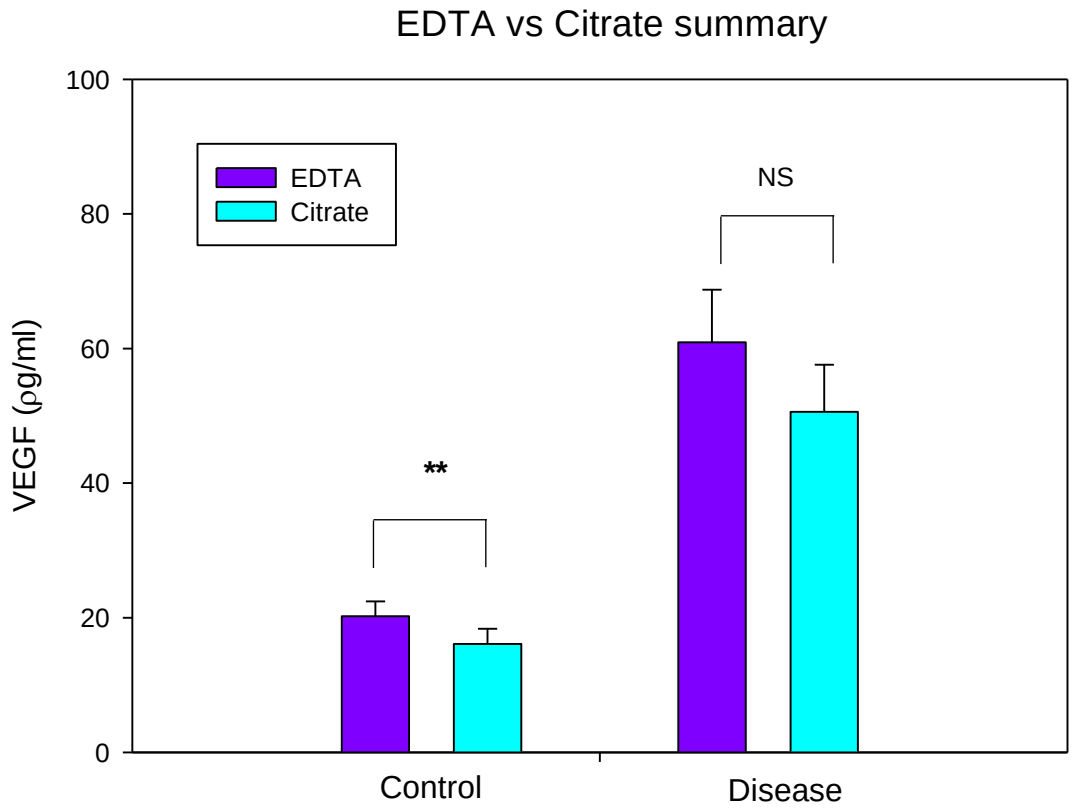


Figure 3-3 Comparison of plasma VEGF taken from EDTA versus Citrate tubes. VEGF concentrations measured in blood collection tubes containing EDTA were compared to ones containing sodium citrate, for women both with and without active cancer (disease). Mean + SEM is graphed.
NS = not significant; ** *P* < 0.031 (paired t-test)

Plots of the difference between the two measurements in the two anticoagulants against the mean of each pair are shown in Figure 3-4. The bias (mean of EDTA minus citrate) and the limits of agreement (± 2 SD of the mean differences) are graphed in this type of analysis [138]. The bias ± 2 SD is 7 ± 51 ρg/ml for all the samples together or separately, 3 ± 12 (mean of the difference ± 2SD) ρg/ml for the control group (full circle, Figure 3-4) and 10 ± 72 ρg/ml for the disease group (empty triangles,

Figure 3-4). The positive value of the mean differences (overall, and separately in both groups) indicates that VEGF levels were higher in blood with EDTA as anticoagulant than citrate. Note that the limits of agreement were also wider in the disease group compared to the control group, and there is increased variability as the concentration of VEGF increases. To determine whether this increase in variability was a consequence of the higher VEGF levels, raw EDTA and citrate VEGF values were $\log_{10}$ transformed and graphed on a second Bland-Altman analysis (Figure 3-5). In this plot the increase in variation for the disease group is not evident, which provides proof that for the untransformed values, the increase of variability of VEGF (absolute differences, EDTA – citrate) from left to right is related to the increase of concentration of VEGF measured.

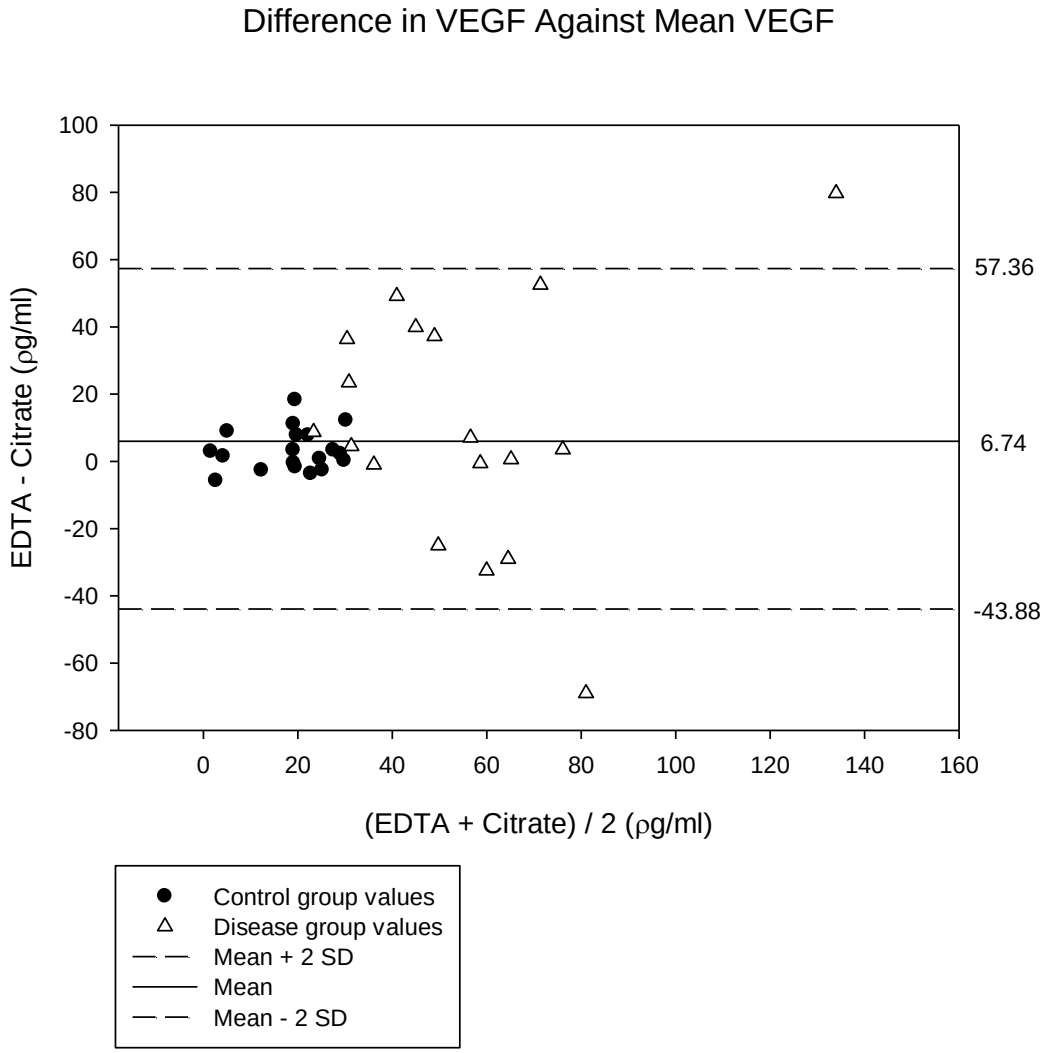


Figure 3-4 Bland-Altman analysis showing agreement of VEGF concentrations between EDTA plasma and citrate plasma, raw data. The VEGF difference between the two samples (EDTA - citrate) was plotted against their average (EDTA + Citrate) / 2. The bias (mean difference, solid line) and limits of agreement (2 x SD, two dashed lines) are shown. Values for the parameters are shown or beside the line.

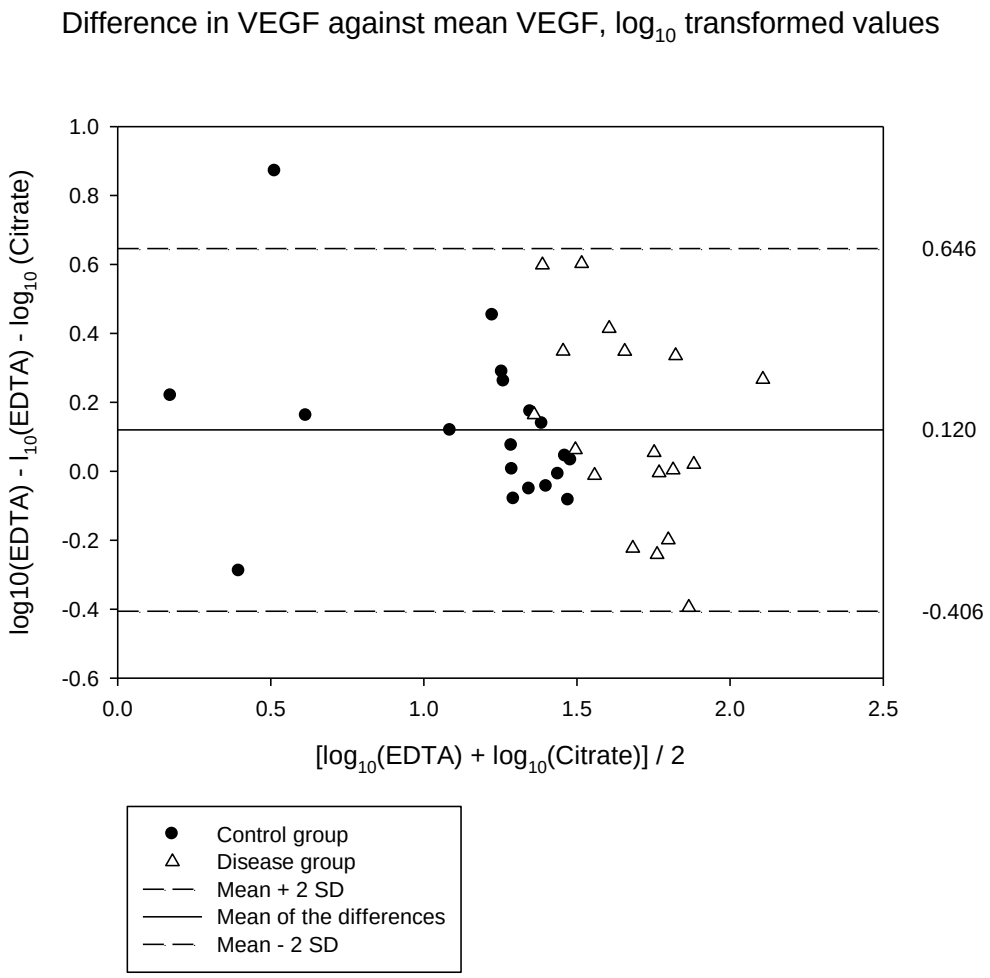


Figure 3-5 Bland-Altman analysis showing agreement of VEGF concentrations between EDTA plasma and citrate plasma, log_{10} transformed data. The VEGF difference between the two samples (EDTA - citrate) was plotted against their average (EDTA + Citrate) / 2 in log_{10} transformed data. The bias (mean difference, solid line) and limits of agreement (2 x SD, two dashed lines) are shown. Values for the parameters are shown or beside the line.

3.3.1 Discussion

Measurement of VEGF in the plasma is the best approximate of *in vivo* circulating levels, because serum contains VEGF that is platelet-derived and released into the serum as the blood clots in the collection tube. Plasma however is collected into tubes containing anticoagulant and prepared by centrifugation of the blood. Coagulation and release of platelet-derived VEGF is minimised. Plasma VEGF therefore may reflect circulating levels in the blood. This process, however, is currently not standardised internationally and there is debate about which anticoagulant should be used for the measurement of VEGF in investigations that aim to study plasma VEGF. EDTA is one of the most widely used anticoagulants to prepare plasma. However, several investigators had found higher VEGF concentrations in samples with EDTA compared to matched samples with sodium citrate [44, 59]. Measuring platelet activation alongside growth factor release, it was found that citrate inhibited platelet activation more so than EDTA [60], therefore the higher concentrations were presumably due to higher degree of platelet activation, which would provide inaccurate estimates of the soluble, circulating levels.

In order to decide on the appropriate anticoagulant for use in our studies measurement of matched samples taken in EDTA and citrate tubes was performed. Comparing EDTA and citrate plasma is effectively comparing two methods of clinical measurement of the same substance. To clearly assess and describe the between-method differences, Bland and Altman proposed a plot of the difference against the mean [138], which has the advantage over Figure 3-3 of showing both the

direction and magnitude of the differences for each individual pair. The limits of agreement are set at two standard deviations on either side of the mean differences, which would include 96% of differences [138].

From the Bland-Altman plots, as seen in Figure 3-4 and Figure 3-5, overall the VEGF concentration was higher when measured in EDTA than citrate, with a bias (mean difference) 7 ρg/ml or 32% ($10^{0.120}$). Interestingly, although the bias was greater in the disease group (10 versus 3 ρg/ml), where higher VEGF concentrations were observed, the mean percentage difference, were similar, and they were 31.8% ($10^{0.120}$) and for the control group and 31.5% ($10^{0.119}$). This is consistent with the observations made by Banks' group [44] and Hetland's group [59]. EDTA had been shown, by measuring the amount Platelet Factor 4, an *in vitro* indicator of activation to be a weaker inhibitor of platelet activation than citrate [60].

Raw data plots (Figure 3-4) appeared to be of a "classic fan shape" type distribution – the data points became more spread out from left to right, which implied that the variability increased as the plasma VEGF measure increased. The visual scattering of the data plots became markedly reduced, after $\log_{10}$ transformation of the raw values (Figure 3-5), which further confirmed that the variability was a proportion of the measured values, rather than a fixed constant. It appears likely therefore that the variability in measurement of plasma VEGF is a proportion of the absolute level. This apparent variability would be greater in those with higher VEGF levels. To our knowledge, the above analysis had not been reported before when VEGF was measured in plasma prepared from various anticoagulants.

The increased variability that was associated with higher plasma VEGF was consistent with previous reports. It has been shown that cancer patients not only have higher plasma VEGF concentrations than people without cancer [20, 49], their platelets also contain more VEGF [51]. EDTA had not only been shown to be less effective in inhibiting *in vitro* platelet activation, there was high inter-individual variability in the degree of activation [139]. Therefore it is plausible that the high variability of the difference between levels measured in citrate and EDTA plasma at higher VEGF concentrations (disease group) reflected platelet-released VEGF; the release of VEGF from incomplete platelet inhibition by EDTA is accentuated because platelets from cancer patients contain more VEGF. . The observations of Lee and colleagues [51] would also explain how the difference between EDTA and citrate plasma was more pronounced in our disease group than in the control group but interestingly the proportion difference was similar between the two groups. It is not surprising that some authors considered EDTA plasma unsuitable for measuring plasma angiogenic growth factors which are also released upon platelet activation [139].

The best anticoagulant, however, has been suggested to be CTAD (citrate-theophylline-dipyridamole-adenosine) [139]. Dittadi and Zimmermann showed using different markers respectively that CTAD produced lowest platelet activation [57, 60]. While CTAD is reported to be the preferred anticoagulant for VEGF measurement in plasma, the purpose of this study was to compare those anticoagulants clinically available in Christchurch.

In summary, we observed there were differences between EDTA plasma and citrate plasma, with VEGF in citrated samples lower in magnitude and variability compared to EDTA samples in women with and without cancer. This leads us to question to what extent VEGF in plasma is in fact an artifact, and not a true measurement of circulating levels. We therefore consider that sodium citrate may offer closer approximation to circulating VEGF levels. This is likely to be due to reduced inhibition of platelet activation by EDTA. Our data support the conclusion that citrate is a superior anticoagulant for the measurement of plasma VEGF. Hence subsequent measurements of plasma VEGF concentrations were performed with citrated samples. In addition, variability appears to be proportional to the absolute value of VEGF. Therefore in order to facilitate making comparisons between high and low VEGF concentrations, in subsequent analysis VEGF data were transformed by taking the logarithm to the base 10, unless otherwise specified.

3.4 Relationships: Serum VEGF, plasma VEGF, platelets and CRP

Debate still exists as which specimen represents the best measure of *in vivo* circulating VEGF levels. Serum is the liquid component of blood after *in vitro* coagulation in tubes, where as plasma is prepared from blood to which anticoagulant(s) has been added. Both serum VEGF and plasma VEGF have been shown to correlate with cancer progression. In VEGF studies of ovarian and endometrial cancer, serum

had been widely adopted, however, relatively little is known about the relationship between serum measurements and plasma measurements. Serum VEGF is higher than plasma VEGF as serum contains VEGF degranulated from α-granules during *in vitro* activation of platelets. Inflammation had been associated with increased serum and plasma VEGF, and this may be independent of cancer disease progression, hence we investigated the correlation of VEGF levels with a short-term inflammatory marker, C-Reactive Protein (CRP).

The results of the first blood specimen from all participants of Cohorts 1, 2, 3, and 4 were combined for this analysis. Each specimen contributed a set of plasma VEGF, serum VEGF, platelet count, haematocrit and C-Reactive Protein (Cohort 1, 2 and 3 only) (Please refer to Methods Chapter 2 for more details on participant selection). Where participants had taken part in more than one Cohort (such as Cohort 2 and Cohort 4), the first chronological specimen was used, so each participant provided no more than one set of data. All blood tests from Cohort 3 were pre-surgery specimens. In two cases (one from Cohort 1, one from Cohort 2), the second week sample was used to obtain complete haematological data. 18 sets were available from Cohort 1, 7 from Cohort 2, 24 from Cohort 3, and 12 from Cohort 4, giving 61 sets in total.

3.4.1 Serum VEGF, plasma VEGF and platelets

Since both serum VEGF and plasma VEGF were skewed, logarithm-transformed data was used. Serum VEGF was higher than plasma VEGF, geometric mean 366 vs 20 ρg/ml, $P < 0.001$, n = 61 [two-sample t-test (equal variances), log transformed data].

Figure 3-6 below illustrates the correlation between serum VEGF and plasma VEGF. Serum VEGF concentrations correlated with plasma VEGF concentrations (log transformed data, Pearson Product Moment Correlation, r = 0.51, $P < 0.00003$).

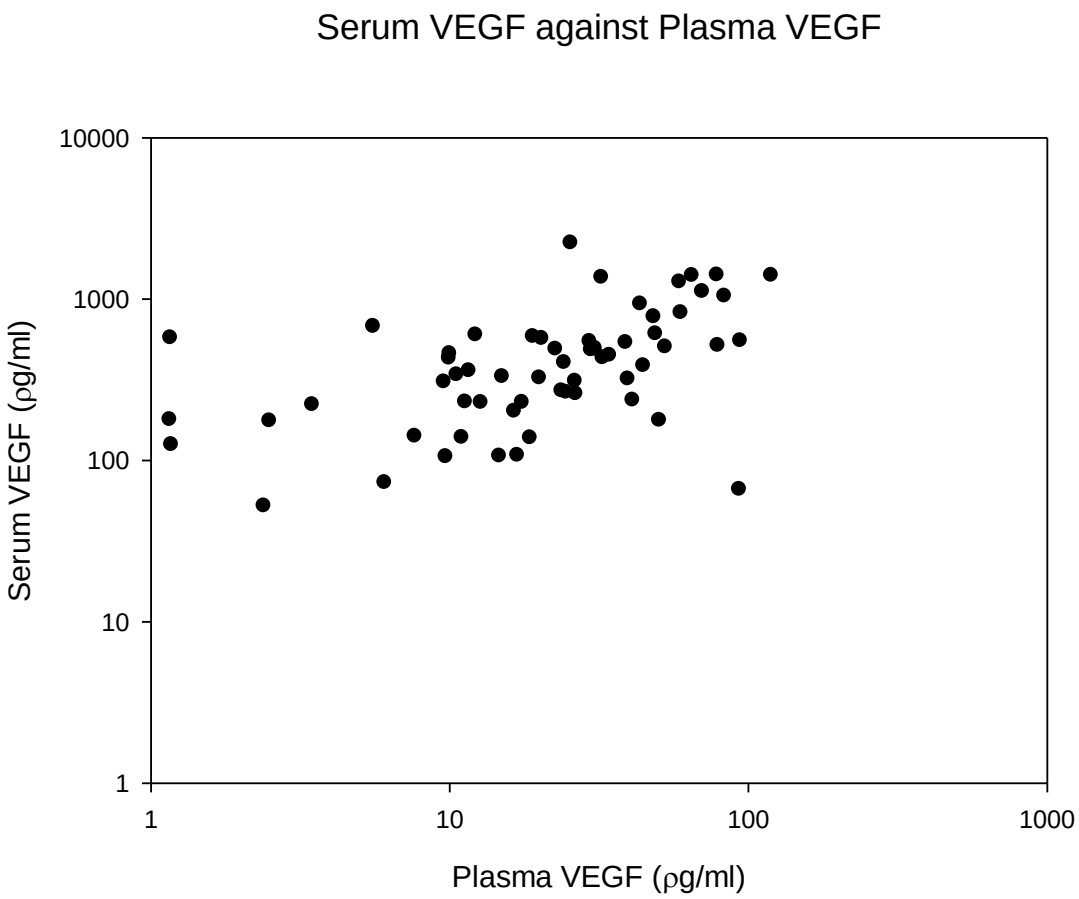


Figure 3-6 Correlation between Serum VEGF and Plasma VEGF. Matched serum and plasma VEGF concentrations from 61 women were plotted against each other. Both axis are $\log_{10}$ scales.

A plot of the difference against the mean (Bland-Altman analysis) is shown below in Figure 3-7. Serum VEGF was higher than plasma VEGF, on average by 18 fold ($10^{1.26}$), with limits of agreement (95% of values) between 128 ($10^{2.11}$) and 3 ($10^{0.41}$) folds.

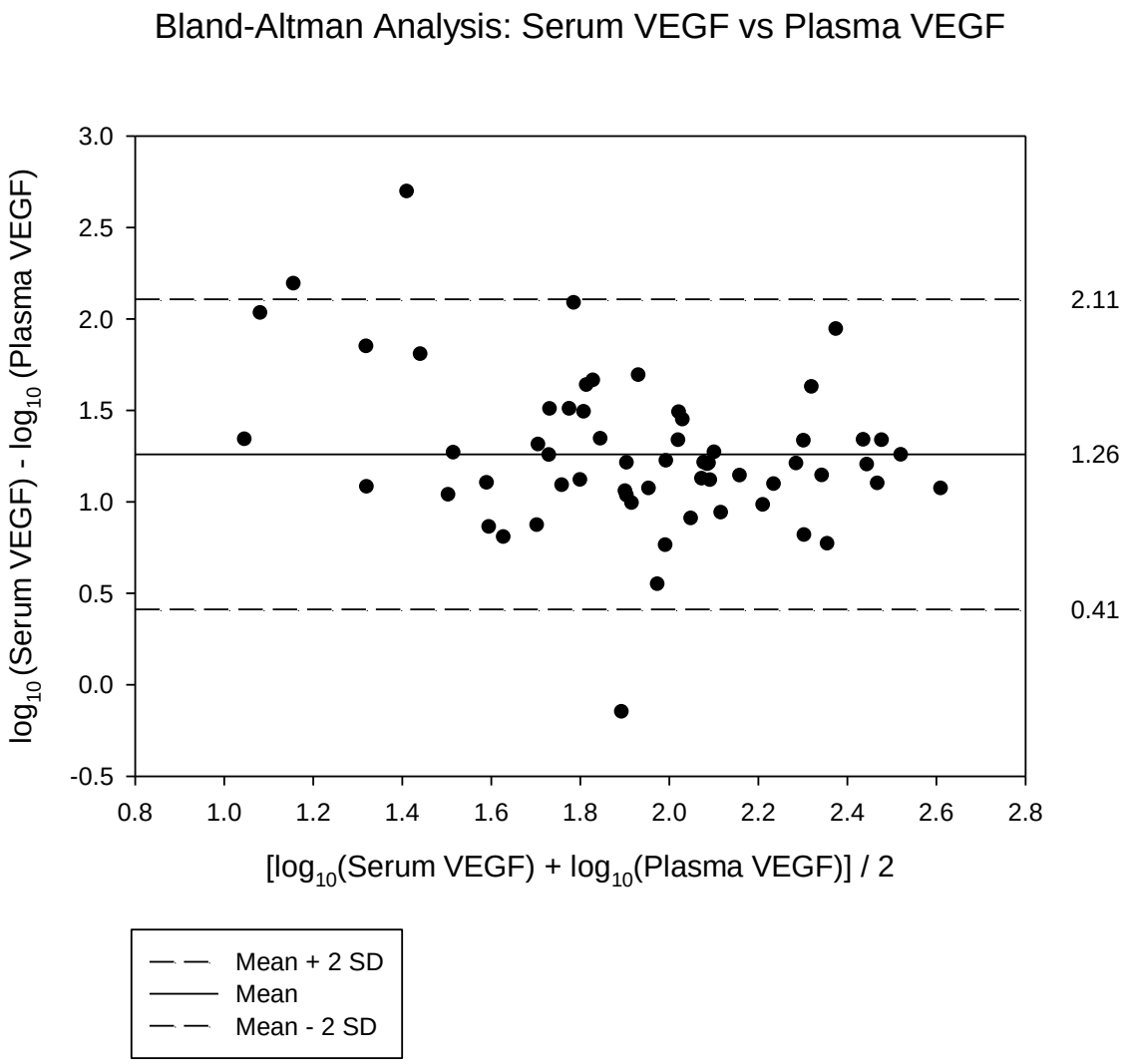


Figure 3-7 Bland-Altman analysis showing agreement between serum VEGF and plasma VEGF. One blood specimen from each of 61 individual women across the entire project was processed to obtain serum and plasma. The difference (log_{10}Serum – log_{10}Plasma) was plotted against the average (log_{10}Serum + log_{10}Plasma) / 2. The bias (mean difference, solid line) and limits of agreement, (2 x SD, two dashed lines) are shown, with their values beside the corresponding line. Note one point had a difference below zero, this was due to one woman having higher plasma VEGF than serum VEGF.

A platelet count was obtained with every blood test. As the platelet count data was also skewed, logarithmic transformation was carried out. Serum VEGF concentrations ($\log_{10}$ transformed) correlated with platelet count ($\log_{10}$ transformed) (Pearson Product Moment Correlation, r = 0.44, $P < 0.0004$, n = 61), which is shown in Figure 3-8 below. Plasma VEGF ($\log_{10}$ transformed), on the other hand, did not correlate with platelet count ($\log_{10}$ transformed) (Pearson Product Moment Correlation, r = 0.14, $P < 0.29$), shown in Figure 3-9 below.

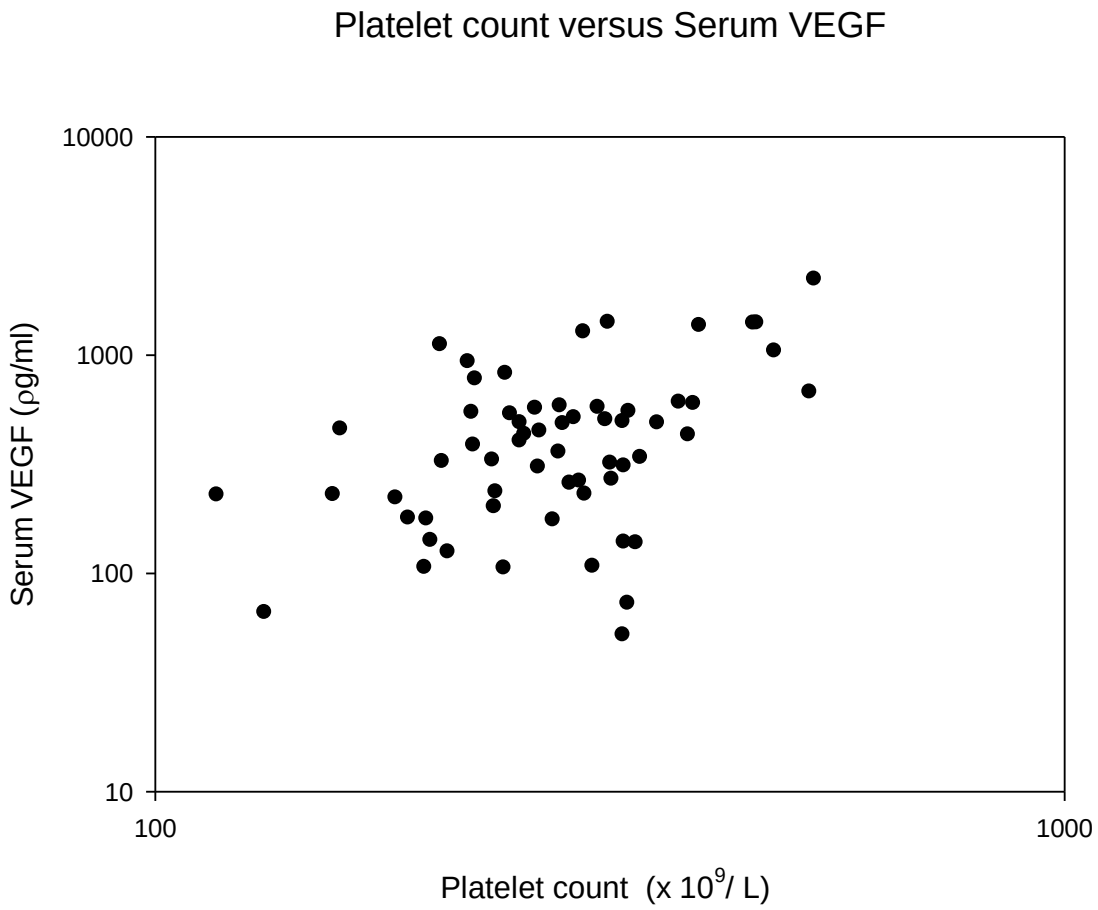


Figure 3-8 Correlation between platelet count and serum VEGF. One blood specimen from 61 women across the project was processed to obtain serum VEGF and platelet count. They were plotted against each other to show correlation. Serum VEGF is on a $\log_{10}$ scale.

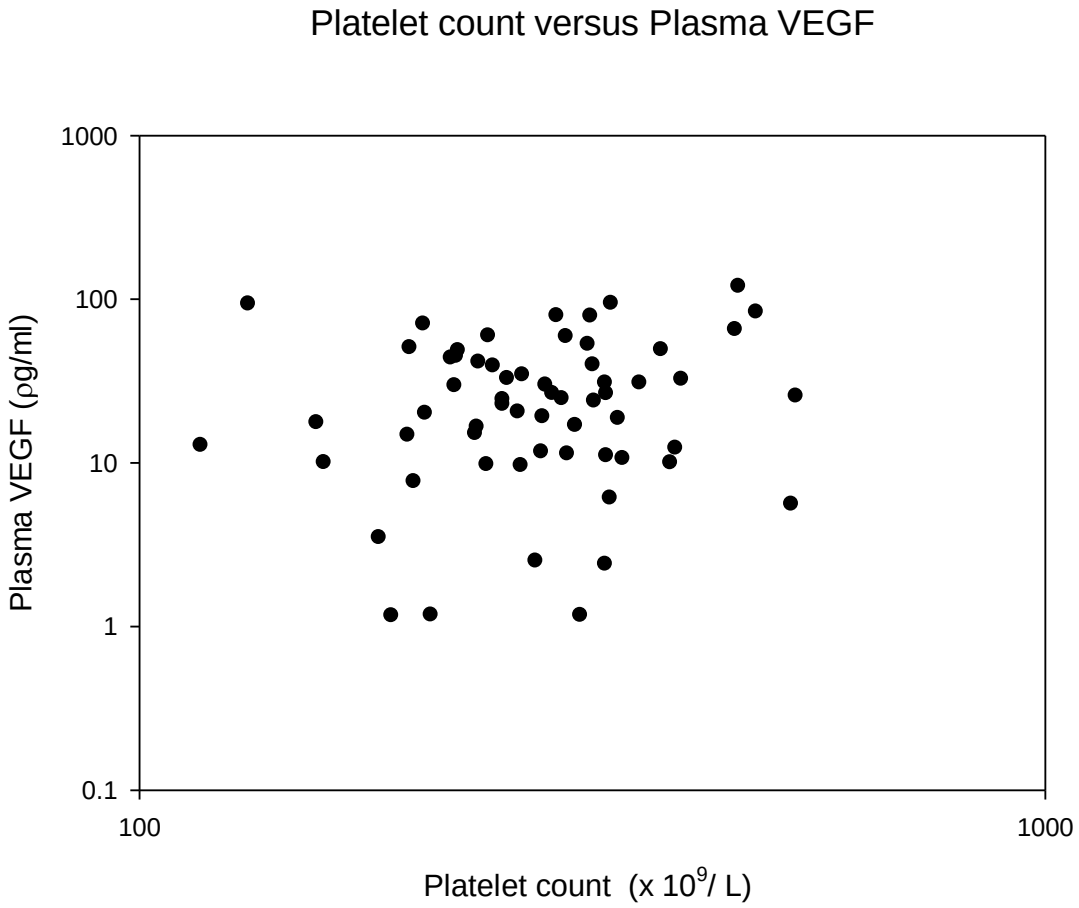


Figure 3-9 Correlation between platelet count and plasma VEGF. One blood specimen from 61 women across the project was processed to obtain plasma VEGF and platelet count. They were plotted against each other to show correlation. Plasma VEGF is on a $\log_{10}$ scale.

CRP was available in 49 specimens of Cohort 1, 2 and 3. As the CRP data were skewed, logarithmic transformation was also carried out. CRP (log_{10} transformed) correlated with serum VEGF (log_{10} transformed) ($r = 0.35$, $P < 0.01$, Pearson Product Moment Correlation, however CRP (log_{10} transformed) did not correlate with plasma VEGF (log_{10} transformed) ($r = 0.09$, $P < 0.52$). CRP (log_{10} transformed) also correlated with platelet count (log_{10} transformed) ($r = 0.33$, $P < 0.02$).

Although we did not measure the VEGF content in platelets directly, an indirect calculation of VEGF per platelet can be obtained using the formula: VEGF (ρg) / 10^6 platelets = (VEGF serum – VEGF plasma) × (1 – haematocrit) / platelet number [136]. Using the 61 specimens as described earlier, they were separated into one group of women with cancer and one group of women without cancer. 24 and 37 specimens were from women without and with cancer, respectively. Of the non-cancer group, one specimen from a Cohort 1 participant (1-16) was excluded because she had polyarteritis nodosa and metabolic syndrome, two conditions that might have contributed to her high serum VEGF, leaving a total of 23 available for analysis. The cancer group consisted of 37 specimens, of which one was excluded because the plasma VEGF was higher than the corresponding serum VEGF, which would result in a negative value.

VEGF per platelet was 1.17 ± 0.77 (mean ± SD) ρg / 10^6 platelets in cancer patients and 0.75 ± 0.56 ρg / 10^6 platelets in non-cancer patients. It was significantly higher in cancer patients compared to non-cancer patients (mean difference = 0.42 ρg / 10^6 platelets, 95% confidence intervals 0.046 to 0.79, $P < 0.03$, t-test equal variance).

3.4.2 Discussion

Previous reports had shown that serum VEGF is much higher than plasma VEGF, presumable due to the addition of platelet derived VEGF that happens during *in vitro* coagulation to prepare serum. In ovarian cancer, serum VEGF has emerged to be an important marker of prognosis; preoperative serum VEGF, in a pooled data of 314 cases, had been shown to be an independent prognostic parameter after multivariate analysis in all stages of the disease [32]. The majority of studies on circulating VEGF in ovarian cancer used serum, although both serum and plasma had been shown to be indicators of prognosis in studies of other cancers [20].

Our results showed that consistent with previous reports, serum VEGF was much higher than plasma VEGF and they correlated with each other. Using the Bland-Altman analysis (Figure 3-7) there appeared to be a constant multiplicative relationship between serum VEGF and plasma VEGF, and on average serum VEGF was 18 times the value of plasma VEGF. This had not been reported in literature previously. Most anticoagulants, including sodium citrate used in our investigation, have been shown to activate platelets to some extent [57]. Therefore plasma and serum measurements may be similar in that both reflect platelet VEGF, just to different extents. While we showed a positive correlation between serum VEGF and platelet count, in contrary to this hypothesis, plasma VEGF did not correlate with platelet count, and this might be related to the inherent low levels of plasma VEGF, which meant they were more susceptible to random errors.

Platelets have been hypothesised as scavengers of VEGF, which take up and store excess VEGF produced by solid tumours [52]. This tumour derived VEGF is then released into serum upon *in vitro* coagulation, or exists in equilibrium with plasma, or "free" VEGF (with minimal release during blood taking process), therefore both may still be valid as an estimate of the local VEGF secretion of tumours.

In measuring blood VEGF, we hope to obtain an estimate local production of VEGF by tumours and gain insight to tumour behaviour and activity. As platelet count can contribute to variation in serum VEGF levels and indeed using $\log_{10}$ transformed data our results indicated that they correlate positively (r=0.44), one criticism of using serum VEGF as estimate of circulating VEGF is that it simply reflects platelet count [44, 48]. Using an indirect measure of VEGF content in platelets, standardised by platelet count, Lee and colleagues demonstrated that in platelet-derived VEGF was higher in cancer patients (with normal platelet counts) than in controls [51]. Using the same method, we have confirmed that platelet-derived VEGF tended to be higher in patients with endometrial and/or ovarian cancer than in women without cancer. This supports Lee and colleagues' conclusion that cancer influences the variation in platelet-derived VEGF contents and therefore the use of serum VEGF does to some extent reflect cancer biology. As a cautionary note, plateletpheresis (lysis of platelets) using detergents have been reported to release much more growth factors [140] (although VEGF was not investigated) than *in vitro* clotting process, of which serum is obtained from blood. There exists a possibility that VEGF content in platelets may be similar irrespective of the presence of cancer, however tumours may influence platelets (for example releasing chemicals that act on the bone marrow), causing them to release more VEGF during

coagulation. Direct analysis of platelet VEGF contents in cancer patients is needed.

The measurement of serum VEGF has the practical advantage over plasma in that values are higher and well above the detection limit of the current ELISA assay, thus more reliable. It has been shown that serum VEGF is stable after two hours clotting time (presumably "complete" platelet release), and therefore may be less subject to variability due to processing procedures such as repeated freezing and thawing, which appeared to affect plasma but not serum VEGF [59].

A CRP was obtained with each blood test in order because acute viral and bacterial infections, as well as inflammatory conditions such as rheumatoid arthritis had been associated with elevated serum VEGF [62, 75] and be the cause of markedly elevated VEGF levels. We found that CRP correlated with serum VEGF ($r=0.35$), which is possibly explained by an increase in platelet numbers and/or platelet activation associated with the acute phase reaction in inflammation. This also raises the question that increases in VEGF in cancer is mediated via inflammatory processes.

One outlier of note is a pair of plasma and serum measurements of which the plasma is higher than serum. No apparent technical error was detected after checking individual values of the duplicate readings as well as results that were analysed on the same ELISA plate. The woman's (participant 4-012) clinical file was reviewed; she had no co-morbidity and she was not on any medications other than supplements (please refer to Table 5-4 in the Appendix VEGF Levels of All Participants for more information). Therefore we were not able to provide an explanation as to why her

plasma VEGF level was higher than her serum VEGF level.

In summary we have shown for the first time that there is multiplicative relationship between plasma VEGF and serum VEGF. While serum VEGF correlated with both plasma VEGF and platelet count, platelet count did not correlate with plasma VEGF. Although both measurements might reflect tumour activity, the inherent low values of plasma VEGF renders it less practical than serum VEGF. Serum VEGF is more influenced by platelet-derived VEGF, however this may also reflect cancer biology as we have also shown indirectly that platelet-derived VEGF is greater in women with cancer. CRP also correlated with serum VEGF and indicates inflammation as a possible mechanism in increased blood VEGF levels observed in cancer patients.

3.5 Reference Ranges

Participants from all cohorts were separated into two categories – women who currently had malignant disease and women who did not have evidence of cancer, or the controls. The control group included Cohort 3 women who were subsequently found to have benign diseases. Reference ranges of the two groups were constructed using the first set of plasma and serum VEGF of from each participant. Because VEGF values in these groups were not normally distributed, the 5^{th} and 95^{th} percentile values were chosen to describe the data.

The plasma levels of VEGF in 24 controls varied between 1 ρg/ml and 79 ρg/ml (median 15 ρg/ml; 5-95th percentile 1-61 ρg/ml). Serum levels ranged between 52 ρg/ml and 1407 ρg/ml (median 297 ρg/ml; 5-95th percentile 90-1375 ρg/ml). For the 37 cancer patients, plasma VEGF varied between 6 ρg/ml and 119 ρg/ml (median 31 ρg/ml; 5-95th percentile 7-94 ρg/ml). Serum VEGF ranged between 66 ρg/ml to 2221 ρg/ml (median 488 ρg/ml; 5-95th percentile 86-1399 ρg/ml).

3.6 Serial Measurements of VEGF

3.6.1 Cohort 1

Serial measurements of serum and plasma VEGF were taken, over three months from women attending clinic for follow-up after treatments for ovarian and/or endometrial cancer, and who were without evidence of disease during the study period. These patients were recruited to establish a reference range of circulating VEGF levels in women without cancer, to assess variation over time, and to identify potential factors that influence VEGF levels.

Of the eighteen women, ten had been treated for endometrial cancer, of which eight were endometrioid adenocarcinoma, one was mixed serous and endometrioid adenocarcinoma and one was carcinosarcoma of the uterus. For the eight women with

past history of ovarian cancer, there were two clear cell ovarian carcinoma, five serous ovarian carcinoma (two borderline) and one mucinous borderline ovarian carcinoma.

A total of 99 specimens were available. Serum VEGF was significantly higher than plasma VEGF (296.433 (median) vs 19.322 pg/ml, $P < 0.001$, Wilcoxon signed rank test), and serum VEGF correlated with plasma VEGF ($r = 0.67$, $P < 0.0001$, Spearman rank order correlation). Plasma and serum VEGF of each participant are shown below in Figure 3-10 and Figure 3-11.

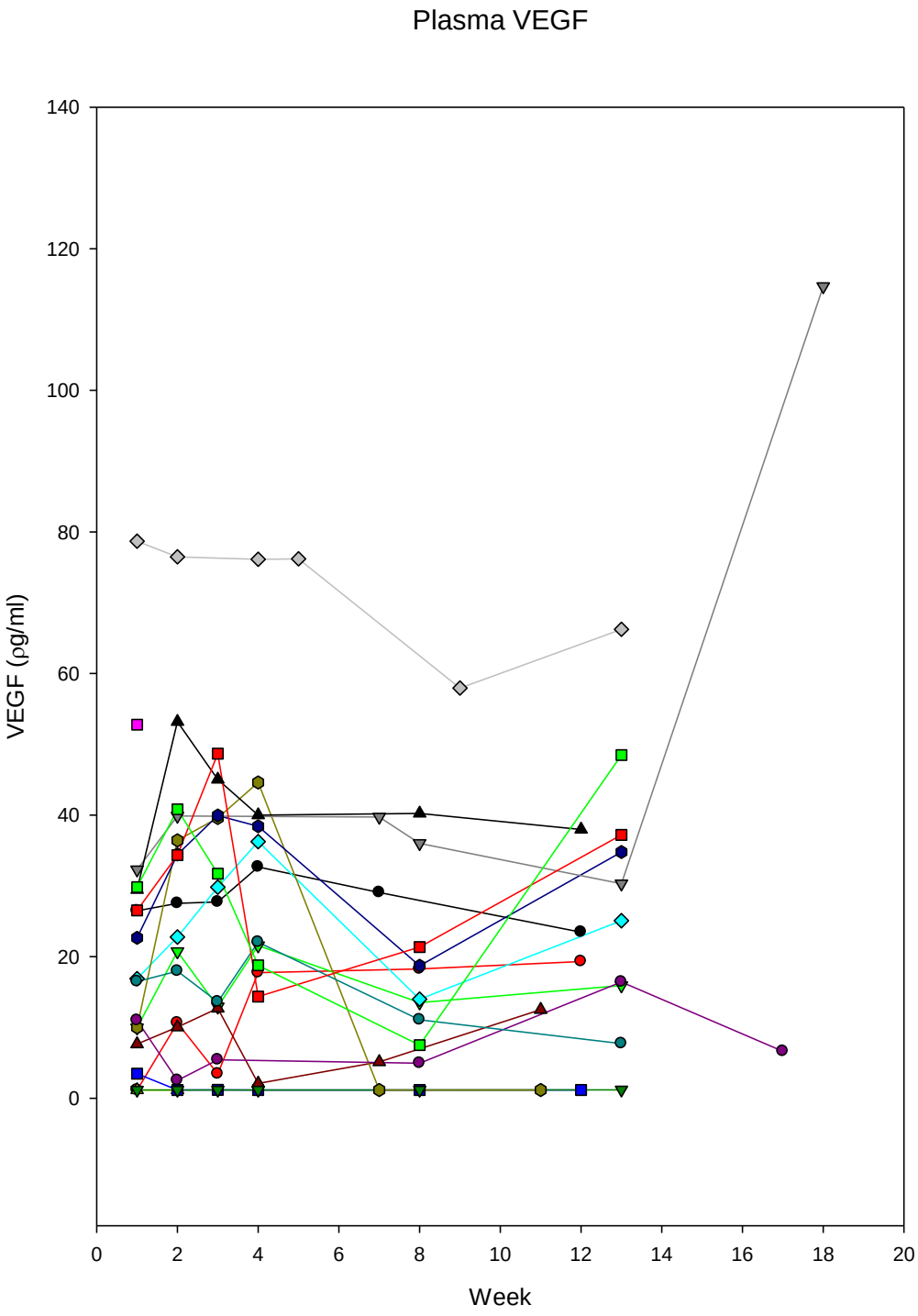


Figure 3-10 Serial VEGF measurements of women without evidence of cancer in plasma. Serial blood tests were performed in 18 women after treatment for ovarian or endometrial cancer, without evidence of disease, with a total of 99 specimens. Week 1 was defined as the first blood test, and after four days, every seven days were "bundled" together into subsequent weeks. VEGF concentration was analysed in plasma using ELISA. Each unique combination of line colour and symbol represents one participant.

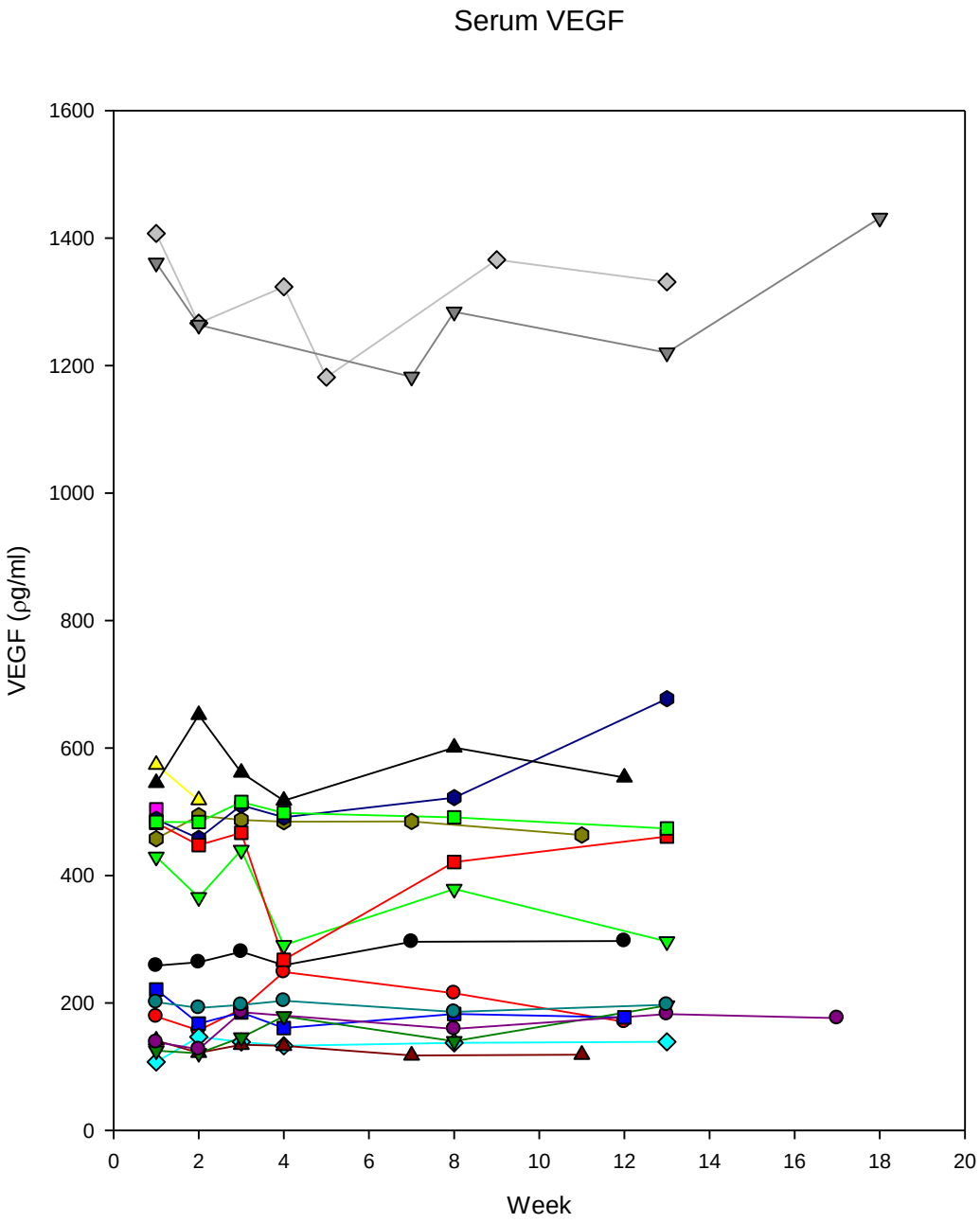


Figure 3-11 Serial VEGF measurements of women without evidence of cancer in serum. Serial blood tests were performed in 18 women after treatment for ovarian or endometrial cancer, without evidence of disease, with a total of 99 specimens. Week 1 was defined as the first blood test, and after four days, every seven days were "bundled" together into subsequent weeks. VEGF concentration was analysed in serum using ELISA. Each unique combination of line colour and symbol represents one participant.

Using all weekly VEGF data from Cohort 1, the geometric mean of plasma VEGF was 13 ρg/ml (5-95% interval: 1-65). Serum VEGF was 299 ρg/ml (5-95% interval: 123-1254).

3.6.2 Cohort 2

In order to establish a baseline level of blood VEGF in women with endometrial and ovarian cancer, observe its short term variability, and correlate VEGF with clinical features, up to six serial measurements of serum and plasma VEGF were taken over three months for women in Cohort 2. These women had clinical evidence of persistent or recurrent endometrial and ovarian cancer. Excluding the participants from whom no samples were drawn, this cohort consisted of 7 women and the number of paired serum and plasma for each woman ranged from 2 to 6, with a total of 32 paired samples. 1 participant had endometrial cancer and the rest were patients with ovarian cancer. Individual measurements of plasma and serum VEGF over time are shown below in Figure 3-12 and 3-13.

Using all VEGF data from Cohort 2, the geometric mean of plasma VEGF was 46 ρg/ml (5-95% interval: 19-118). The mean of serum VEGF was 822 ρg/ml (5-95% interval: 503-1766).

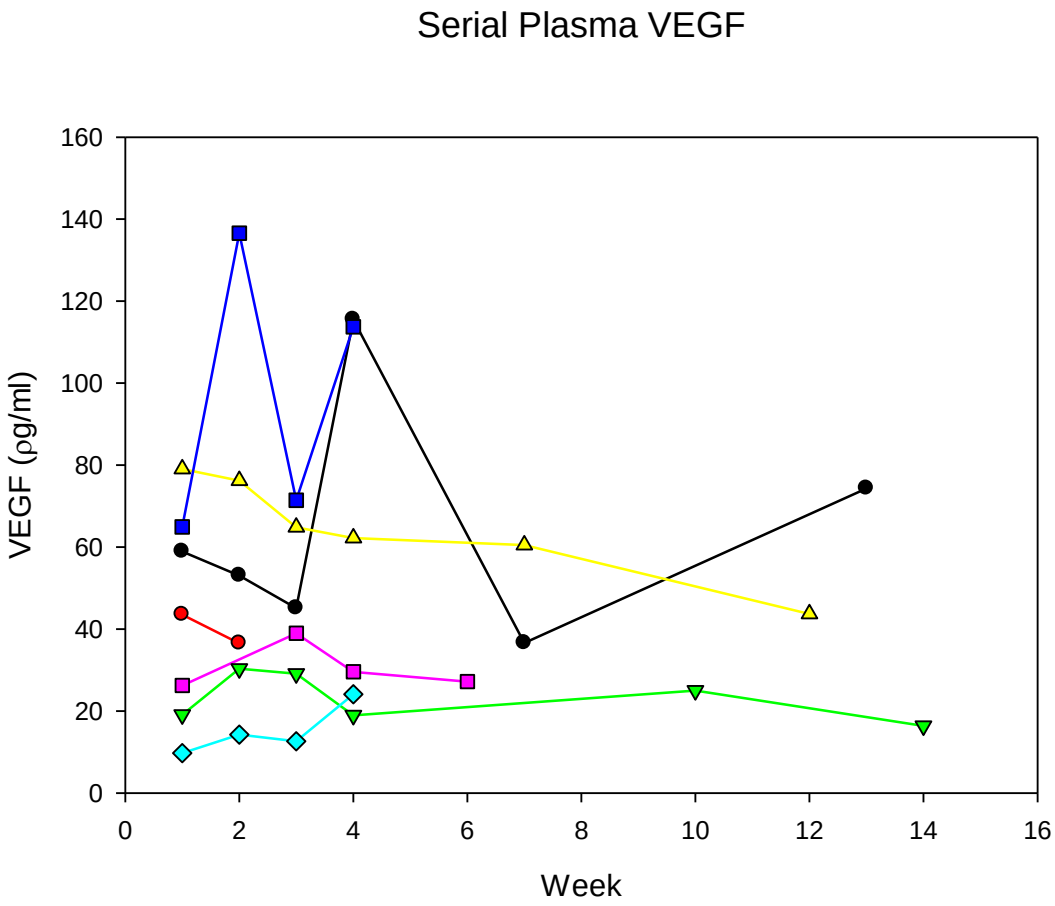


Figure 3-12 Serial plasma VEGF measurements of women with persistent or recurrent endometrial and ovarian cancer. Serial blood tests of 7 women with active ovarian (n = 6) or endometrial cancer (n = 1). Week 1 was defined as the day of the first blood test, and every 7 days, starting from the 5th day, were "bundled" into subsequent weeks. Each line represents one participant and is identical across both graphs.

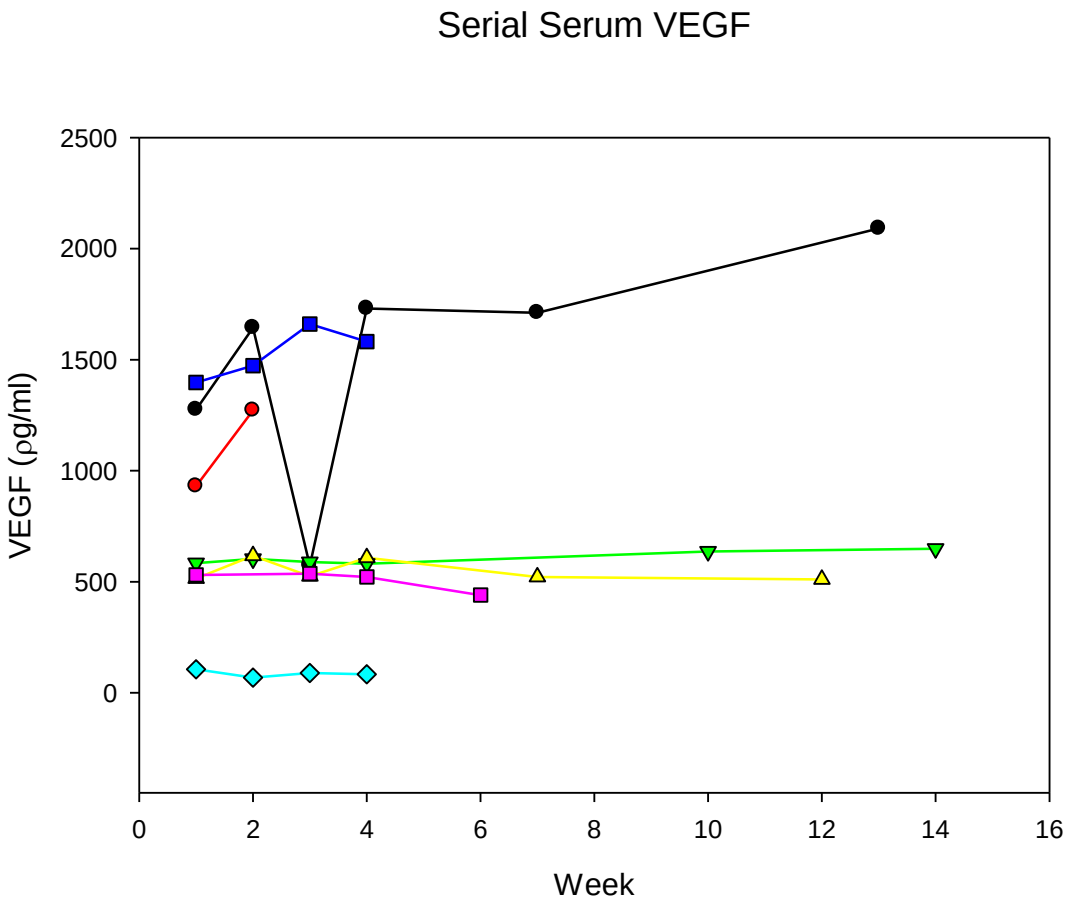


Figure 3-13 Serial serum VEGF measurements of women with persistent or recurrent endometrial and ovarian cancer. Serial blood tests of 7 women with active ovarian (n = 6) or endometrial cancer (n = 1). Week 1 was defined as the day of the first blood test, and every 7 days, starting from the 5th day, were "bundled" into subsequent weeks. Each line represents one participant and is identical across both graphs.

3.6.3 Variation

In Cohort 1, the absolute values of VEGF varied from 1 to 115 ρg/ml for plasma, and from 107 to 1431 ρg/ml for serum. The median week to week coefficient of variation (CV) for each Cohort 1 participant was 34% (range: 0-91%) for plasma VEGF, 8% (3-20%) for serum VEGF. In Cohort 2, VEGF concentrations ranged from 10 to 137 ρg/ml for plasma and from 28 to 2091 ρg/ml for serum and the median week to week CV for each participant was 25% (range: 12-44%) for plasma VEGF, 9% (range: 5-35%) for serum VEGF. In both cohorts, there was no evidence of a rising trend in plasma and serum VEGF over a fourteen week period.

3.6.4 Discussion

A number of studies have reported elevated levels of circulating VEGF in ovarian cancer and endometrial cancer patients compared to healthy subjects, and these values provided prognostic information. However, these correlations were made typically using one-off measurements of VEGF concentrations. In fact, this was also true for studies of other cancer types and few data were available on the variation of VEGF with time (please refer to 1.4.2 Assumptions and Limitations, p19). Specifically it has not been shown previously whether levels of blood VEGF in women with ovarian or endometrial cancer increases over time. Therefore, the repeatability of a single serum and/or plasma VEGF has yet to be demonstrated. The temporal pattern of circulating VEGF is important if it is to be used in clinical practice as a marker of tumour progression.

For Cohort 1 participants, who acted as controls to provide baseline levels, plasma VEGF values were low and relatively constant, with a geometric mean of 13 ρg/ml, and a median coefficient of variation of 34%, which was comparable to plasma levels of healthy volunteers in another study which reported a median of 45 ρg/ml and a day to day CV of 39-56% for 5 serial measurements of 32 participants. [59]. Serum VEGF was on average 23 fold higher than plasma, with a mean of 299 ρg/ml, and was also stable across eighteen weeks with median week-to-week CV of 8%, which was relatively less variable than plasma VEGF. The higher CV in plasma levels may be due to low mean values, which were often below sensitivity levels of the assay and therefore more sensitive to random variation. We found higher variability in plasma than another study which looked at biological variation of VEGF in 18 healthy volunteers ages 42-61 for which the within-subject coefficient of variation was 14.1% for CTAD plasma VEGF and 10.7% for serum [141]. In this study, the CV reported incorporated all the VEGF values, however, because the varying number of measurements taken for each participant, we were unable to calculate this CV using the same statistical method (nested analysis of variance) and therefore the CV for each participant was reported instead. Kraft and colleagues found that there were "no increases" in weekly serum VEGF in 7 healthy volunteers "above the upper limit" of the reference range calculated from 145 healthy controls [62], however no further analysis was done.

In our analysis of Cohort 2 – women with persistent or recurrent cancer without treatment, VEGF values were higher than those of women without cancer, but stayed

relatively constant over the study period and did not show an increasing trend, which was contrary to our hypothesis. The geometric means of plasma and serum VEGF levels were higher in this group, which were 46 and 822 ρg/ml respectively. Levels in both fluids did not show significant variation over time and the CV observed were similar to ones of the control group, with a median CV of 25% for plasma VEGF and 9% for serum VEGF (versus 34% and 8%, respectively). With a relatively small degree of variation, it would appear that a single measurement of either plasma and serum sufficiently reliable. In addition, the graphs Figure 3-12 and Figure 3-13 did not indicate an upward trend in either plasma or serum VEGF. This phenomenon differed from our expectation that VEGF would rise over time. There is evidence which pointed to the tumour as a significant source of elevated VEGF in blood of cancer patients (1.4.1 Circulating VEGF p17). Therefore, in a group of women with active cancer who were not undergoing treatment, for which we could assume tumour growth VEGF would theoretically rise. This lack of a rising trend in ovarian and endometrial tumour progression has not been reported previously. However, a major limitation of our study was the small number of participants and the relative short duration – data was available from seven women, and they were largely confined to ovarian cancer (only one had endometrial cancer). It was likely that there was not enough power and/or time to detect a small or variable trend, or that other histological subtypes of ovarian and endometrial cancer may behave differently and larger studies are warranted in order to confirm this.

Several women had VEGF concentrations which did not seem consistent with their disease status. They were participants 1-008 and 1-016 who were represented by the grey and dark grey line in Figure 3-11, and had consistently high serum VEGF,

which were in the approximate range of 1200-1400 ρg/ml. Participant 1-008's mean plasma VEGF of 72 ρg/ml was also higher than the 95th percentile concentration of 61 ρg/ml. The values were more compatible with those observed in women with active cancer. Upon reviewing the medical notes, participant 1-008 remained in remission after treatment for Stage 4 ovarian serous adenocarcinoma. Neither her medications nor her co-morbidities (hypertension, depression, constipation, osteoarthritis of knees) provided explanation. While intermittent inflammation may be a feature of osteoarthritis [142], there was no evidence of an acute infectious/inflammatory process as her C-Reactive Protein levels remained between 3-6 throughout and therefore we were uncertain the cause of her high serum VEGF. Participant 1-016 was also without clinical evidence of disease but was found to have polyarteritis nodosa, an inflammatory condition that has been reported to cause elevated serum VEGF [77], however, her CRP was also low and stayed below 5 throughout the duration of the study. In hindsight, she may not have been appropriate for inclusion in the study and for the purpose of constructing a reference interval, although her CRP indicated that she may be in remission. Nevertheless, she highlighted the fact that VEGF levels need to be interpreted in the context of the participant's other medical conditions, especially ones with an inflammatory component.

Another outlier appeared to be the week 3 serum measurement for participant 2-001, shown in the black line in Figure 3-13. It was significantly lower than ones before and after. Reanalysis of the stored sample gave the same result, and therefore it was not due to handling error, unless it had occurred during sample processing. No single event was identified at the time from the clinical notes that may have contributed to this drop. This result also accounted for the highest CV in Cohort 2 and when it was

removed from analysis, the CV for this participant was lowered from 35% to 17%, hence changing the range of the weekly CV for Cohort to 5-22%.

In summary we have analysed the variability of serial plasma and serum VEGF measurements and showed that these concentrations appear to be relatively stable. Moreover, in women with persistent disease progression, there was no evidence of an increasing trend. Hence our observations supported the use of single measurements in assessing VEGF levels, although data on women with disease were limited due to small numbers. High serum and plasma VEGF may be present even in women without cancer and co-existing diseases must be considered.

3.7 VEGF before and after surgical excision of cancer

Women who were to have primary surgery for removal of known ovarian and endometrial cancer or pelvic masses were recruited into Cohort 3. The histological subtypes of the tumours were verified by pathologists postoperatively. A blood test was taken preoperatively, usually one to two weeks prior to surgery and another was obtained at least four weeks after surgery. Twenty-four women were recruited and preoperative blood specimens were obtained. Six were subsequently shown to have benign disease and therefore excluded from this analysis. Three declined the postoperative blood test, and in another postoperative specimen only the serum sample was obtained due to difficulty in venepuncture so 14 pairs of plasma VEGF and 15 pairs

of serum VEGF were available. Out of the 15 women, 7 had ovarian cancer and the histological subtypes were as follows: papillary serous (3), endometrioid (2), mucinous (1), and granulosa (1). Histology of the 8 endometrial cancer patients were of the following: serous (1), endometrioid (5), clear-cell (1), mixed clear-cell and endometrioid (1) and carcinosarcoma (1).

Plasma and serum VEGF of the 15 participants before and after surgery are shown in Figure 3-14 and Figure 3-15, respectively.

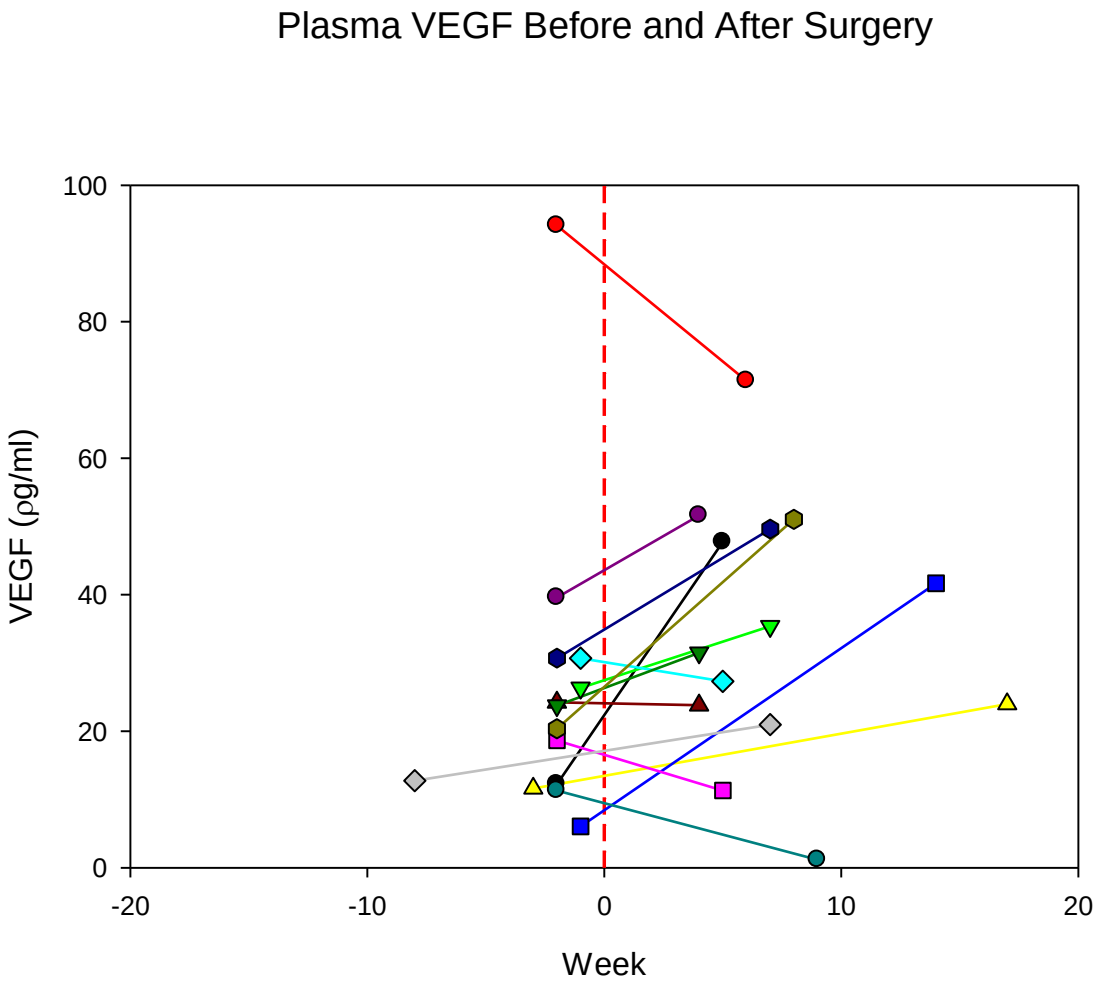


Figure 3-14 Plasma VEGF before and after surgery. 15 women were to have surgery for confirmed ovarian (n=7) or endometrial cancer (n=8). Blood specimens were taken before, and at least four weeks after surgery, and VEGF was measured in plasma. Each line represents a single participant and is the same across both graphs. Week 0 is defined as the period of 3 days before and after the day of surgery (indicated by dashed red line)

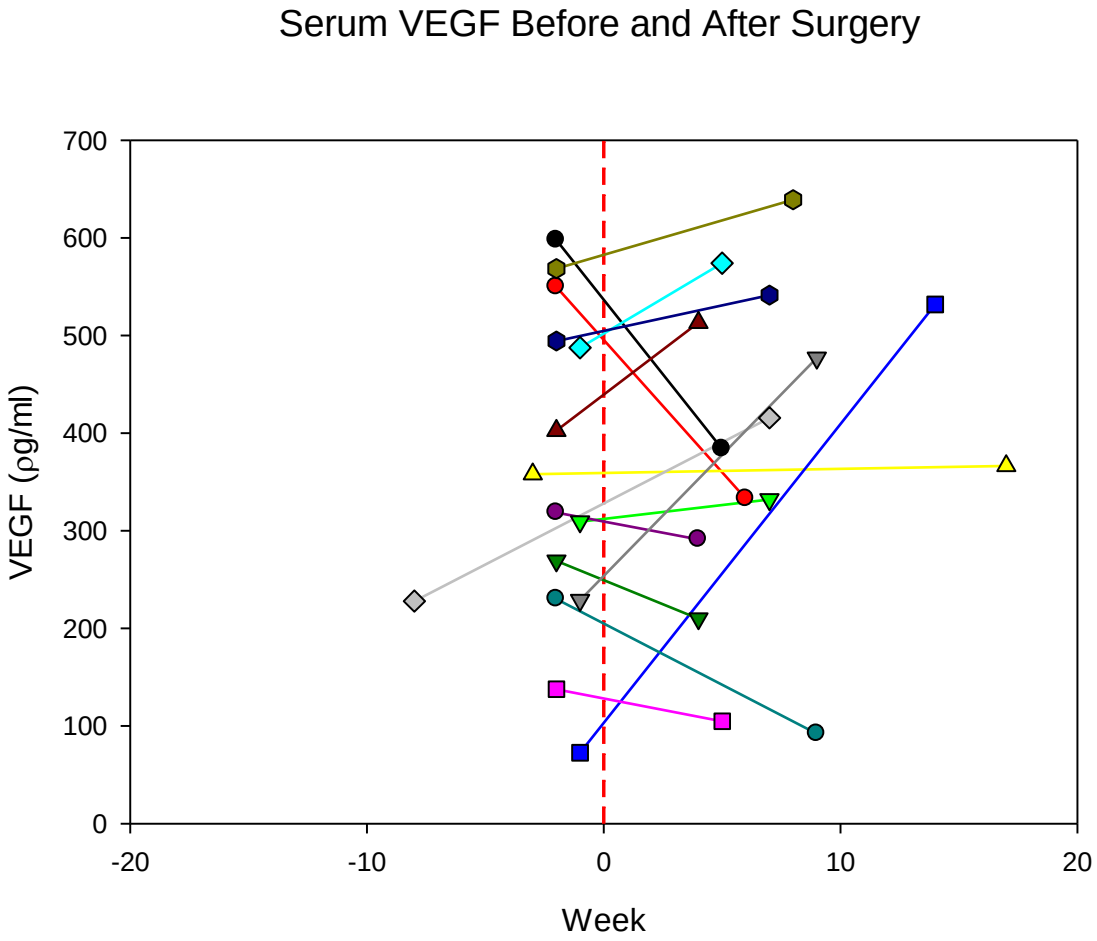


Figure 3-15 Serum VEGF before and after surgery. 15 women were to have surgery for confirmed ovarian (n=7) or endometrial cancer (n=8). Blood specimens were taken before, and at least four weeks after surgery, and VEGF was measured in serum. Each line represents a single participant and is the same across both graphs. Week 0 is defined as the period of 3 days before and after the day of surgery (indicated by dashed red line).

Plasma VEGF decreased in 3/7 ovarian cancer patients and 1/8 endometrial patients, and serum VEGF decreased in 4/7 and 2/8 women, respectively. Levels were higher in the post-operative samples for the rest.

Comparison of VEGF levels before and after surgery did not show a significant difference, although there was a trend to increase in both plasma VEGF (mean difference = +9 ρg/ml, 95% Confidence interval: -1 to 19 ρg/ml, $P = 0.073$, paired t-test) and serum VEGF (mean difference, 37 ρg/ml; 95% CI, -60 to 134 ρg/ml; $P = 0.431$, paired t-test).

Platelet count data was not of normal distribution, even after $\log_{10}$ transformation, therefore, the raw data was used. Platelet was not significantly different before and after surgery (mean difference, 26×10^9 / L; 95% CI, -3 to 54×10^9 / L, $P = 0.073$).

The inflammatory marker C-Reactive Protein (CRP) was not significantly different before and after surgery (mean difference, -2 mg/L; 95% CI, -8 to 3 mg/L, $P = 0.34$, paired t-test).

3.7.1 Discussion

Serum VEGF has been proposed as a tumour marker in clinical monitoring of ovarian cancer, partly because levels decreased after successful tumour removal. Yamamoto and colleagues reported decreases in serum VEGF down to the normal range in 11 ovarian cancer patients 1-2 months after optimal debulking [25]. In addition, in

one patient re-elevation of serum VEGF correlated with subsequent relapse [25]. In another study of ovarian cancer patients, Oehler and Caffier found that levels not only decreased after cytoreductive surgery, they were lower in patients with no residual disease, suggesting that serum VEGF might indicate treatment efficacy [29]. This decline in serum VEGF had also been reported in endometrial cancer patients after hysterectomy and bilateral oophorectomy, although values were still above those of controls [34].

Our results did not show a significant change in circulating VEGF after surgery and therefore could not confirm the previous findings. Acute inflammatory states such as bacterial infections had been associated with increased serum VEGF levels and may have been a possible confounder, however, using CRP measurement there was no evidence that the inflammatory states were different before or after surgery. Paradoxically, there was an increasing trend and this might be due to the small sample size and random variation. Of note, there was also an increasing trend in platelet count which may have contributed to the raised serum VEGF levels. VEGF levels observed in these women before surgery were not high in comparison to the reference range we obtained from women without cancer (paragraph 3.5). The only plasma level (94 ρg/ml, indicated by the red line in Figure 3-14 above the 95th percentile of 61 ρg/ml decreased to 71 ρg/ml, and none of the serum levels were above the 95th percentile of 1375 ρg/ml. There is the possibility that reduction in blood VEGF after surgery is only limited to those with high levels.

In summary in 7 women with ovarian cancer and 8 with endometrial cancer, we did not find changes in plasma and serum VEGF levels, at least four weeks after

surgical resection of the disease. We could not confirm previous findings suggesting that levels drop after cytoreductive surgery, and contrary to these, there was an increasing, but non-significant trend in both plasma and serum VEGF, which might have corresponded to the increasing trend in platelets. A potential source of confounding may be the effect of surgical menopause in premenopausal women who had their ovaries removed during the cancer surgery. Older, postmenopausal women have been shown to have higher VEGF than their premenopausal counterparts [67]. Although information on menopausal status was not requested, only three women were between 40-50 years of ages, two of which had decreased levels of VEGF after surgery, and therefore menopausal status is unlikely to be a confounder. Again the numbers were small in our cohort and most VEGF levels were below the range observed in our control cohort, which may also be why we did not detect a decline in VEGF.

3.8 VEGF and Disease Status

Subgroup analyses of blood VEGF levels of women with active ovarian and endometrial cancer were performed according to cancer type, FIGO stage and histology. The same data was used, as outlined in paragraph 3.5: one set of plasma and serum results from the first weekly samples from Cohort 2, the pre-surgery samples in Cohort 3 (from those who had malignant disease, as proven by histology), and the pre-administration samples for Cohort 4. Control samples included participants from Cohort 1 as well as those in Cohort 3 who had non-malignant disease.

3.8.1 Cancer Type

There were a total of 26 patients with ovarian cancer and 11 patients with endometrial cancer. Individual plots of plasma VEGF (Figure 3-16) and serum VEGF (Figure 3-17) are shown below. Differences between patient cohorts were tested with non parametric Mann-Whitney *U* test.

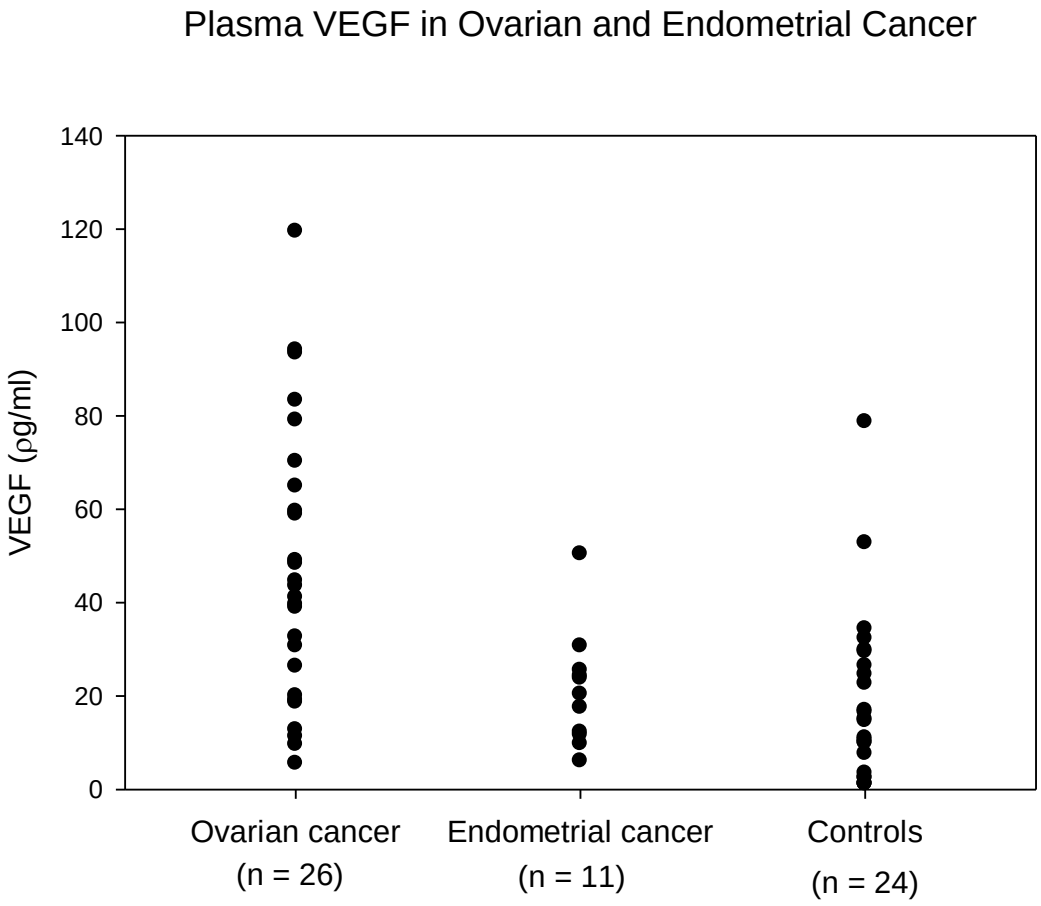


Figure 3-16 Plasma VEGF in malignant diseases. VEGF concentrations in plasma of women with ovarian cancer (n = 26) and endometrial cancer (n = 11) and controls who had past history of one of these cancers, but were currently free of disease (n = 24).

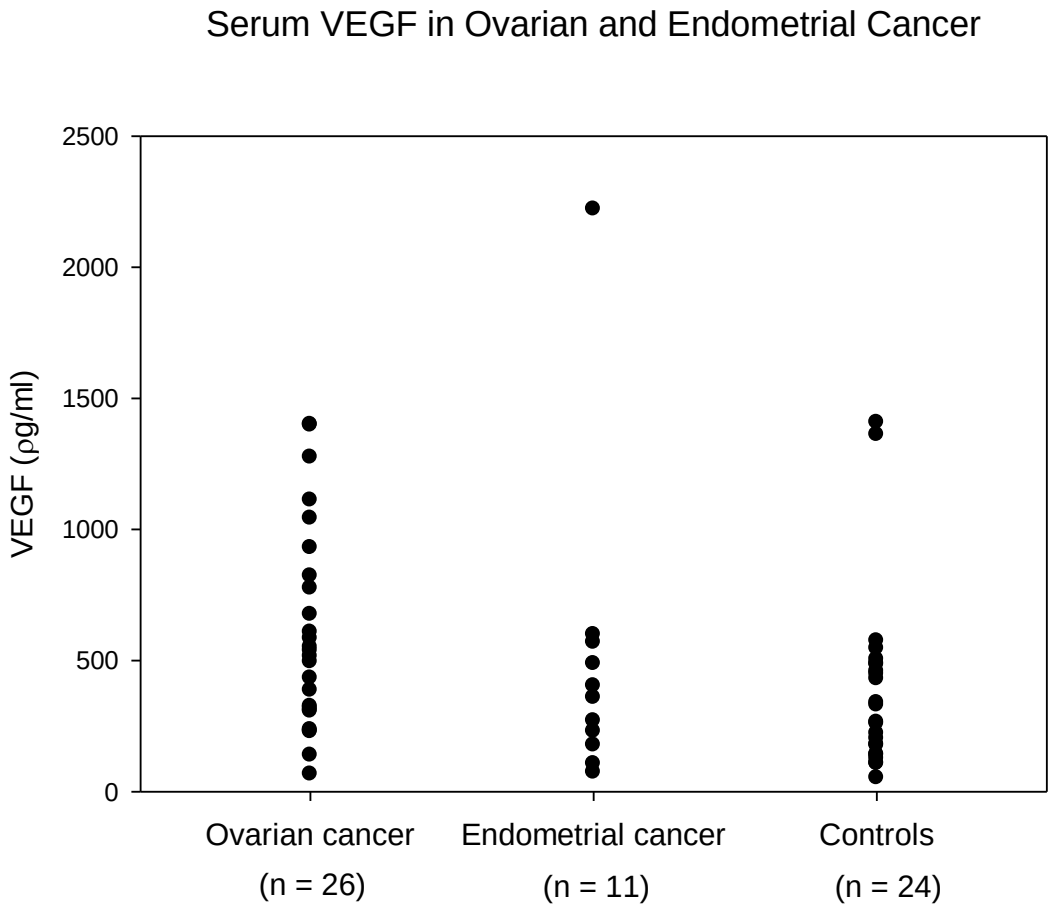


Figure 3-17 Serum VEGF in malignant diseases. VEGF concentrations in serum of women with ovarian cancer (n = 26) and endometrial cancer (n = 11) and controls who had past history of one of these cancers, but were currently free of disease (n = 24).

6/26 (23%) of plasma VEGF levels in women with ovarian cancer and 1/11 (9%) women with endometrial cancer exceeded the 95th percentile (61 ρg/ml) in the controls. For serum, 2/26 (8%) and 1/11 (9%) women with ovarian and endometrial cancer, respectively, had higher levels than the 95th percentile (1375 ρg/ml) observed in controls.

There was no significant difference between plasma VEGF in endometrial cancer patients (median, 20 ρg/ml; range, 6-50 ρg/ml) and controls (median, 15 ρg/ml; range, 1-75 ρg/ml), $P = 0.404$. However, plasma VEGF was significantly higher in ovarian cancer patients, compared to controls (median, 42 ρg/ml; range, 6-119 ρg/ml), $P < 0.001$.

Similarly, there was no significant difference between serum VEGF in endometrial cancer patients (median, 358 ρg/ml; range, 73-2221 ρg/ml) and controls (median, 297 ρg/ml; range, 52-1407 ρg/ml), $P = 0.683$, while ovarian cancer patients (median, 526 ρg/ml; range, 66-1400 ρg/ml) had higher levels than controls (median, 297 ρg/ml; range, 52-1407 ρg/ml), $P = 0.016$).

3.8.2 Stage

International Federation of Gynaecologists and Obstetricians (FIGO) stage information of women with ovarian and endometrial cancer were collected and classified as “early” – stages 1 and 2 and “advanced” – stages 3, 4 and disease recurrence, irrespective of the stage at diagnosis.

Individual plasma and serum VEGF plots broken down into Early and Advanced stages in ovarian and endometrial cancer are shown in Figure 3-18 and Figure 3-19, respectively. Because the VEGF data in these subgroups were of normal distribution, t-test was used for the differences between early and advanced disease stages.

Early ovarian cancer patients had significantly lower plasma VEGF (mean, 13 ρg/ml; SEM, 3 ρg/ml) than advanced ovarian cancer patients (mean, 51 ρg/ml; SEM, 6 ρg/ml), with a mean difference of 38 ρg/ml, 95% CI (3-73 ρg/ml), P = 0.035. However, there were only three early ovarian cancer samples and therefore the results must be viewed with caution. There was no difference between plasma VEGF in early endometrial cancer patients (mean, 22 ρg/ml; SEM, 5 ρg/ml) and advanced endometrial cancer patients (mean, 20 ρg/ml; SEM, 5 ρg/ml), 95% CI (-18-21 ρg/ml), P = 0.826. There were eight samples and three samples from early and advanced endometrial cancer patients, respectively.

Serum VEGF concentrations in ovarian cancer patients showed a similar trend to plasma VEGF, with early stage patients (mean, 225 ρg/ml; SEM, 49 ρg/ml) having a lower mean than advanced stage patients (mean, 653 ρg/ml; SEM, 80 ρg/ml). However, this difference was not significant, a mean difference 428 ρg/ml, 95% CI (-893-36 ρg/ml), P = 0.069. Similarly, serum VEGF in endometrial cancer patients were no different in patients with early stage (mean, 362 ρg/ml; SEM, 190 ρg/ml) or advanced stage disease (mean, 865 ρg/ml; SEM, 1177 ρg/ml), 95% CI (-384-1391 ρg/ml), P = 0.231.

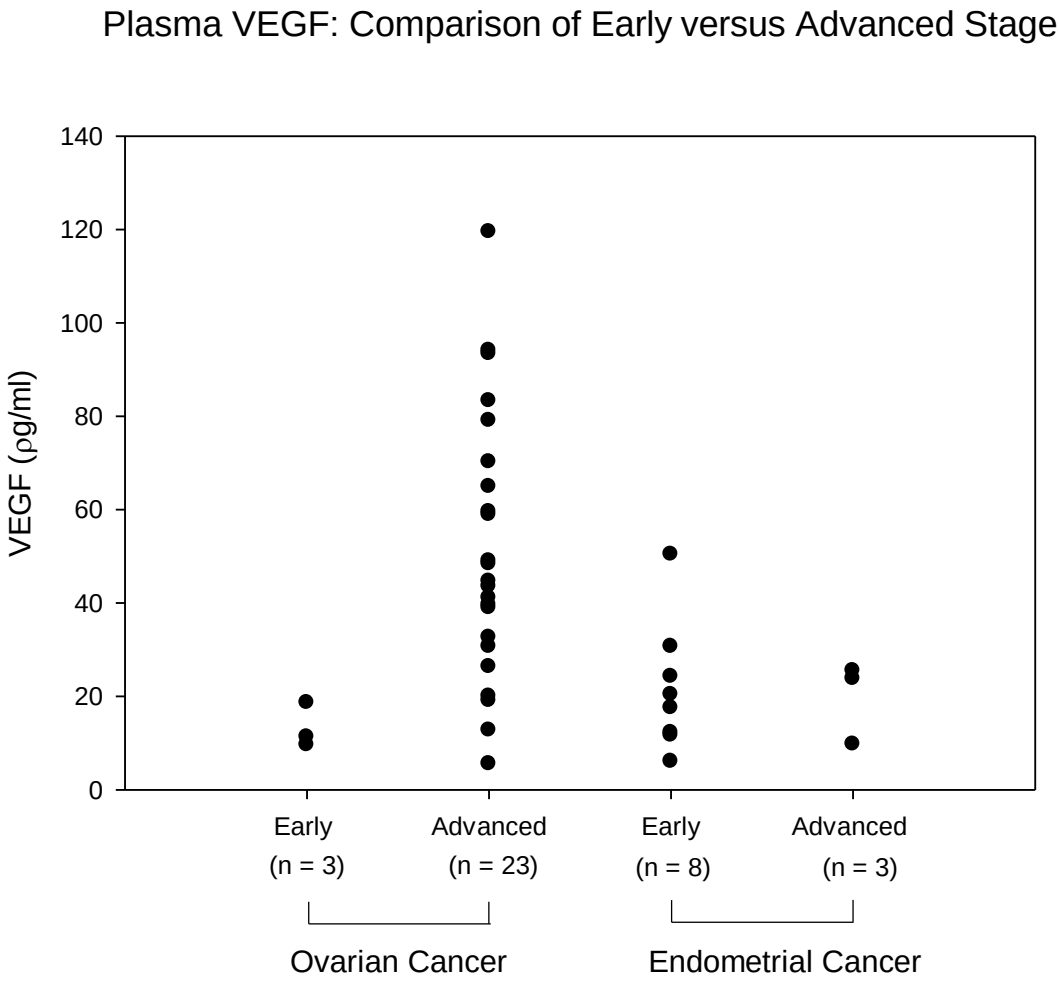


Figure 3-18 Plasma VEGF levels in Early and Adanced Stages of Ovarian and Endometrial cancer. Individual levels of plasma VEGF taken from the first blood specimen are shown for participants with Early (FIGO stages 1 or 2) and Advanced (FIGO stage 3, 4 or recurrent disease) ovarian and endometrial cancer.

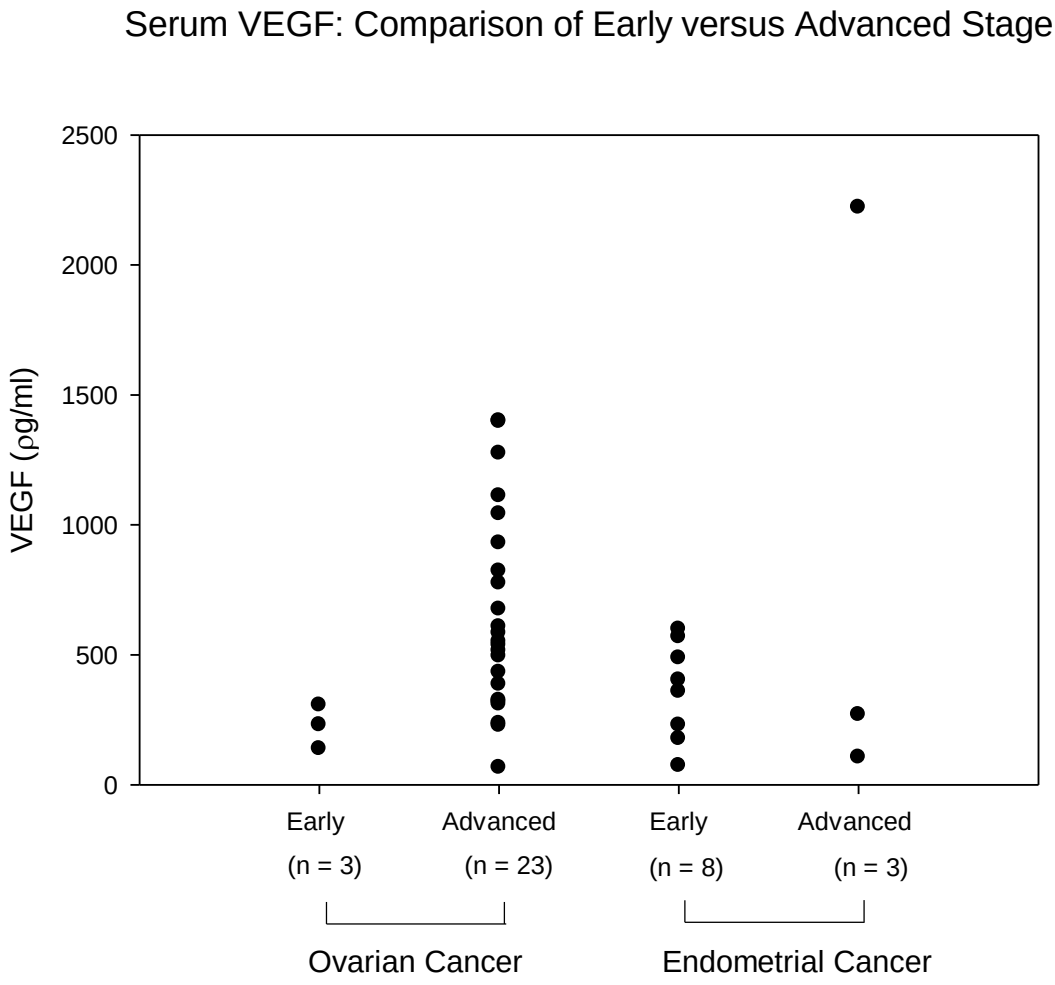


Figure 3-19 Serum VEGF levels in Early and Adanced Stages of Ovarian and Endometrial cancer. Individual levels serum VEGF taken from the first blood specimen are shown for participants with Early (FIGO stages 1 or 2) and Advanced (FIGO stage 3, 4 or recurrent disease) ovarian and endometrial cancer.

3.8.3 Histological Subtype

The histological subtype of current tumours from all participants were obtained from the CDHB (Canterbury District Health Board) database and/or patient notes. Blood VEGF levels broken into these subtypes are shown below in Figure 3-20 (plasma) and Figure 3-21 (serum) for endometrial cancer; Figure 3-22 (plasma) and Figure 3-23 (serum) for ovarian cancer.

The Endometrioid type accounted for the single largest group in endometrial cancer, with serous, clear cell, mixed (endometrioid and clear cell) and carcinosarcoma each having one sample. The plasma VEGF in Endometrioid type ranged from 6 to 50 ρg/ml and the other types were all within that range Figure 3-20. For serum, the Serous type had the highest VEGF concentration (2221 ρg/ml), followed by the Mixed type (598 ρg/ml) while the rest were within the range of the Endometrioid type (73-569 ρg/ml) (Figure 3-21).

In ovarian cancer patients the majority of participants had Serous adenocarcinoma (17/26) and their plasma VEGF ranged from 6 to 93 ρg/ml. The two highest concentrations were observed in women with the Endometrioid type (119 ρg/ml, 94 ρg/ml) (Figure 3-22). For, serum VEGF, again the Endometrioid type accounted for two of the highest levels (1400 ρg/ml and 1111ρg/ml), while high concentrations were also observed in the Clear Cell type (1397 ρg/ml and 1275 ρg/ml) (Figure 3-23). Interestingly all three women with early stage ovarian cancer, despite having different histology (one serous, one endometrioid and one mucinous type), had relatively low

levels of both plasma and serum VEGF.

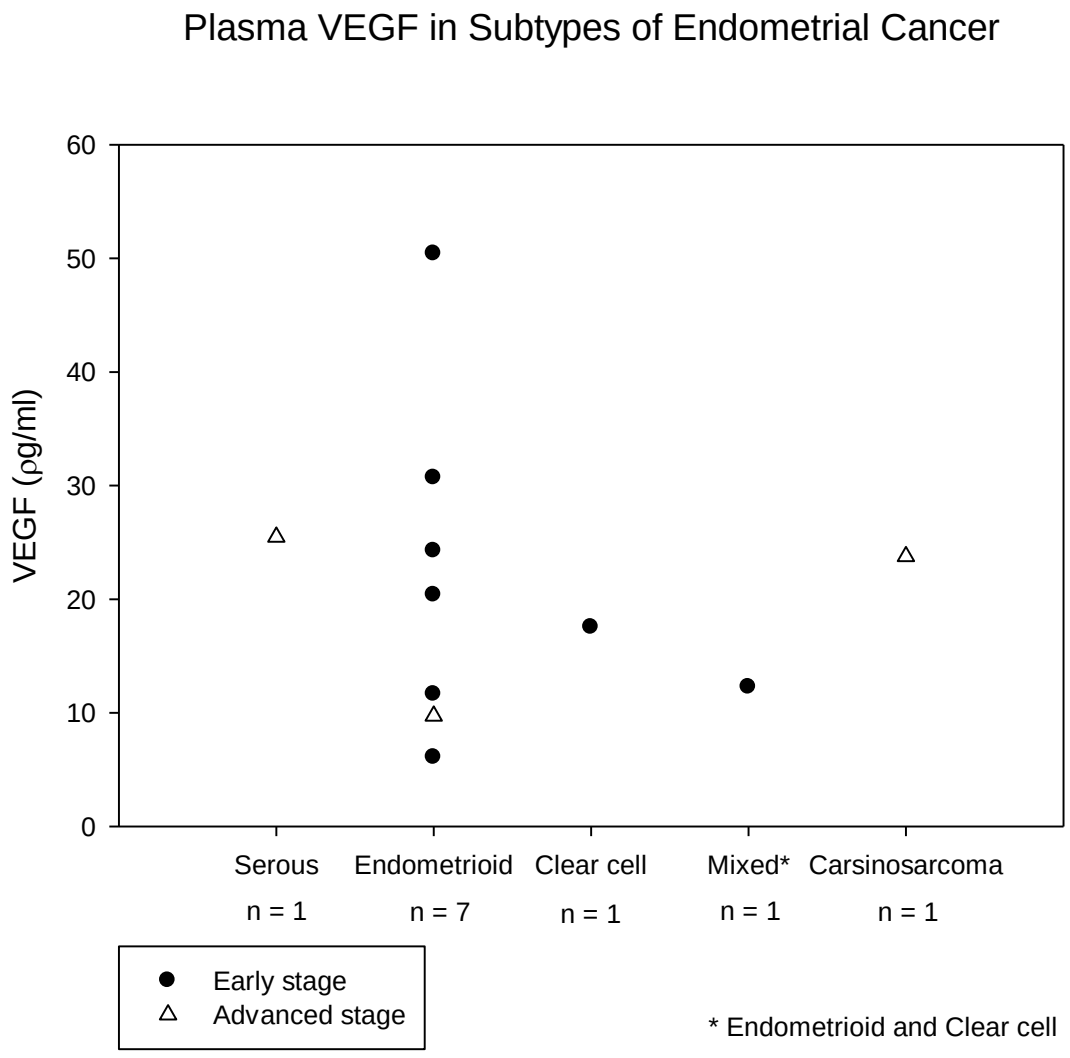


Figure 3-20 Plasma VEGF levels in Histological Subtypes Endometrial Cancer. Individual levels of plasma VEGF and taken from the first blood specimen are shown for participants with endometrial cancer, according to the histology of the tumour. Early and advanced stages are indicated for reference.

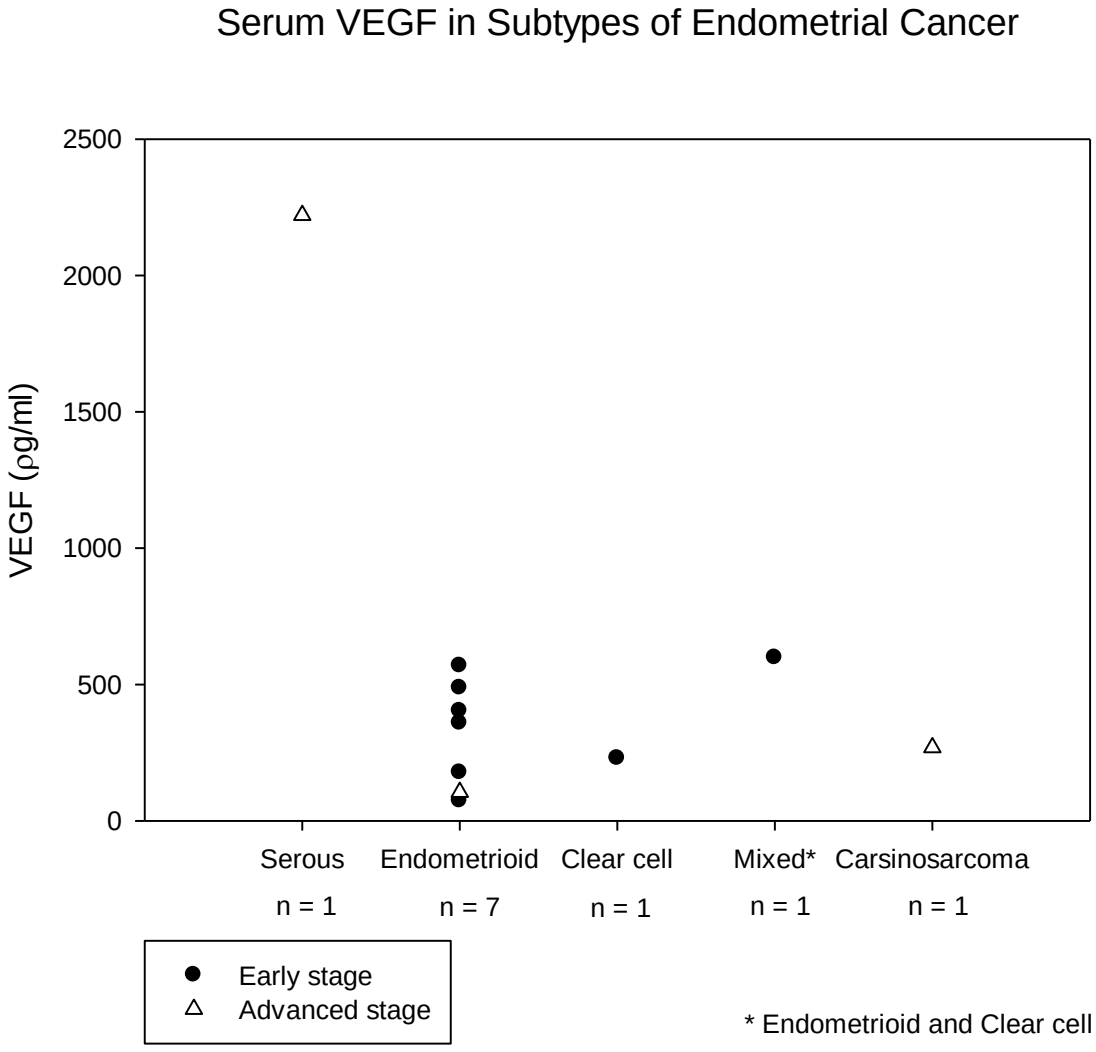


Figure 3-21 Serum VEGF levels in Histological Subtypes Endometrial Cancer. Individual levels of serum VEGF taken from the first blood specimen are shown for participants with endometrial cancer, according to the histology of the tumour. Early and advanced stages are indicated for reference.

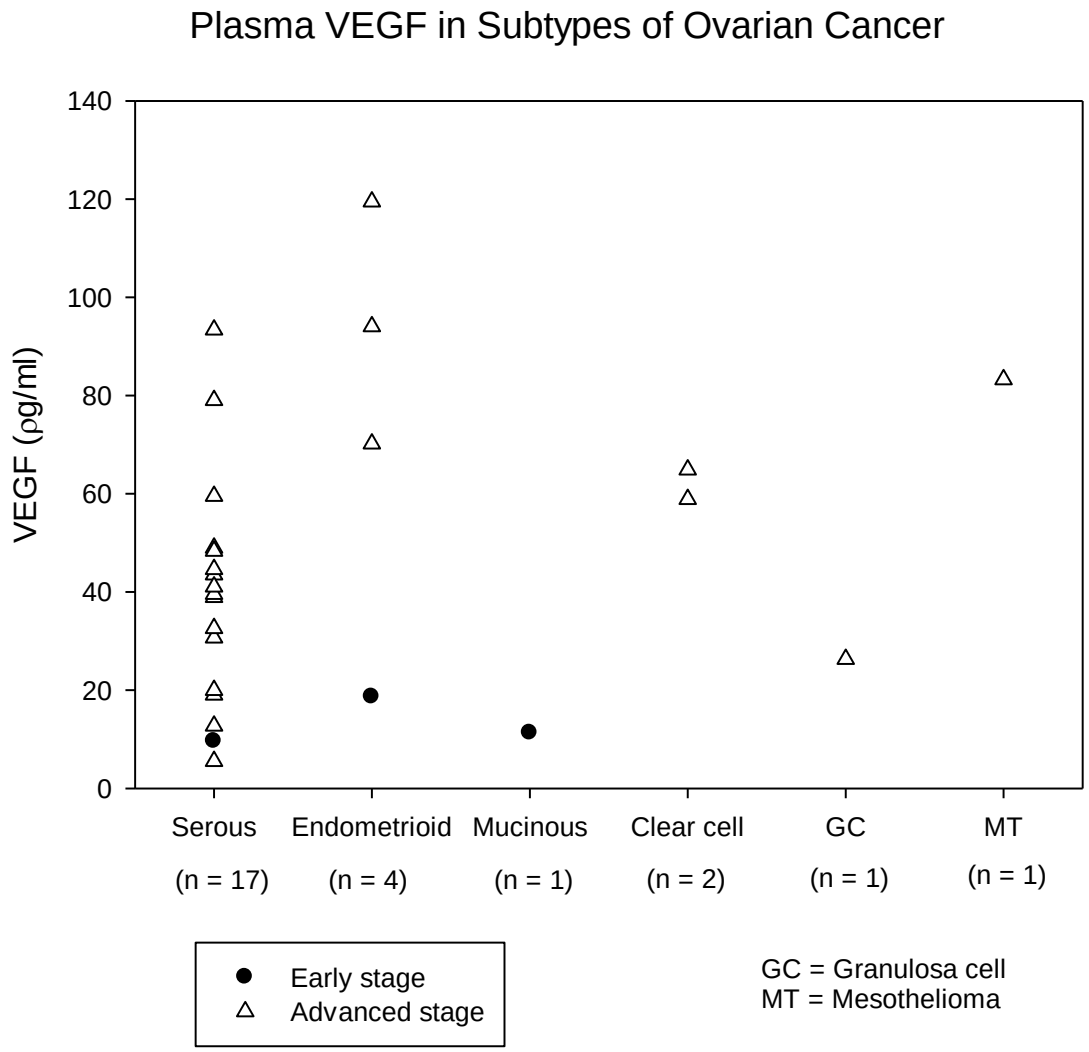


Figure 3-22 Plasma VEGF levels in Histological Subtypes of Ovarian Cancer. Individual levels of plasma VEGF VEGF taken from the first blood specimen are shown for participants with ovarian cancer, according to the histology of the tumour. Early and advanced stages are indicated for reference.

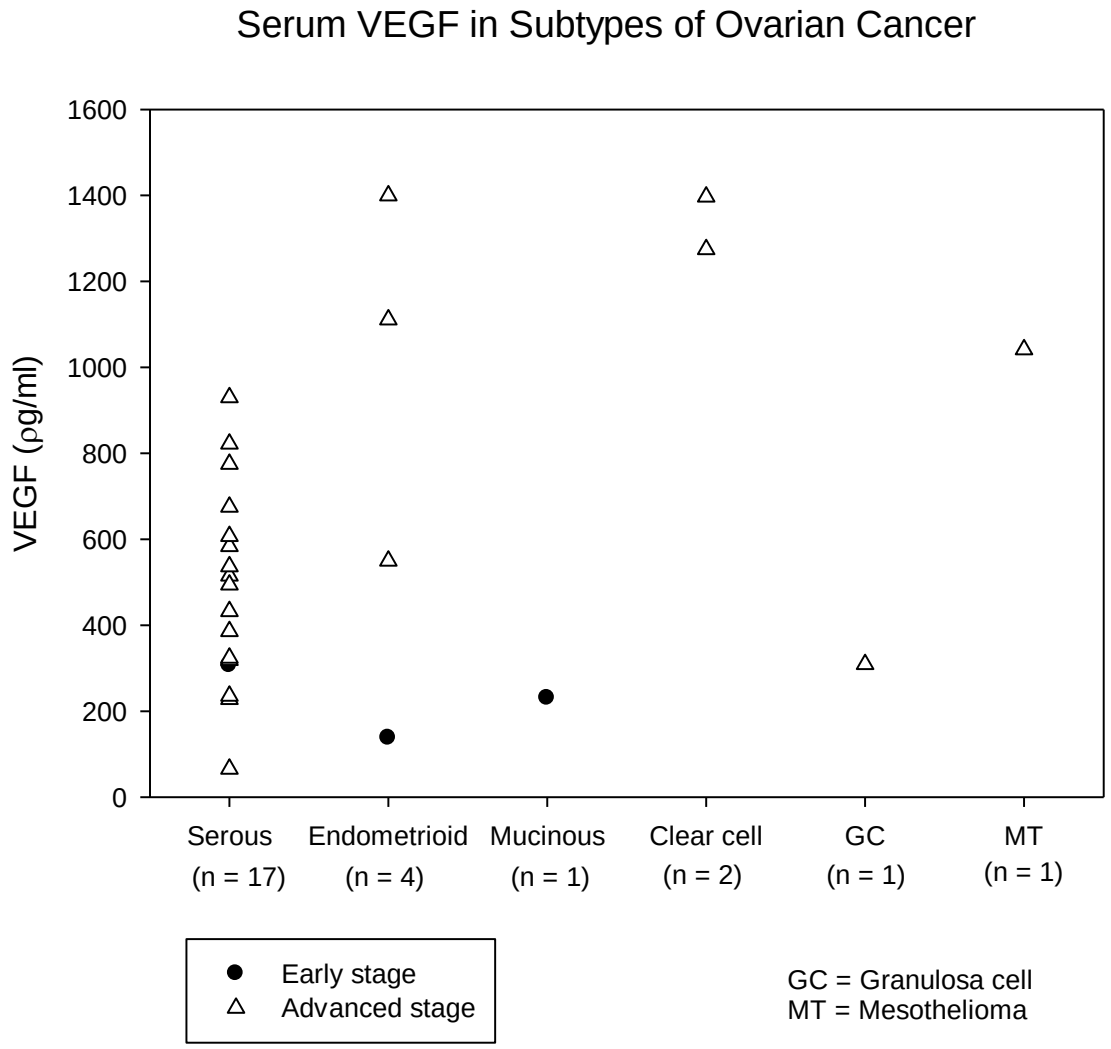


Figure 3-23 Serum VEGF levels in Histological Subtypes of Ovarian Cancer. Individual levels of serum VEGF taken from the first blood specimen are shown for participants with ovarian cancer, according to the histology of the tumour. Early and advanced stages are indicated for reference

3.8.4 Discussion

Consistent with previous reports [32], we found elevated plasma and serum VEGF in ovarian cancer patients compared to controls. However, there was considerable overlap in terms of the concentrations observed in women with cancer, to those without. There was no difference between endometrial cancer patients and controls. This might be due to the small sample and/or the fact that 8 out of 11 of the women had early stage disease. Shaarawy and El-Sharkawy found that disease stage correlated with serum VEGF in endometrial cancer, of which 7% and 37% of stage I and stage II endometrial cancer patients, respectively had elevated levels compared to controls, as opposed to 100% for stage III and IV [34]. However, we did not find a difference between VEGF levels in early versus advanced endometrial cancer and this was consistent with another study [143]. Although statistically advanced stage ovarian cancer patients had higher VEGF than early stage the disproportionately low numbers of participants with early stage disease prevented meaningful interpretation. Previous studies did not find differences in serum VEGF between different stages [32]. Data on subgroup analysis of histological subtypes were limited, especially for endometrial cancer. We did not observe significant discrepancies between different disease histology and this was consistent with previous reports [27, 32].

In summary we have performed sub-group analyses in plasma and serum VEGF levels in terms of cancer type, stage and disease histology. While women with ovarian cancer had higher levels than controls, those with endometrial cancer did not, and there was significant overlap between women with disease and those without. We did not

find differences between early and advanced stage disease, and between histological subtypes, however, our data was limited in numbers and therefore could not confirm previous findings.

3.9 Effect of Oral Green Tea Extracts on VEGF

There were two sub-cohorts for this pilot clinical trial; a disease group and a control group. The disease group comprised of women who had evidence of persistent endometrial and/or ovarian cancer, who were not receiving treatment for their cancer. These women were to take 1350mg of Green Tea Extract (equivalent of 900mg EGCG) daily for a period of six days. Plasma and serum VEGF was measured before and on the day the Green Tea Extract was finished. A control group, which was to take the same regimen, was recruited, and they were women who had past history of endometrial or ovarian cancer but were without evidence of disease.

For the disease group, 15 women ages between 31 and 77 were recruited of which 14 had ovarian cancer and one had endometrial cancer. Of the ovarian cancer patients, 11 had serous adenocarcinoma, 2 had endometrioid adenocarcinoma and 1 had epithelioid mesothelioma. The endometrial cancer patient had endometrioid adenocarcinoma. The control group consisted of 6 women, 48 to 77 years of age, of which 2 women had a past history of ovarian cancer and 4 with past history of endometrial cancer.

For Cohort 4, one participant (4-013) began taking the Green Tea Extract 11 days after the first blood test, and for the rest they started taking the Green Tea Extract within 48 hours of the first blood test. For the controls, the Green Tea Extracts were started within one day after the first blood test. The second blood tests were performed on the Day 6, for both Cohort 4 and the control group irrespective of whether they had finished the whole course. In Cohort 4, one participant (4-005) could not tolerate the capsules and withdrew, then subsequently restarted but only to withdraw again, therefore her results were excluded from graphing and analysis. Another participant (4-008) withdrew after taking the Green Tea Extracts for three and a half days and one in the control group (4-21) stopped after five days.

3.9.1 Results

Plasma and serum VEGF concentrations before and after taking Green Tea Extract containing 900mg of EGCG daily for six days are shown below in Figure 3-24 and Figure 3-25 for women with persistent ovarian and/or endometrial cancer. The change in plasma VEGF and serum VEGF after taking Green Tea Extract is shown in Figure 3-26 and Figure 3-27, respectively. There was no difference between plasma VEGF before and after (mean difference, 1 ρg/ml; 95% CI, -10-13 ρg/ml, P = 0.826, paired t-test). The mean serum VEGF was significantly higher after taking Green Tea Extract, however the 95% confidence intervals were close to zero (mean difference, 55 ρg/ml; 95% CI, 0-109 ρg/ml, P = 0.048, paired t-test).

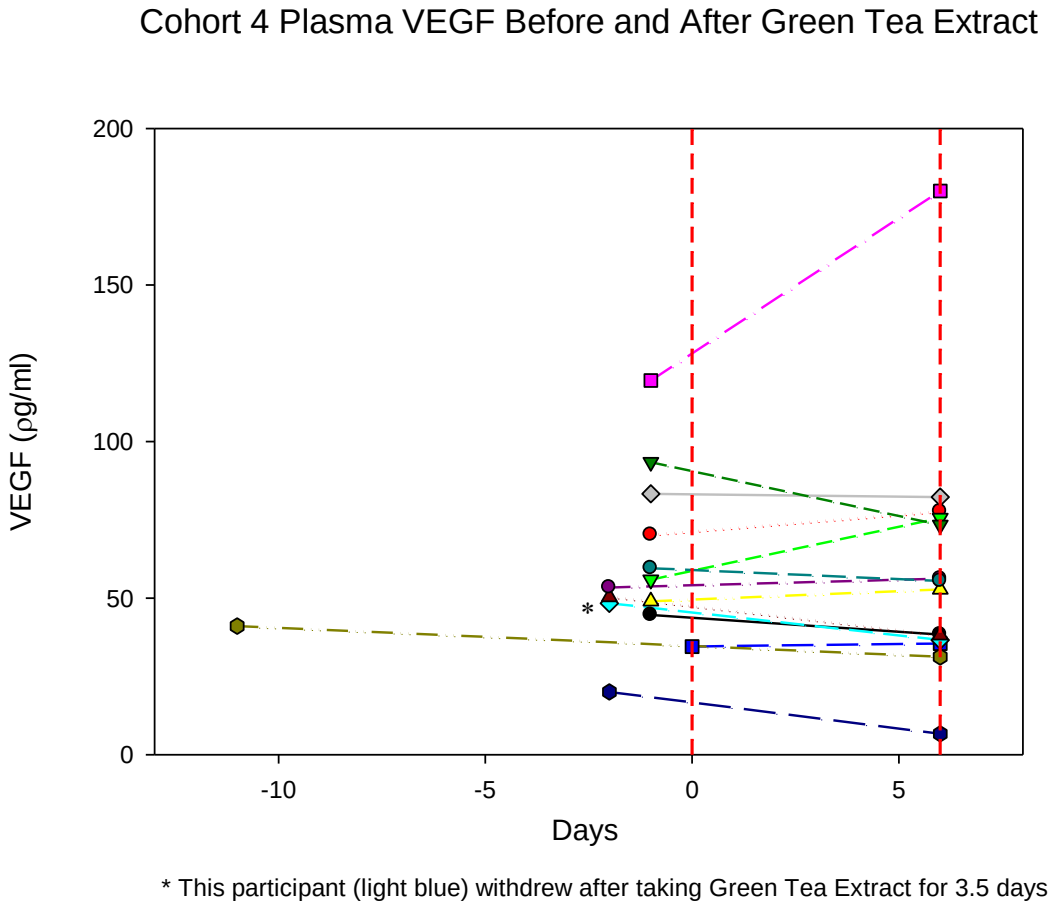


Figure 3-24 Plasma VEGF before and after taking Green Tea Extract in women with persistent ovarian and/or endometrial cancer Individual levels plasma VEGF concentrations are shown before and after taking Green Tea Extract, for participants with ovarian and/or endometrial cancer. Day 0 to Day 6 represents the period oral Green Tea Extract were taken (for the individual woman), as indicated by the vertical red lines, unless specified otherwise.

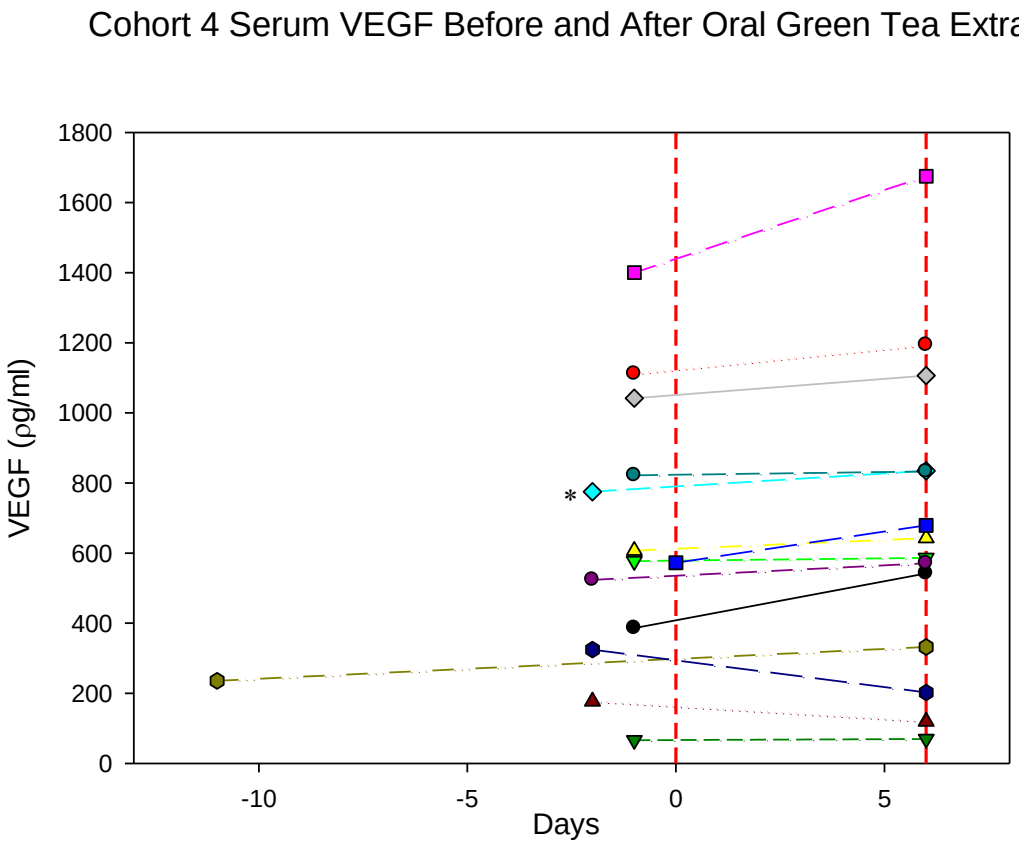
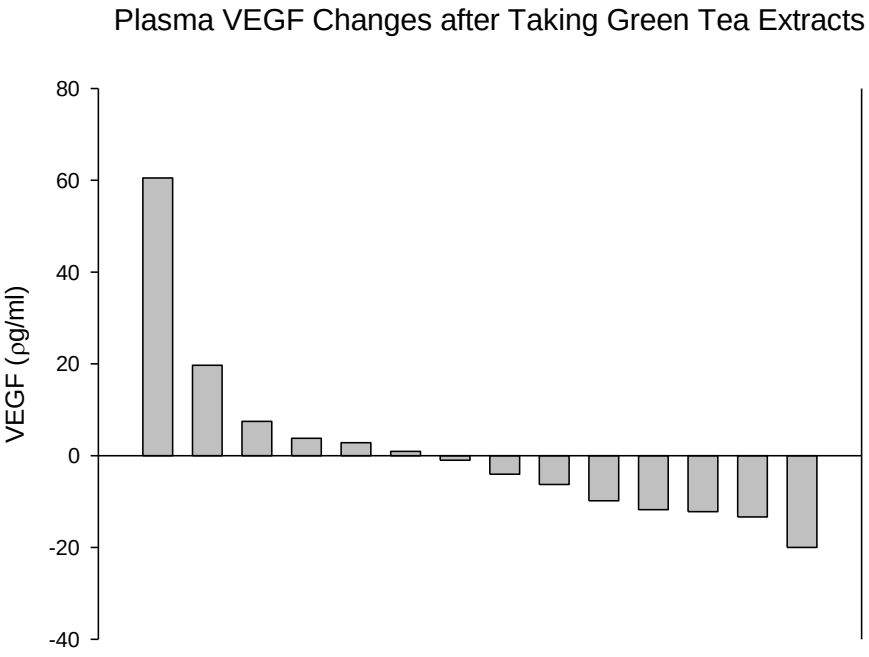


Figure 3-25 Serum VEGF before and after taking Green Tea Extract in women with persistent ovarian and/or endometrial cancer Individual levels of serum VEGF concentrations are shown before and after taking Green Tea Extract, for participants with ovarian and/or endometrial cancer. Day 0 to Day 6 represents the period oral Green Tea Extract were taken (for the individual woman), as indicated by the vertical red lines, unless specified otherwise.



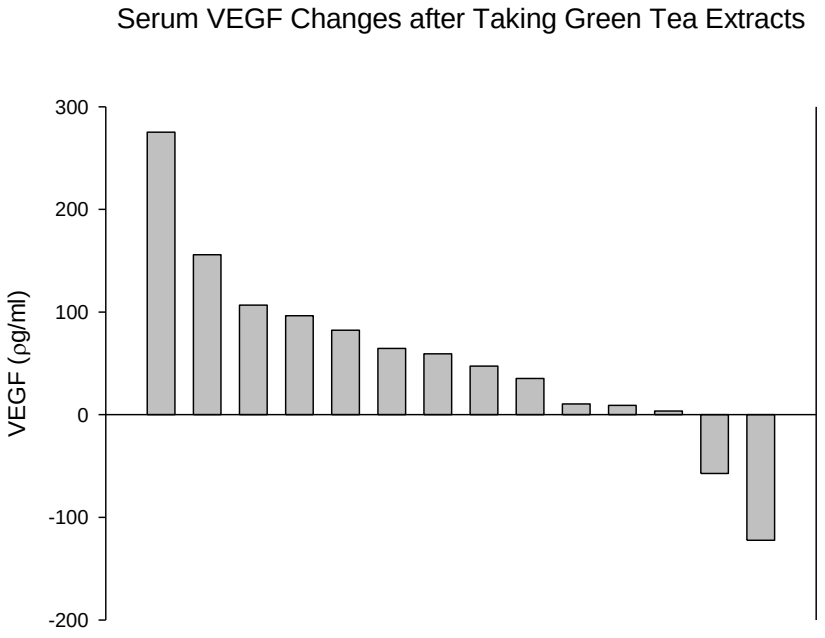


Figure 3-26 Changes in plasma VEGF levels after taking Green Tea Extract

Plasma VEGF level changes, ordered from the most positive. Each bar represents an individual woman.

Figure 3-27 Changes in serum blood VEGF levels after taking Green Tea Extract

Serum VEGF level changes, ordered from the most positive. Each bar represents an individual woman.

For control participants without evidence of cancer, plasma and serum VEGF before and after taking Green Tea Extract containing 900mg of EGCG daily for six days are shown below in Figure 3-28 and Figure 3-29. There was no change in plasma VEGF before and after taking Green Tea Extract (mean difference, 9 ρg/ml; 95% CI, -18-36 ρg/ml; P = 0.436; paired t-test). Similarly there were no difference between serum VEGF concentrations before and after taking Green Tea Extract (mean difference, 28 ρg/ml; 95% CI, -58-116 ρg/ml; P = 0.431).

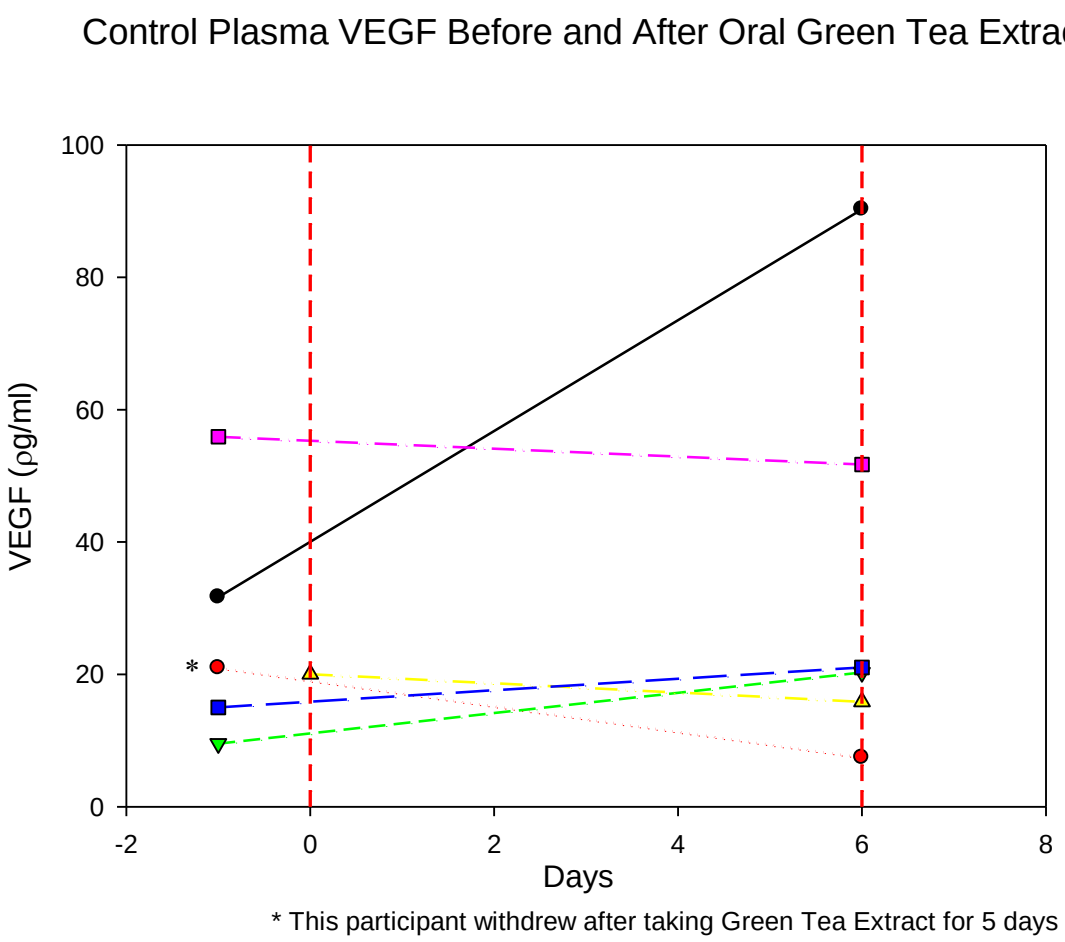


Figure 3-28 Plasma VEGF before and after taking Green Tea Extract in controls. Individual levels of plasma VEGF concentrations are shown before and after taking Green Tea Extract, for participants without evidence of cancer. Day 0 to Day 6 represents the period oral Green Tea Extract were taken (for the individual woman), as indicated by the vertical red lines, unless specified otherwise

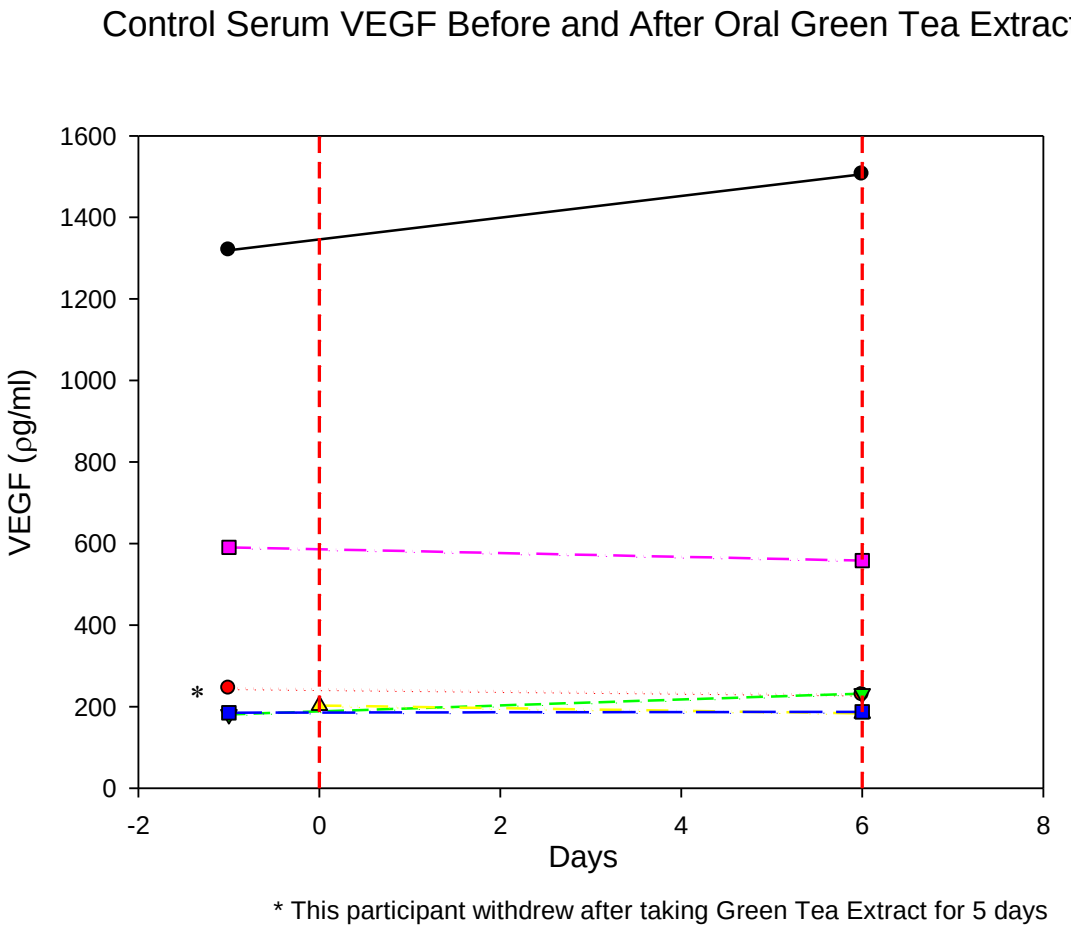


Figure 3-29 Serum VEGF before and after taking Green Tea Extract in controls. Individual levels of serum VEGF concentrations are shown before and after taking Green Tea Extract, for participants without evidence of cancer. Day 0 to Day 6 represents the period oral Green Tea Extract were taken (for the individual woman), as indicated by the vertical red lines, unless specified otherwise

There was no significant change in platelet count before and after taking Green Tea Extract, either the disease group (mean difference, -2 $\times$ 10^9 / L; 95% CI, -26 to 29 $\times$ 10^9 / L, P = 0.903, paired t-test) or the control group (mean difference, 5 $\times$ 10^9 / L; 95% CI, -23 to 13 $\times$ 10^9 / L, P = 0.5, paired t-test).

3.9.2 Safety

Recently there has been case reports of liver damage with use of green tea-containing products and therefore a Liver Function Test was requested with the blood tests, and participants were given a questionnaire to report side effects.

Green Tea Extracts (Bio Concepts, Queensland, Australia) equivalent to 900mg of EGCG daily appeared to produce some side effects and were generally well tolerated. 12/21 women reported side effects and the ones that resulted in withdrawal from the studies were: abdominal pain associated with diarrhoea and vomiting (1/12), nausea, fatigue and insomnia (2/12). Side effects reported by other women were nausea (4/12), insomnia (3/12), fatigue (2/12), headache (2/12), diarrhoea (1/12), exacerbation of dermatitis (1/12), and polydipsia (1/12).

A liver function test was requested along with the blood tests. Participants with liver function tests outside the normal range before and/or after taking the Green Tea Extract are show below in Table 3-1. Elevation of the liver enzymes after taking the Green Tea Extract occurred in four women, of which two already had abnormal levels before commencing the capsules. In every case only one enzyme marker was affected and elevations were within one times the upper limit of normal.

Table 3-1 Green Tea Trial (Cohort 4): Liver Function Tests Before and After Taking Green Tea Extract

ID	Cohort	Before LFT	After LFT
4-003	4	TP 85, GGT 53	TP 83, GGT 47
4-004	4	TP 86, GGT 53	TP 88, GGT 51
4-007	4	ALB 33, ALP 312, GGT 166	ALB 31, ALP 246, GGT 102
4-012	4	Normal	GGT 48
4-013	4	GGT 70	GGT 68
4-017	4	ALT 36	Normal
4-018	Control	Normal	ALT 35
4-021	Control	GGT 54	GGT 66
4-024	Control	ALT 33	ALT 34

Keys and reference range: TP, Total Protein (64-83 g/L); ALB, Albumin (35-50 g/L); BIL, Bilirubin (2-20 μmol/L); ALP, Alkaline Phosphatase (30-150 U/L); GGT, Gamma-Glutamyl Transpeptidase (10-35 U/L); AST, Aspartate Transaminase (10-50 U/L); ALT, Alanine Transaminase (0-30 U/L).

3.9.3 Discussion

Results of our pilot study indicated that there was no evidence that a short course of oral Green Tea Extract at our dosing regimen suppressed blood levels of VEGF in women with endometrial and ovarian cancer. Interestingly, contrary to our hypothesis, the direction of change appeared to be positive for both plasma and serum VEGF, in either women with persistent cancer or controls. This change was borderline significant for serum VEGF in women with cancer. The reason for this is not clear; there was no significant change in platelet count. *In vitro* studies have shown that EGCG reduced ovarian cancer cell proliferation via inhibiting endothelin A receptor /endothelin-1 axis [117]. Oral administration of green tea to mice with xenograft also inhibited tumour growth and VEGF expression [117]. Similarly, EGCG inhibited VEGF secretion in endometrial cancer cells *in vitro* [118].

To our knowledge, there has been no prior data with regards to the effect of oral green tea extract/EGCG on plasma and serum VEGF in endometrial and/or ovarian cancer. In a trial of similar design, 25 men with stage I to III prostate cancer were given Polyphenon E, a tea polyphenol supplement daily before radical prostatectomy, and serum VEGF was measured before and after administration [126]. A significant decrease in serum VEGF was observed, after a median of 34.5 days and 6 men had >25% percent decrease in magnitude. Although the daily dose of green tea extract with respect to the EGCG content was similar to that used in our trial (800mg versus 900mg), the duration of the study was much longer in McLarty and colleague's study, which ranged from 12 to 214 days,. In contrast, the duration was 6 days in our trial.

Green tea extracts at the doses used in our study appeared to be well tolerated in women with ovarian and endometrial cancer. Similar side effects profile had been reported in cancer patients, and they were gastrointestinal (bloating, dyspepsia, nausea and vomiting) and neurological (dizziness, insomnia, tremor, restlessness) [100]. These were possibly related to caffeine [100] and unfortunately caffeine levels in the Green Tea Extract capsules were not available from the manufacturer. There has been a number of case reports of liver toxicity associated with green tea products [129], and there was no evidence of such toxicity in our trial.

EGCG has low bioavailability and exists as free form in the plasma and the half-life is roughly 3-4 hours at 800mg as a mixed extract [127]. Therefore we split the daily dose of Green Tea Extract into two (morning and night) in order to maintain a steady plasma level. Taking green tea extracts in the fasting state or with food had been shown to be different. There was a 3.5 fold decrease in average the maximum plasma concentration of EGCG when Polyphenon E was taken with food than when taken after an overnight fast [133]. We added a clause to the participants' information sheet advising participants to take the EGCG with food in the protocols, in line with the recommendation by the US Pharmacopeia due to concerns over the recent toxicity reports [134]. A possibility exists that the plasma concentration of the catechins achieved in our study may not be high enough to exert a detectable effect on VEGF.

In summary, a daily dose of Green Tea Extracts containing 900mg ECGC over six days, did not reduce plasma and serum VEGF levels in women with endometrial and ovarian cancer. There was an increased trend, for which we could not identify the cause of. The Green Tea Extracts at this dose were generally well-tolerated by the

women and we did not observe severe liver toxicity.

4 Conclusion

Although serum VEGF has been shown to be a prognostic indicator in ovarian cancer, blood VEGF data in the field of gynaecological cancer have been scarce. In addition, there has been debate as to which is the optimal specimen to use in measuring blood VEGF. In our series of investigations, we aimed to describe and compare the properties of serum and plasma VEGF, clarify their relationships with platelets and investigate their correlation with disease activity in women with ovarian or endometrial cancer.

Various types of anti-coagulants have been used to prepare plasma from blood for VEGF measurement. In our investigation, we found that VEGF concentrations were higher when measured in EDTA-supplemented samples, in comparison to those with sodium citrate. They differed by an average of approximately 32%, in both low and high concentrations. Further analysis with Bland-Altman plot showed that the variability of this difference increased in proportion to the absolute concentrations measured. Therefore logarithmic transformation was used in comparative statistics where high and low values were grouped together.

Platelets have been shown to be a major *in vitro* source of blood VEGF and EDTA has been shown to be a weaker inhibitor of VEGF release from platelets than citrate. This would imply that plasma extracted from EDTA is perhaps contaminated by VEGF released from platelets to a greater extent than plasma extracted from citrate. We would therefore support the use of citrate over EDTA as anticoagulant as it

probably offers a closer approximation to “circulating” levels.

An important finding of this study is the previously unreported Bland-Altman analysis that reveals a constant multiplicative relationship between plasma and serum VEGF. We found positive correlations between serum VEGF and platelet count, which support previous findings that a large proportion of serum VEGF is derived from platelets during the clotting process. However, plasma VEGF did not correlate with platelets, and it is not clear to what extent plasma VEGF is independent of serum VEGF and to what extent it reflects circulating VEGF. A possibility exists that there is minimal circulating VEGF and like serum VEGF, plasma VEGF represents leakage of platelet VEGF that occurs in blood that has been taken. Irrespective of the exact mechanisms, the positive correlation between serum and plasma VEGF found in our cohorts suggest that both may be valid measurements.

Indirect analysis suggests that VEGF content in platelets was higher in women with endometrial and/or ovarian cancer than in women without disease, hence support the notion that serum VEGF, albeit contaminated by platelet VEGF, reflects cancer biology. For the purpose of a disease marker, serum VEGF may also offer a practical advantage as all levels measured in our investigation were above the sensitivity of the assay, while plasma VEGF levels in general were close to or below that sensitivity. We also showed serum VEGF correlated with C-Reactive Protein, a marker of acute inflammation, and which gives rise to the question whether cancer-induced increase in VEGF is mediated by inflammation.

Using a single plasma and serum VEGF measurement we were able to construct a reference range for a group of women with cancer, and for a group of women without cancer who either had had endometrial/ovarian cancer or had benign disease of the pelvis. The median plasma and serum VEGF were higher in women with cancer versus the controls, (30 vs 15 ρg/ml, 488 vs 297 ρg/ml, respectively) but significant overlap in the 5-95th range was observed between the two groups. Although reference ranges of VEGF levels have been published previously, age, sex and co-morbidities have been shown to have influence and our cohort of controls would provide a relevant comparison in terms of those, and other potential confounders of blood VEGF levels.

Most investigations of blood VEGF that have been undertaken used a single measurement of VEGF and therefore we attempted to assess the reliability of this by using serial measurements. The week to week variability (denoted by the coefficient of variation) in plasma measurements were about 34% in controls and 25% in the cohort with persistent endometrial/ovarian cancer, which were higher than serum coefficient of variation of the corresponding cohorts. Serum measurements varied by approximately 10% from week to week and in our view this variability is sufficiently small to warrant the use of single measurements. The higher variation observed in plasma may be due to its inherent low values in relation to the sensitivity of our assay. Interestingly, we found consistently high levels of serum VEGF in two women without cancer. One has an inflammatory condition that is known to cause elevated VEGF but we could not identify the cause in the other woman.

In our cohort of women with endometrial/ovarian cancer, surgical resection of

disease did not correlate with a decrease in VEGF level and there was a trend towards an increase. We could not identify the reason for this; however, all women before surgery had serum levels which were within the 95th percentile of the reference range established from women without cancer, therefore a reduction in VEGF may only be observed in women with higher levels.

Sub-group analysis was performed in women with active endometrial and ovarian cancer based on cancer type, stage, and histology. There was a high overlap between levels observed in cancer patients and levels in women without cancer and only ovarian cancer patients on average had significantly higher plasma and serum levels than controls. We could not identify any definitive associations between stage and histological subtypes, possibly because of small sample size.

Lastly, after obtaining a better understanding of VEGF levels in women with gynaecological cancer, we undertook a pilot clinical trial, giving women with and without cancer, a short a course of oral Green Tea Extracts, and measuring the changes in plasma and serum VEGF. EGCG, a catechin in green tea, has been found to have anti-cancer and anti-angiogenic properties including inducing down regulation of VEGF. The Green Tea Extracts used in the trial were shown to be safe and generally well-tolerated although there were a number of side effects reported. We observed a paradoxical increase in both serum and plasma VEGF, although both relationships failed to reach significance. The study was relatively short in comparison to other trials that used oral green tea extracts and a longer trial is warranted.

We have conducted a number of investigations of different aspects of blood measurement of VEGF in women with endometrial or ovarian cancer. The observations will contribute to the existing knowledge on circulating VEGF Pre-analytical factors such as anticoagulant type can affect plasma levels and would need to be better defined and standardized. Blood VEGF reflects cancer biology but it is not clear to what extent platelet count and inflammation contribute to circulating VEGF and further studies are clearly needed. The use of serum versus plasma VEGF continues to be controversial and more work, such as direct analysis of platelet VEGF, is needed to understand the source of VEGF in these samples. Some but not all women with cancer have elevated VEGF levels, while others without cancer can have elevated levels. Together with lack of correlation with tumour mass, our investigations would suggest that VEGF is not suitable as a blood marker for routine clinical monitoring of disease. While no significant difference was observed, the effect of green tea extracts in producing the rise in blood VEGF is interesting and deserves further study.

5 Appendix VEGF Levels of All Participants

Vascular Endothelial Growth Factor (VEGF) levels in serum and plasma are shown below for all participants, in tables according to their designated cohorts. Women who had given an adequate amount of blood on at least one occasion are included. Hence those who had given consent yet withdrew before a blood specimen was obtained are not included.

Medication and co-morbidity data had also been collected, however due to the small sample size we were unable to perform meaningful correlations of these data with VEGF concentrations, hence they are not analysed.

Endometrial cancer (cancer of the uterus) and ovarian cancer staging is according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria (1988 and 2002, respectively).

All plasma results from the tables below are from citrated plasma, and calculated from formula to take account for haematocrit and citrate volume (please refer to Methods **)**. ID: 1.12 denotes Cohort 1, number 12. Women who had participated in more than one cohort are allocated two IDs which are shown.

Table 5-1 Cohort 1: Participant information and VEGF Levels 18 women who had past history of ovarian or endometrial cancer that had been treated, and were without evidence of current disease were recruited. Peripheral blood specimens were obtained, whenever possible, weekly for four weeks, and monthly for two months.

Past Tumour Histology (n)	ID	Stage when diagnosed	Age	Number of specimens	Serum Mean VEGF (SD)/ ρg/ml	Plasma Mean VEGF (SD)/ ρg/ml	Mean platelet count (SD)/ $\times 10^6$	Co-Morbidities
Ovarian								
Papillary serous (5)	1-1	1C	47	6	276(18)	28(3)	286 (6)	Post-menopausal symptoms, rheumatoid arthritis in remission
	1-3	3B	57	6	367(63)	16(5)	354 (22)	Depression
	1-4	3A	57	2	546(39)	1(--)*	312 (6)	Suspected psychosis
	1-8	4	77	6	1313(79)	72(8)	306 (9)	Hypertension, depression, constipation, osteoarthritis knees
	1-10	3C	61	6	478(14)	22(20)	175 (9)	Asthma
Clear cell (2)	1-2	1C	47	6	193(33)	12(8)	199 (14)	Single Kidney

	1-19	1C	32	6	491(14)	30(15)	316 (21)	Stable sub-pleural nodules on CT
Mucinous (1)	1-9	1C	49	6	128(10)	8(4)	202 (7)	None
Endometrial								
Endometrioid (8)	1-5	2B	69	6	182(21)	2(0.9)	184 (6)	Hypertension, BMI > 25
	1-6	1B	63	1	504(--)	53(--)	313 (--)	BMI > 25
	1-7	1A	54	6	134(14)	24(8)	314 (13)	Surgical menopause, depression
	1-12	3A	56	6	151(30)	1(--)*	211 (28)	Hypertension
	1-13	1A	53	6	524(78)	31(9)	251 (19)	Right coronary artery disease
	1-14	1B	68	6	162(24)	9(5)	294 (19)	Hypertension, cellulitis, hypothyroidism, early osteoarthritis hip

	1-15	1B	80	6	196(6)	15(5)	250 (13)	Gastro-oesophageal reflux disease, left hemi-diaphragm defect, osteoporosis
	1-16	1A	46	6	1290(92)	32(13)	371 (35)	Epilepsy, metabolic syndrome (hypertension, impaired fasting glucose, hyperlipidaemia), polyarteritis nodosa, anxiety/panic disorder
Mixed – serous and endometrioid (1)	1-17	2A	48	6	572(48)	41(8)	251 (18)	None
Carcinosarcoma (1)	1-18	3C	74	6	424(80)	30(12)	259 (13)	None

* All weekly plasma VEGF concentrations measured were below the sensitivity of Quantikine ELISA assay (please refer to Methods).

Table 5-2 Cohort 2: Participant Information and VEGF Levels 7 women who had evidence of ovarian/endometrial cancer and were not on treatment were recruited. Peripheral blood tests were performed weekly for four weeks, and monthly for two months.

Tumour Histology (n)	ID	Stage at diagnosis	Tumour status: Recurrent (R) / Persistent (P)	Age	Number of specimens	Serum Mean VEGF (SD)/ ρg/ml	Plasma Mean VEGF (SD)/ ρg/ml	Mean platelet count (SD)/ $\times 10^6$	Co-Morbidities
Ovarian									
Papillary serous (4)	2-2	3	R	84	2	1101(242)	64(28)	254(47)	Hypertension
	2-3	3	R	76	6	607(29)	23(6)	281(25)	None
	2-4	3C	R	73	6	549(49)	64(13)	306(27)	Hypertension, urinary urgency, early satiety
	2-6	2C	R	59	6	1313(79)	72(8)	245(13)	Cerebrovascular accident
Clear cell (2)	2-1	3	R	60	6	1504(525)	362(76)	362(76)	Hypercholesterolaemia, diarrhoea, faecal urgency
	2-5	3C	R	59	4	1528(116)	97(34)	478(29)	None

Endometrial									
Endometrioid (1)	2-7	3A	R	65	4	87(15)	15(6)	212(20)	Hypothyroidsim, atrial fibrillaition, BMI > 25

Table 5-3 Cohort 3: Participant Details and VEGF Before and After Surgery. 24 participants were to undergo surgery for confirmation and/or treatment for (suspected) endometrial or ovarian cancer. VEGF levels and platelet counts were measured before and after surgery.

Tumour Histology (n)	**ID**	**Stage at diagnosis**	**Tumour status: Recurrent (R) / Persistent (P)**	**Age**	**Before Surgery serum VEGF (S), plasma VEGF (P) / ρg/ml, platelet count (pl) / ×10^6**	**After Surgery serum VEGF (S), plasma VEGF (P) / ρg/ml, platelet count (pl) / ×10^6**	**Co-Morbidities**
Ovarian							
Epithelial							
Papillary serous (5)	3-6	3C	P	69	S = 675, P = 15 pl = 525	NA^0	None
	3-9	1A	P	84	S = 306, P = 10 pl = 264	NA^0	Hypertension
	3-21	3C	P	77	S = 494, P = 30 pl = 327	S = 541, P = 50 pl = 358	Right femoral artery pseudoaneurysm, osteoarthritis left hip, rheumatoid arthritis, constipation

	3-12	3	R	81	S = 228, P = 13 pl = 117	S = 416, P = 21 pl = 284	Hypertension, hyperlipidaemia, bronchiectasis, renal compromise secondary to ureteric obstruction
	3-24	3C	P	84	S = 319, P = 40 pl = 531	S = 291, P = 52 pl = 376	Hyptertension
Endometrioid (2)	3-3	4	P	52	S = 550, P = 94 pl = 332	S = 333, P = 71 pl = 288	Irritable bowel syndrome, opiod allergy
	3-8**	1C	P	74	S = 138, P = 19 pl = 338	S = 105, P = 11 pl = 349	Emphysema, hypertension
Mucinous (1)	3-25	1C	P	43	S = 230, P = 11 pl = 297	S = 92, P = 1 pl = 302	Depression
Non-Epithelial							
Granulosa (1)	3-4	1	R	42	S = 309, P = 26 pl = 328	S = 332, P = 35 pl = 378	Mild asthma, depression, migraines, fibromyalgia, Type II diabetes, obesity
Benign							

Thecoma and serous adenofibroma (1)	3-2	N/A	N/A	73	S = 329, P = 15 pl = 235	Not collected	Idiopathic thrombocytopenic purpura, essential hypertension, past history malignant melanoma
Mucinous cystadenoma (1)	3-10	N/A	N/A	31	S = 174, P = 2 pl = 274	Not collected	Epilepsy
Hydrosalpinx (1)	3-15	N/A	N/A	72	S = 52, P = 2 pl = 327	Not collected	Asthma
Mucinous cystadenoma and endometriosis (1)	3-17	N/A	N/A	62	S = 106, P = 15 pl = 198	Not collected	Diabetes, hypertension, past bowel cancer
Endometriomatous ovarian cysts (1)	3-19	N/A	N/A	42	S = 339, P = 11 pl = 342	Not collected	None
Serous cystadenoma (1)	3-22	N/A	N/A	84	S = 264, P = 25 pl = 293	S = 226, P = 14 pl = 280	Hypertension, glaucoma, osteoarthritis, depression
Endometrial							
Epithelial							

Serous (1)	3-23	4	P	74	S = 2221, P = 25 pl = 531	NA[1]	Hypertension, Ischaemic heart disease
Endometrioid (5)	3-5	1B	P	61	S = 358, P = 12 pl = 278	S = 366, P = 24 pl = 297	Hypertension, hiatus hernia
	3-7	2B	P	85	S = 73, P = 6 pl = 331	S = 532, P = 42 pl = 314	Renal failure, cerebrovascular accident
	3-11	1C	P	67	S = 488, P = 31 pl = 357	S = 574, P = 27 pl = 348	Hypertension, obesity (BMI=38)
	3-13	1C	P	63	S = 402, P = 24 pl = 252	S = 513, P = 24 pl = 274	Hypertension, indigestion
	3-20	1B	P	66	S = 569, P = 20 pl = 262	S = 639, P = 51 pl = 369	None
Clear-cell (1)	3-16	2B	P	66	S = 229, P = 18 pl = 157	S = 477, P = NA[2] pl = NA[2]	Hypertension, hypothyroidism, cerebrovascular accident
Mixed – clear-cell and endometrioid (1)	3-1	1B	P	72	S = 598, P = 12 pl = 391	S = 384, P = 48 pl = 389	Ulcerative colitis, pituitary adenoma causing pan-hypopituiterism, asthma
Non-Epithelial							
Carsinosarcoma of the	3-14	3C	P	83	S = 269, P = 24 pl = 318	S = 210, P = 31 pl = 369	Past history breast cancer, dyslipidaemia

uterus (1)

** = Concomitant endometrioid adenoma of the endometrium.

R = Recurrent disease

N/A = Not applicable

NA^0 = Participant declined the second blood test.

NA^1 = During laparotomy surgical resection of disease did not proceed due to advanced disease.

NA^2 = Due to difficulty in accessing veins, only enough blood was obtained for serum analysis.

Table 5-4 Cohort 4: Pilot Clinical Trial of Green Tea Extract in Patients with Ovarian and/or Endometrial Cancer, Disease Group

15 women with evidence of endometrial or ovarian cancer were to take oral Green tea capsules (equivalent of 900mg EGCG a day) for 6 days. VEGF and platelets were measured before and on the 6th days, after the last capsules were taken.

Tumour Histology (n)	**ID**	**Stage**	**Age**	**Tumour status: Recurrent (R) / Persistent (P)**	**Before serum VEGF/ plasma VEGF (ρg/ml), platelet count (×10^6)**	**Number of days taking Green Tea Extracts**	**After serum VEGF / plasma VEGF (ρg/ml), platelet count (×10^6)**	**Reported Adverse Effects**	**Co-Morbidities**	**Medications**
Ovarian										
Epithelial										
Papillary serous (11)	4-1	3B	69	R	386 / 45, 224	6	542 / 38, 123	None	None	Hyocine butylbromide, fentanyl, coloxyl, bisacodyl, omeprazole
	4-3/ 2-4	3C	73	R	577 / 56, 299	6	586 / 76, 276	Insomnia, diarrhoea	Hypertension, urinary urgency, early satiety	None
	4-4	3C	77	P	607 / 48, 377	6	642 / 53, 288	Deterioration of	Hypertension, hypercholsterolemia,	Tamoxifen, metoproplol,

							dermatitis	osteoporosis, chonic musculo-skeletal pain, hyponatremia	aspirin, cholecalciferol, alendronate, calcium carbonate
4-5*	3C	67	P	432 / 33, 255	14	692 / 53, 349 601 / 5, 259	Abdominal pain, diarrhoea, vomiting	None	None
4-6/2-3	3	76	P	572 / 35, 280	6	679 / 35, 320	None	None	None
4-8	4	78	P	775 / 48, 225	3.5	834 / 37, 237	Fatigue, nausea, insomnia	Hypertension	Omeprazole, cyclizine, amizide, morphine
4-12**	3C	59	P	66 / 93, 132	6	69 / 73, 137	Insomnia	None	None
4-13	3C	61	P	236 / 41, 237	6	332 / 31, 270	Fatigue	None	Fluoxetine, paracetamol, zopiclone, aprepitant, omeprazole, domperidone,

										dexamethasone
	4-15	3C	56	P	324 / 20, 307	6	202 / 6, 164	None	None	None
	4-17/2-6	3C	59	R	524 / 53, 232	6	570 / 56, 246	None	Cerebrovascular accident	Metoprolol
	4-23	3C	54	P	822 / 60, 243	6	832 / 55, 240	Nausea, polydipsia	Hypothyroidism	Thyroxine
Endometrioid (2)	4-2	4	73	P	1111 / 70, 206	6	1193 / 78, 220	None	Hypertension, mild asthma, spinal fusion, bilateral carpal tunnel syndrome	Bendrofluazide
	4-7	4	38	P	1400 / 119, 459	6	1674 / 180, 515	None	None	Amitriptyline, estrofem, fluoxetine, cyclizine, paracetamol, ranitidine, morphine
Non-Epithelial										

Epithelioid Mesothelioma (1)	4-9	4	31	P	1042 / 83, 480	6	1106 / 82, 519	None	None	Enoxaparin
Endometrial Endometrioid (1)	4-10	2	57	P	177 / 50, 199	6	120 / 38, 223	Nausea	Hiatus hernia	Omeprazole, metoclopramide, fluoxetine

*** = The participant stopped taking the capsules after one day due to side effects and on a later occasion resumed on half the dose (equivalent of 450mg EGCG) of Green Tea Extracts for four days. An additional blood test was performed after the second period of ingesting Green Tea Extracts.**

**** = Concomitant endometrial endometrioid cancer, stage 1B**

Table 5-5 Cohort 4: Pilot Clinical Trial of Green Tea Extract in Patients with Ovarian and/or Endometrial Cancer, Control Group

6 women who had had endometrial or ovarian cancer were to take oral Green tea capsules (equivalent of 900mg EGCG a day) for 6 days. VEGF and platelets were measured before and on the 6th days, after the last capsules were taken.

Past Tumour Histology (n)	ID	Stage when diagnosed	Age	Before serum VEGF/ plasma VEGF (ρg/ml), platelet count ($\times 10^6$)	Number of days taking Green Tea Extracts	After serum VEGF / plasma VEGF (ρg/ml), platelet count ($\times 10^6$)	Reported Adverse Effects	Co-Morbidities	Medications
Ovarian									
Epithelial									
Papillary serous (1)	4-22/1-8	4	77	1319 / 32, 308	6	1506 / 90, 334	Nausea, headache	Hypertension, depression, constipation, osteoarthritis knees	Labetolol, omeprazole, paracetamol, metamucil, coloxyl
Clear-cell (1)	4-21/1-2	1C	48	245 / 21, 216	5	229 / 7, 215	Nausea, insomnia	Single kidney	None
Endometrial									

Endometrioid (4)	4-18/1-7	1A	54	181 / 10, 314	6	232 / 20, 332	Nausea, headache	Surgical menopause, depression	Estrofem, citalopram
	4-19/1-12	3A	56	202 / 20, 224	6	183 / 16, 235	None	Hypertension	Quinapril
	4-20/1-14	1B	68	185 / 15, 311	6	187 / 21, 308	None	Hypertension, cellulitis, hypothyroidism, early osteoarthritis hip	Felodipine, thyroxine, paracetamol, fluazide, glucosamine
	4-24/1-13	1A	53	590 / 56, 269	6	558 / 52, 248	Insomnia	Right coronary artery disease	Aspirin

6 Bibliography

1. Ministry of Health. *Cancer: New registrations and deaths 2005: Revised edition*. 2009 [cited 2009 17 July]; Available from: http://www.moh.govt.nz.

2. Holschneider, C.H. and J.S. Berek. *Ovarian cancer: epidemiology, biology, and prognostic factors*. 2000: John Wiley & Sons, Inc. New York.

3. Cannistra, S.A., *Cancer of the Ovary.* N Engl J Med, 1993. **329**(21): p. 1550-1559.

4. Bell, R., M. Petticrew, and T. Sheldon, *The performance of screening tests for ovarian cancer: results of a systematic review.* Br J Obstet Gynaecol, 1998. **105**(11): p. 1136-47.

5. Ozols, R.F., *Future directions in the treatment of ovarian cancer.* Semin Oncol, 2002. **29**(1 Suppl 1): p. 32-42.

6. Parkin, D.M., F. Bray, J. Ferlay, and P. Pisani, *Global cancer statistics, 2002.* CA Cancer J Clin, 2005. **55**(2): p. 74-108.

7. Amant, F., P. Moerman, P. Neven, D. Timmerman, E. Van Limbergen, and I. Vergote, *Endometrial cancer.* Lancet, 2005. **366**(9484): p. 491-505.

8. Bakkum-Gamez, J.N., J. Gonzalez-Bosquet, N.N. Laack, A. Mariani, and S.C. Dowdy. *Current issues in the management of endometrial cancer*. 2008: Mayo Clinic.

9. Shang, Y., *Molecular mechanisms of oestrogen and SERMs in endometrial carcinogenesis.* Nat Rev Cancer, 2006. **6**(5): p. 360-8.

10. Montejo, M., T.L. Werner, and D. Gaffney, *Current challenges in clinical*

management of endometrial cancer. Adv Drug Deliv Rev, 2009. **61**(10): p. 883-9.

11. Ferrara, N., *VEGF and the quest for tumour angiogenesis factors.* Nature Reviews Cancer, 2002. **2**(10): p. 795-803.

12. Sandison, J.C., *Observations on the growth of blood vessels as seen in the transparent chamber introduced into the rabbit's ear.* American Journal of Anatomy, 1928. **41**(3).

13. Algire, G.H. and H.W. Chalkley, *Vascular reaction of normal and malignant tissue in vivo. I. Vascular reactions of mice to wounds and to normal and neotic implants.* J. nat. Cancer Inst, 1945. **6**: p. 73.

14. Folkman, J., *Tumor angiogenesis: therapeutic implications.* N Engl J Med, 1971. **285**(21): p. 1182-6.

15. Folkman, J., *What is the evidence that tumors are angiogenesis dependent?* 1990, © Oxford University Press. p. 4-7.

16. Zetter, B.R., *Angiogenesis and tumor metastasis.* Annual review of medicine, 1998. **49**: p. 407.

17. Hicklin, D.J. and L.M. Ellis, *Role of the Vascular Endothelial Growth Factor Pathway in Tumor Growth and Angiogenesis.* J Clin Oncol, 2005. **23**(5): p. 1011-1027.

18. Ferrara, N., H.P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors.* Nature medicine, 2003. **9**(6): p. 669-676.

19. Dvorak, H.F., L.F. Brown, M. Detmar, and A.M. Dvorak, *Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis.* American Journal of Pathology, 1995. **146**(5): p. 1029-1039.

20. Poon, R.T.P., S.T. Fan, and J. Wong, *Clinical implications of circulating angiogenic factors in cancer patients.* Journal of Clinical Oncology, 2001. **19**(4): p. 1207.

21. Kondo, S., M. Asano, K. Matsuo, I. Ohmori, and H. Suzuki, *Vascular endothelial growth factor/vascular permeability factor is detectable in the sera of tumor-bearing mice and cancer patients.* Biochimica et biophysica acta. Molecular cell research, 1994. **1221**(2): p. 211-214.

22. Salven, P., H. Manpaa, A. Orpana, K. Alitalo, and H. Joensuu, *Serum vascular endothelial growth factor is often elevated in disseminated cancer.* Clinical Cancer Research, 1997. **3**(5): p. 647-651.

23. Peng, X.P., J.D. Li, M.D. Li, X.M. Ye, and W.C. Yan, *Clinical significance of vascular endothelial growth factor in sera of patients with gynaecological malignant tumors.* Ai zheng= Aizheng= Chinese journal of cancer, 2002. **21**(2): p. 181.

24. Li, L.I., L. Wang, W.E.I. Zhang, B. Tang, J. Zhang, H. Song, D. Yao, Y. Tang, X. Chen, and Z. Yang, *Correlation of Serum VEGF Levels with Clinical Stage, Therapy Efficacy, Tumor Metastasis and Patient Survival in Ovarian Cancer.* Anticancer Research, 2004. **24**(3B): p. 1973.

25. Yamamoto, S., I. Konishi, M. Mandai, H. Kuroda, T. Komatsu, K. Nanbu, H. Sakahara, and T. Mori, *Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels.* British journal of cancer, 1997. **76**(9): p. 1221.

26. Gadducci, A., M. Ferdeghini, A. Fanucchi, C. Annicchiarico, B. Ciampi, C. Prontera, and A.R. Genazzani, *Serum preoperative vascular endothelial growth factor(VEGF) in epithelial ovarian cancer: Relationship with prognostic variables and clinical outcome.* Anticancer research, 1999. **19**(2 B): p. 1401-1405.

27. Cooper, B.C., J.M. Ritchie, C.L.W. Broghammer, J. Coffin, J.I. Sorosky, R.E. Buller, M.J.C. Hendrix, and A.K. Sood, *Preoperative Serum Vascular Endothelial Growth Factor Levels Significance in Ovarian Cancer 1*. 2002, AACR. p. 3193-3197.

28. Harlozinska, A., P. Sedlaczek, J. Kulpa, M. Grybos, E. Wojcik, A. Van Dalen, and R. Einarsson, *Vascular endothelial growth factor(VEGF) concentration in*

sera and tumor effusions from patients with ovarian carcinoma. Anticancer research, 2004. **24**(2 C): p. 1149-1157.

29. Oehler, M.K. and H. Caffier, *Prognostic relevance of serum vascular endothelial growth factor in ovarian cancer.* Anticancer research, 2000. **20**(6 D): p. 5109-5112.

30. Tempfer, C., A. Obermair, L. Hefler, G. Haeusler, G. Gitsch, and C. Kainz, *Vascular endothelial growth factor serum concentrations in ovarian cancer.* acogjnl, 1998. **92**(3): p. 360-363.

31. Chen, C.A., W.F. Cheng, C.N. Lee, T.M. Chen, C.C.S. Kung, F.J. Hsieh, and C.Y. Hsieh, *Serum vascular endothelial growth factor in epithelial ovarian neoplasms: correlation with patient survival.* Gynecologic Oncology, 1999. **74**(2): p. 235-240.

32. Hefler, L.A., R. Zeillinger, C. Grimm, A.K. Sood, W.F. Cheng, A. Gadducci, C.B. Tempfer, and A. Reinthaller, *Preoperative serum vascular endothelial growth factor as a prognostic parameter in ovarian cancer.* Gynecologic oncology, 2006. **103**(2): p. 512-517.

33. Duncan, T.J., A. Al-Attar, P. Rolland, I.V. Scott, S. Deen, D.T.Y. Liu, I. Spendlove, and L.G. Durrant, *Vascular endothelial growth factor expression in ovarian cancer: a model for targeted use of novel therapies?* Clinical Cancer Research, 2008. **14**(10): p. 3030.

34. Shaarawy, M. and S.A. El-Sharkawy, *Biomarkers of intrinsic angiogenic and anti-angiogenic activity in patients with endometrial hyperplasia and endometrial cancer.* Acta Oncologica, 2001. **40**(4): p. 513-518.

35. McMeekin, D.S., M.W. Sill, D. Benbrook, K.M. Darcy, D.J. Stearns-Kurosawa, L. Eaton, and S.D. Yamada, *A phase II trial of thalidomide in patients with refractory endometrial cancer and correlation with angiogenesis biomarkers: a Gynecologic Oncology Group study.* Gynecologic oncology, 2007. **105**(2): p. 508-516.

36. Folkman, J., *Clinical applications of research on angiogenesis.* New England Journal of Medicine, 1995. **333**(26): p. 1757-1763.

37. Des Guetz, G., B. Uzzan, P. Nicolas, M. Cucherat, J.F. Morere, R. Benamouzig, J.L. Breau, and G.Y. Perret, *Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature.* British journal of cancer, 2006. **94**(12): p. 1823-1832.

38. Yamamoto, Y., M. Toi, S. Kondo, T. Matsumoto, H. Suzuki, M. Kitamura, K. Tsuruta, T. Taniguchi, A. Okamoto, and T. Mori, *Concentrations of vascular endothelial growth factor in the sera of normal controls and cancer patients.* Clinical Cancer Research, 1996. **2**(5): p. 821-826.

39. Poon, R.T.P., C.P.Y. Lau, S.T. Cheung, W.C. Yu, and S.T. Fan, *Quantitative correlation of serum levels and tumor expression of vascular endothelial growth factor in patients with hepatocellular carcinoma*. 2003, AACR. p. 3121-3126.

40. Salgado, R., I. Benoy, R. Weytjens, D. Van Bockstaele, E. Van Marck, P. Huget, M. Hoylaerts, P. Vermeulen, and L.Y. Dirix, *Arterio-venous gradients of IL-6, plasma and serum VEGF and D-dimers in human cancer.* British journal of cancer, 2002. **87**(12): p. 1437-1444.

41. Werther, K., S. Bulow, P. Hesselfeldt, N.F.K. Jespersen, M.N. Svendsen, and H. Nielsen, *VEGF concentrations in tumour arteries and veins from patients with rectal cancer.* Apmis, 2002. **110**(9): p. 646-650.

42. Berse, B., L.F. Brown, L. Van de Water, H.F. Dvorak, and D.R. Senger, *Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors.* Molecular biology of the cell, 1992. **3**(2): p. 211.

43. Webb, N.J.A., C.R. Myers, C.J. Watson, M.J. Bottomley, and P.E.C. Brenchley, *Activated human neutrophils express vascular endothelial growth factor (VEGF).* Cytokine, 1998. **10**(4): p. 254-257.

44. Banks, R.E., M.A. Forbes, S.E. Kinsey, A. Stanley, E. Ingham, C. Walters, and P.J. Selby, *Release of the angiogenic cytokine vascular endothelial growth factor(VEGF) from platelets: significance for VEGF measurements and cancer biology.* British journal of cancer, 1998. **77**(6): p. 956-964.

45. Verheul, H.M., K. Hoekman, S. Luykx-de Bakker, C.A. Eekman, C.C. Folman, H.J. Broxterman, and H.M. Pinedo, *Platelet: transporter of vascular endothelial growth factor.* Clinical cancer research, 1997. **3**(12): p. 2187-2190.

46. Maloney, J.P., C.C. Silliman, D.R. Ambruso, J. Wang, R.M. Tuder, and N.F. Voelkel, *In vitro release of vascular endothelial growth factor during platelet aggregation.* American Journal of Physiology- Heart and Circulatory Physiology, 1998. **275**(3): p. 1054-1061.

47. Salgado, R., I. Benoy, J. Bogers, R. Weytjens, P. Vermeulen, L. Dirix, and E. Van Marck, *Platelets and vascular endothelial growth factor(VEGF): A morphological and functional study.* Angiogenesis(London), 2001. **4**(1): p. 37-43.

48. Jelkmann, W., *Pitfalls in the measurement of circulating vascular endothelial growth factor.* Clinical Chemistry, 2001. **47**(4): p. 617-623.

49. Fuhrmann-Benzakein, E., M.N. Ma, L. Rubbia-Brandt, G. Mentha, D. Ruefenacht, A.P. Sappino, and M.S. Pepper, *Elevated levels of angiogenic cytokines in the plasma of cancer patients.* International Journal of Cancer, 2000. **85**(1).

50. Salven, P., A. Orpana, and H. Joensuu, *Leukocytes and Platelets of Patients with Cancer Contain High Levels of Vascular Endothelial Growth Factor 1*. 1999, AACR. p. 487-491.

51. Lee, J.K., Y.J. Hong, C.J. Han, D.Y. Hwang, and S.I. Hong, *Clinical usefulness of serum and plasma vascular endothelial growth factor in cancer patients: which is the optimal specimen?* International journal of oncology, 2000. **17**(1): p. 149.

52. George, M.L., S.A. Eccles, M.G. Tutton, A.M. Abulafi, and R.I. Swift, *Correlation of plasma and serum vascular endothelial growth factor levels with platelet count in colorectal cancer: clinical evidence of platelet scavenging?* 2000, AACR. p. 3147-3152.

53. Vermeulen, P.B., P. Salven, I. Benoy, G. Gasparini, and L.Y. Dirix, *Blood platelets and serum VEGF in cancer patients.* British journal of cancer, 1999.

79(2): p. 370.

54. Klement, L., *Platelets actively sequester angiogenesis regulators.* Blood, 2009. **113**(12): p. 2835.

55. Kusumanto, Y.H., W.A. Dam, G.A.P. Hospers, C. Meijer, and N.H. Mulder, *Platelets and granulocytes, in particular the neutrophils, form important compartments for circulating vascular endothelial growth factor.* Angiogenesis, 2003. **6**(4): p. 283-287.

56. Werther, K., I.J. Christensen, and H.J. Nielsen, *Determination of vascular endothelial growth factor (VEGF) in circulating blood: significance of VEGF in various leucocytes and platelets.* Scandinavian Journal of Clinical and Laboratory Investigation, 2002. **62**(5): p. 343-350.

57. Dittadi, R., S. Meo, F. Fabris, G. Gasparini, D. Contri, M. Medici, and M. Gion, *Validation of blood collection procedures for the determination of circulating vascular endothelial growth factor(VEGF) in different blood compartments.* The International journal of biological markers, 2001. **16**(2): p. 87-96.

58. Hormbrey, E., P. Gillespie, K. Turner, C. Han, A. Roberts, D. McGrouther, and A.L. Harris, *A critical review of vascular endothelial growth factor (VEGF) analysis in peripheral blood: is the current literature meaningful?* Clinical and Experimental Metastasis, 2002. **19**(8): p. 651-663.

59. Hetland, M.L., I.J. Christensen, T. Lottenburger, J.S. Johansen, M.N. Svendsen, K. Hørslev-Petersen, L. Nielsen, and H. Jørgen Nielsen, *Circulating VEGF as a biological marker in patients with rheumatoid arthritis? Preanalytical and biological variability in healthy persons and in patients.* Disease Markers, 2008. **24**(1): p. 1-10.

60. Zimmermann, R., J. Koenig, J. Zingsem, V. Weisbach, E. Strasser, J. Ringwald, and R. Eckstein, *Effect of specimen anticoagulation on the measurement of circulating platelet-derived growth factors*. 2005, Am Assoc Clin Chem. p. 2365-2368.

61. Hyodo, I., T. Doi, H. Endo, Y. Hosokawa, Y. Nishikawa, M. Tanimizu, K.

Jinno, and Y. Kotani, *Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer.* European Journal of Cancer, 1998. **34**(13): p. 2041-2045.

62. Kraft, A., K. Weindel, A. Ochs, C. Marth, J. Zmija, P. Schumacher, C. Unger, D. Marme, and G. Gastl, *Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease.* Cancer, 1999. **85**(1): p. 178-187.

63. Skopi ski, P., E. Skopi ska-Ró ewska, L. Jung, E. Sommer, J. Chorostowska-Wynimko, and A. Wasiuty ski, *Age-dependence of angiogenic activity of human serum.* Centr Eur J Immunol, 2009. **34**: p. 53-56.

64. Berrahmoune, H., J.V. Lamont, B. Herbeth, P.S. FitzGerald, and S. Visvikis-Siest, *Biological determinants of and reference values for plasma interleukin-8, monocyte chemoattractant protein-1, epidermal growth factor, and vascular endothelial growth factor: results from the STANISLAS cohort.* Clinical Chemistry, 2006. **52**(3): p. 504.

65. Kimura, K., T. Hashiguchi, T. Deguchi, S. Horinouchi, T. Uto, H. Oku, S. Setoyama, I. Maruyama, M. Osame, and K. Arimura, *Serum VEGF—As a prognostic factor of atherosclerosis.* Atherosclerosis, 2007. **194**(1): p. 182-188.

66. Kong, S.Y., H.L. Lee, H.S. Eom, W.S. Park, T. Yun, H.J. Kim, B.H. Nam, M.W. Seong, and D.H. Lee, *Reference intervals for circulating angiogenic cytokines.* Clinical Chemistry & Laboratory Medicine, 2008. **46**(4): p. 545.

67. Malamitsi-Puchner, A., J. Tziotis, A. Tsonou, E. Protonotariou, A. Sarandakou, and G. Creatsas, *Changes in serum levels of vascular endothelial growth factor in males and females throughout life.* Reproductive Sciences, 2000. **7**(5): p. 309.

68. Lieb, W., R. Safa, E.J. Benjamin, V. Xanthakis, X. Yin, L.M. Sullivan, M.G. Larson, H.M. Smith, J.A. Vita, and G.F. Mitchell, *Vascular endothelial growth factor, its soluble receptor, and hepatocyte growth factor: clinical and genetic correlates and association with vascular function.* European Heart Journal, 2009. **30**(9): p. 1121.

69. Heer, K., H. Kumar, V. Speirs, J. Greenman, P.J. Drew, J.N. Fox, P.J. Carleton, J.R.T. Monson, and M.J. Kerin, *VEGF--cyclical variation may have implications in benign breast disease.* The Breast, 1997. **6**(6): p. 396.

70. Kusumanto, Y.H., G.A. Hospers, W.J. Sluiter, W.A. Dam, C. Meijer, and N.H. Mulder, *Circulating vascular endothelial growth factor during the normal menstrual cycle.* Anticancer research, 2004. **24**(6): p. 4237.

71. McIlhenny, C., W.D. George, and J.C. Doughty, *A comparison of serum and plasma levels of vascular endothelial growth factor during the menstrual cycle in healthy female volunteers.* British journal of cancer, 2002. **86**(11): p. 1786.

72. Lissoni, P., F. Rovelli, F. Malugani, F. Brivio, L. Fumagalli, and G.S. Gardani, *Changes in circulating VEGF levels in relation to clinical response during chemotherapy for metastatic cancer.* Int J Biol Markers, 2003. **18**(2): p. 152-5.

73. Vincenzi, B., D. Santini, A. Russo, M. Gavasci, F. Battistoni, G. Dicuonzo, L. Rocci, V.M. Rosaria, N. Gebbia, and G. Tonini, *Circulating VEGF reduction, response and outcome in advanced colorectal cancer patients treated with cetuximab plus irinotecan.* Pharmacogenomics, 2007. **8**(4): p. 319-327.

74. Deguchi, T., T. Hashiguchi, S. Horinouchi, T. Uto, H. Oku, K. Kimura, K. Makisumi, and K. Arimura, *Serum VEGF increases in diabetic polyneuropathy, particularly in the neurologically active symptomatic stage.* Diabetic Medicine, 2009. **26**(3): p. 247-252.

75. Zayed, A., H. Amer, H. Nasar, A. Ibrahiem, and M. Hesham, *Some Biochemical Changes in Serum and Synovial Fluid of Patients with Rheumatoid Arthritis.* Journal of Medical Sciences, 2007. **7**(4): p. 526-535.

76. Griga, T., A. Tromm, J. Spranger, and B. May, *Increased serum levels of vascular endothelial growth factor in patients with inflammatory bowel disease.* Scandinavian journal of gastroenterology, 1998. **33**(5): p. 504-508.

77. Kikuchi, K., T. Hoashi, S. Kanazawa, and K. Tamaki, *Angiogenic cytokines in serum and cutaneous lesions of patients with polyarteritis nodosa.* Journal of the American Academy of Dermatology, 2005. **53**(1): p. 57-61.

78. Mitsuyama, H., W. Matsuyama, J. Iwakawa, I. Higashimoto, M. Watanabe, M. Osame, and K. Arimura, *Increased Serum Vascular Endothelial Growth Factor Level in Churg-Strauss Syndrome**. 2006, Am Coll Chest Phys. p. 407-411.

79. Frantz, S., K.A. Vincent, O. Feron, and R.A. Kelly, *Innate immunity and angiogenesis.* Circulation research, 2005. **96**(1): p. 15.

80. Futami, R., M. Miyashita, T. Nomura, H. Makino, T. Matsutani, K. Sasajima, and T. Tajiri, *Increased serum vascular endothelial growth factor following major surgical injury.* Journal of Nippon Medical School, 2007. **74**(3): p. 223-229.

81. Belizon, A., E. Balik, P. Horst, D. Feingold, T. Arnell, T. Azarani, V. Cekic, R. Skitt, S. Kumara, and R.L. Whelan, *Persistent elevation of plasma vascular endothelial growth factor levels during the first month after minimally invasive colorectal resection.* Surgical Endoscopy, 2008. **22**(2): p. 287-297.

82. Ozols, R.F., *Systemic therapy for ovarian cancer: current status and new treatments.* Semin Oncol, 2006. **33**(2 Suppl 6): p. S3-11.

83. Hurwitz, H., L. Fehrenbacher, W. Novotny, T. Cartwright, J. Hainsworth, W. Heim, J. Berlin, A. Baron, S. Griffing, and E. Holmgren, *Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer.* The New England journal of medicine, 2004. **350**(23): p. 2335.

84. Frumovitz, M. and A.K. Sood, *Vascular endothelial growth factor (VEGF) pathway as a therapeutic target in gynecologic malignancies.* Gynecologic oncology, 2007. **104**(3): p. 768-778.

85. Sanchez-Munoz, A., E. Perez-Ruiz, C. Mendiola Fernandez, E. Alba Conejo, and A. Gonzalez-Martin, *Current status of anti-angiogenic agents in the treatment of ovarian carcinoma.* Clin Transl Oncol, 2009. **11**(9): p. 589-95.

86. Yap, T.A., C.P. Carden, and S.B. Kaye, *Beyond chemotherapy: targeted therapies in ovarian cancer.* Nature Reviews Cancer, 2009. **9**(3): p. 167-181.

87. Frederick, P.J., J.M. Straughn, R.D. Alvarez, and D.J. Buchsbaum, *Preclinical*

studies and clinical utilization of monoclonal antibodies in epithelial ovarian cancer. Gynecologic Oncology, 2009. **113**(3): p. 384-390.

88. Kamat, A.A., W.M. Merritt, D. Coffey, Y.G. Lin, P.R. Patel, R. Broaddus, E. Nugent, L.Y. Han, C.N. Landen, and W.A. Spannuth, *Clinical and biological significance of vascular endothelial growth factor in endometrial cancer.* Clinical Cancer Research, 2007. **13**(24): p. 7487.

89. Yance Jr, D.R. and S.M. Sagar, *Targeting angiogenesis with integrative cancer therapies.* Integrative Cancer Therapies, 2006. **5**(1): p. 9.

90. Guilford, J.M. and J.M. Pezzuto, *Natural products as inhibitors of carcinogenesis.* Expert Opin. Investig. Drugs, 2008. **17**(9): p. 1341-1352.

91. Cekic, V., R.L. Whelan, H. Shantha Kumara, D. Feingold, M. Kalady, N. Dujovny, A. Senagore, and N. Hyman, *Colorectal Resection is Associated With Persistent Proangiogenic Plasma Protein Changes: Postoperative Plasma Stimulates In Vitro Endothelial Cell Growth, Migration, and Invasion.* Annals of Surgery, 2009. **249**(6): p. 973.

92. Cassileth, B.R. and A.J. Vickers, *High prevalence of complementary and alternative medicine use among cancer patients: implications for research and clinical care.* Journal of Clinical Oncology, 2005. **23**(12): p. 2590.

93. Peters, U., C. Poole, and L. Arab, *Does Tea Affect Cardiovascular Disease? A Meta-Analysis.* Am. J. Epidemiol., 2001. **154**(6): p. 495-503.

94. Graham, H.N., *Green tea composition, consumption, and polyphenol chemistry.* Preventive medicine, 1992. **21**(3): p. 334.

95. Cabrera, C., R. Artacho, and R. Gimenez, *Beneficial Effects of Green Tea--A Review.* J Am Coll Nutr, 2006. **25**(2): p. 79-99.

96. Khan, N. and H. Mukhtar, *Tea polyphenols for health promotion.* Life sciences, 2007. **81**(7): p. 519-533.

97. Pham-Huy, L.A.N., H. He, and C. Pham-Huy, *Green tea and health: An overview.* JOURNAL OF FOOD AGRICULTURE AND ENVIRONMENT,

2008. **6**(1): p. 6.

98. Thomasset, S.C., D.P. Berry, G. Garcea, T. Marczylo, W.P. Steward, and A.J. Gescher, *Dietary polyphenolic phytochemicals-promising cancer chemopreventive agents in humans? A review of their clinical properties.* International Journal of Cancer, 2007. **120**(3): p. 451-458.

99. Demeule, M., J. Michaud-Levesque, B. Annabi, D. Gingras, D. Boivin, S. Lamy, Y. Bertrand, and R. Beliveau, *Green tea catechins as novel antitumor and antiangiogenic compounds.* Current Medicinal Chemistry-Anti-Cancer Agents, 2002. **2**(4): p. 441-463.

100. Pisters, K.M., R.A. Newman, B. Coldman, D.M. Shin, F.R. Khuri, W.K. Hong, B.S. Glisson, and J.S. Lee, *Phase I trial of oral green tea extract in adult patients with solid tumors.* J Clin Oncol, 2001. **19**(6): p. 1830-8.

101. Yang, C.S., X. Wang, G. Lu, and S.C. Picinich, *Cancer prevention by tea: animal studies, molecular mechanisms and human relevance.* Nature Reviews Cancer, 2009. **9**(6): p. 429-439.

102. Boehm, K., F. Borrelli, E. Ernst, G. Habacher, S.K. Hung, S. Milazzo, and M. Horneber, *Green tea (Camellia sinensis) for the prevention of cancer.* Cochrane database of systematic reviews (Online), 2009(3).

103. Tang, N.P., H. Li, Y.L. Qiu, G.M. Zhou, and J. Ma, *Tea consumption and risk of endometrial cancer: a metaanalysis.* American journal of obstetrics and gynecology, 2009. **201**(6): p. 605.

104. Zhou, B., L. Yang, L. Wang, Y. Shi, H. Zhu, N. Tang, and B. Wang, *The association of tea consumption with ovarian cancer risk: A metaanalysis.* American journal of obstetrics and gynecology, 2007. **197**(6): p. 594.

105. Zhang, M., C.W. Binns, and A.H. Lee, *Tea Consumption and Ovarian Cancer Risk.* Cancer Epidemiology Biomarkers & Prevention, 2002. **11**(8): p. 713.

106. Steevens, J., L.J. Schouten, B.A.J. Verhage, R.A. Goldbohm, and P.A. Van Den Brandt, *Tea and coffee drinking and ovarian cancer risk: results from the Netherlands Cohort Study and a meta-analysis.* British journal of cancer, 2007.

97(9): p. 1291-1294.

107. Song, Y.J., A.R. Kristal, K.G. Wicklund, K.L. Cushing-Haugen, and M.A. Rossing, *Coffee, tea, colas, and risk of epithelial ovarian cancer.* Cancer Epidemiology Biomarkers & Prevention, 2008. **17**(3): p. 712.

108. Zhang, M., A.H. Lee, C.W. Binns, and X. Xie, *Green tea consumption enhances survival of epithelial ovarian cancer.* International Journal of Cancer, 2004. **112**(3): p. 465-469.

109. Chen, D., V. Milacic, M.S. Chen, S.B. Wan, W.H. Lam, C. Huo, K.R. Landis-Piwowar, Q.C. Cui, A. Wali, and T.H. Chan, *Tea polyphenols, their biological effects and potential molecular targets.* Histology and histopathology, 2008. **23**(4): p. 487.

110. Jung, Y.D., M.S. Kim, B.A. Shin, K.O. Chay, B.W. Ahn, W. Liu, C.D. Bucana, G.E. Gallick, and L.M. Ellis, *EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells.* British journal of cancer, 2001. **84**(6): p. 844-850.

111. Sartippour, M.R., Z.-M. Shao, D. Heber, P. Beatty, L. Zhang, C. Liu, L. Ellis, W. Liu, V.L. Go, and M.N. Brooks, *Green Tea Inhibits Vascular Endothelial Growth Factor (VEGF) Induction in Human Breast Cancer Cells.* J. Nutr., 2002. **132**(8): p. 2307-2311.

112. Mantena, S.K., S.M. Meeran, C.A. Elmets, and S.K. Katiyar, *Orally Administered Green Tea Polyphenols Prevent Ultraviolet Radiation-Induced Skin Cancer in Mice through Activation of Cytotoxic T Cells and Inhibition of Angiogenesis in Tumors.* J. Nutr., 2005. **135**(12): p. 2871-2877.

113. Siddiqui, I.A., N. Zaman, M.H. Aziz, S.R. Reagan-Shaw, S. Sarfaraz, V.M. Adhami, N. Ahmad, S. Raisuddin, and H. Mukhtar, *Inhibition of CWR22R{nu}1 tumor growth and PSA secretion in athymic nude mice by green and black teas.* Carcinogenesis, 2006. **27**(4): p. 833-839.

114. Shankar, S., S. Ganapathy, S.R. Hingorani, and R.K. Srivastava, *EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer.* Frontiers in bioscience: a journal and virtual library, 2008. **13**: p. 440.

115. Zhang, Q., X. Tang, Q.Y. Lu, Z.F. Zhang, J. Rao, and A.D. Le, *Green tea extract and (-)-epigallocatechin-3-gallate inhibit hypoxia-and serum-induced HIF-1 protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells*. 2006, AACR. p. 1227-1238.

116. Tang, X.D., X. Zhou, Q.Z. Zhang, A.D. Le, and K.Y. Zhou, *Effects of green tea extract on expression of human papillomavirus type 16 oncoproteins-induced hypoxia-inducible factor-1alpha and vascular endothelial growth factor in human cervical carcinoma cells.* Zhonghua yi xue za zhi, 2008. **88**(40): p. 2872.

117. Spinella, F., L. Rosano, V. Di Castro, S. Decandia, A. Albini, M.R. Nicotra, P.G. Natali, and A. Bagnato, *Green tea polyphenol epigallocatechin-3-gallate inhibits the endothelin axis and downstream signaling pathways in ovarian carcinoma.* Molecular cancer therapeutics, 2006. **5**(6): p. 1483.

118. Dann, J.M., P.H. Sykes, D.R. Mason, and J.J. Evans, *Regulation of Vascular Endothelial Growth Factor in endometrial tumour cells by resveratrol and EGCG.* Gynecol Oncol, 2009. **113**(3): p. 374-8.

119. Ahn, W.S., J. Yoo, S.W. Huh, C.K. Kim, J.M. Lee, S.E. Namkoong, S.M. Bae, and I.P. Lee, *Protective effects of green tea extracts (polyphenon E and EGCG) on human cervical lesions.* European Journal of Cancer Prevention, 2003. **12**(5): p. 383.

120. Bettuzzi, S., M. Brausi, F. Rizzi, G. Castagnetti, G. Peracchia, and A. Corti, *Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study.* Cancer research, 2006. **66**(2): p. 1234.

121. Li, N., Z. Sun, C. Han, and J. Chen, *The chemopreventive effects of tea on human oral precancerous mucosa lesions.* Proceedings of the Society for Experimental Biology and Medicine, 1999. **220**(4): p. 218-224.

122. Shimizu, M., Y. Fukutomi, M. Ninomiya, K. Nagura, T. Kato, H. Araki, M. Suganuma, H. Fujiki, and H. Moriwaki, *Green tea extracts for the prevention of metachronous colorectal adenomas: a pilot study.* Cancer Epidemiology

Biomarkers & Prevention, 2008. **17**(11): p. 3020.

123. Tsao, A.S., D. Liu, J. Martin, X. Tang, J.J. Lee, A.K. El-Naggar, I. Wistuba, K.S. Culotta, L. Mao, and A. Gillenwater, *Phase II randomized, placebo-controlled trial of green tea extract in patients with high-risk oral premalignant lesions.* Cancer Prevention Research, 2009. **2**(11): p. 931.

124. Shanafelt, T.D., T.G. Call, C.S. Zent, B. LaPlant, D.A. Bowen, M. Roos, C.R. Secreto, A.K. Ghosh, B.F. Kabat, and M.J. Lee, *Phase I trial of daily oral polyphenon E in patients with asymptomatic Rai stage 0 to II chronic lymphocytic leukemia.* Journal of Clinical Oncology, 2009. **27**(23): p. 3808.

125. Brausi, M., F. Rizzi, and S. Bettuzzi, *Chemoprevention of human prostate cancer by green tea catechins: two years later. A follow-up update.* European urology, 2008. **54**(2): p. 472.

126. McLarty, J., R.L.H. Bigelow, M. Smith, D. Elmajian, M. Ankem, and J.A. Cardelli, *Tea polyphenols decrease serum levels of prostate-specific antigen, hepatocyte growth factor, and vascular endothelial growth factor in prostate cancer patients and inhibit production of hepatocyte growth factor and vascular endothelial growth factor in vitro.* Cancer Prevention Research, 2009. **2**(7): p. 673.

127. Chow, H.H., Y. Cai, I.A. Hakim, J.A. Crowell, F. Shahi, C.A. Brooks, R.T. Dorr, Y. Hara, and D.S. Alberts, *Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals.* Clin Cancer Res, 2003. **9**(9): p. 3312-9.

128. Ullmann, U., J. Haller, J.P. Decourt, N. Girault, J. Girault, A.S. Richard-Caudron, B. Pineau, and P. Weber, *A single ascending dose study of epigallocatechin gallate in healthy volunteers.* J Int Med Res, 2003. **31**(2): p. 88-101.

129. Jimenez-Saenz, M. and M.C. Martinez-Sanchez, *Camellia Sinensis liver toxicity.* Journal of Hepatology, 2007. **47**(2): p. 297-298.

130. Gloro, R., I. Hourmand-Ollivier, B. Mosquet, L. Mosquet, P. Rousselot, E. SalamÃ©, M.-A. Piquet, and T.n. Dao, *Fulminant hepatitis during*

self-medication with hydroalcoholic extract of green tea. European Journal of Gastroenterology & Hepatology, 2005. **17**(10): p. 1135-1137.

131. Lee, M.J., P. Maliakal, L. Chen, X. Meng, F.Y. Bondoc, S. Prabhu, G. Lambert, S. Mohr, and C.S. Yang, *Pharmacokinetics of Tea Catechins after Ingestion of Green Tea and (-)-Epigallocatechin-3-gallate by Humans Formation of Different Metabolites and Individual Variability 1.* Cancer Epidemiology Biomarkers & Prevention, 2002. **11**(10): p. 1025-1032.

132. Isbrucker, R.A., J.A. Edwards, E. Wolz, A. Davidovich, and J. Bausch, *Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: dermal, acute and short-term toxicity studies.* Food Chem Toxicol, 2006. **44**(5): p. 636-50.

133. Chow, H.H., I.A. Hakim, D.R. Vining, J.A. Crowell, J. Ranger-Moore, W.M. Chew, C.A. Celaya, S.R. Rodney, Y. Hara, and D.S. Alberts, *Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals.* Clin Cancer Res, 2005. **11**(12): p. 4627-33.

134. Sarma, D.N., M.L. Barrett, M.L. Chavez, P. Gardiner, R. Ko, G.B. Mahady, R.J. Marles, L.S. Pellicore, G.I. Giancaspro, and T.L. Dog, *Safety of Green Tea Extracts: A Systematic Review by the US Pharmacopeia.* Drug Safety, 2008. **31**(6): p. 469.

135. Australian Biospecimen Network. *Biorepository Protocols.* 2007 March 2007 [cited 2009 10 March]; Available from: http://www.abrn.net/.

136. Adams, J., P.J. Carder, S. Downey, M.A. Forbes, K. MacLennan, V. Allgar, S. Kaufman, S. Hallam, R. Bicknell, J.J. Walker, F. Cairnduff, P.J. Selby, T.J. Perren, M. Lansdown, and R.E. Banks, *Vascular Endothelial Growth Factor (VEGF) in Breast Cancer: Comparison of Plasma, Serum, and Tissue VEGF and Microvessel Density and Effects of Tamoxifen.* Cancer Res, 2000. **60**(11): p. 2898-2905.

137. Unno, T., Y.M. Sagesaka, and T. Kakuda, *Analysis of tea catechins in human plasma by high-performance liquid chromatography with solid-phase extraction.* Journal of agricultural and food chemistry, 2005. **53**(26): p. 9885.

138. Martin Bland, J. and D.G. Altman, *Statistical methods for assessing agreement between two methods of clinical measurement.* The Lancet, 1986. **327**(8476): p. 307-310.

139. Zimmermann, R., J. Ringwald, and R. Eckstein, *EDTA plasma is unsuitable for in vivo determinations of platelet-derived angiogenic cytokines.* Journal of Immunological Methods, 2009.

140. Zimmermann, R., D. Arnold, E. Strasser, J. Ringwald, A. Schlegel, J. Wiltfang, and R. Eckstein, *Sample preparation technique and white cell content influence the detectable levels of growth factors in platelet concentrates.* Vox Sanguinis, 2003. **85**(4): p. 283-289.

141. Meo, S., R. Dittadi, and M. Gion, *Biological variation of vascular endothelial growth factor.* Clinical Chemical Laboratory Medicine, 2005. **43**(3): p. 342-343.

142. Brooks, P., *Inflammation as an important feature of osteoarthritis.* Bulletin of the World Health Organization, 2003. **81**(9): p. 689.

143. Gornall, R.J., F.W. Anthony, E.J. Coombes, P. Hogston, and R.P. Woolas, *Investigation of women with endometrial carcinoma using serum vascular endothelial growth factor (VEGF) measurement.* International journal of gynecological cancer: official journal of the International Gynecological Cancer Society. **11**(2): p. 164.