The Endotheliome and the Angiome in Colorectal Cancer

by

Khedar Sean Ramcharan

MBBS FRCS

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Abstract

Cancers receive nutrients, convey waste and metastasise by a dynamic vasculature. Heterogeneous vessels are created by angiogenesis and vasculogenesis. The endothelium, via the angiogenic switch, is central to both processes. Assessing its activities in colorectal cancer (CRC) has promising applications, more so recently for monitoring anti-angiogenic therapies. However no single marker has translated to clinical care and many are altered in other tumours and non-cancer disorders. Hence, the 'endotheliome', the multifactorial assessment of endothelial activity and its subset, the 'angiome' (angiogenic activity), are proposed. I hypothesised and tested that together, circulating cellular and plasma biomarkers are more important determinants of stage, prognosis, and treatment outcomes than a single test.

An improved and validated flow cytometry assay quantified circulating endothelial cells (CECs, displaced from blood vessels) and endothelial progenitor cells (EPCs, for vasculogenesis). The plasma markers, measured by ELISA, were: von Willebrand factor (vWf, for endothelial damage/turnover), soluble E-selectin (for endothelial adhesion in tumour migration), vascular endothelial growth factor (VEGF) and angiogenin (for the 'angiogenic switch'). Markers were prospectively quantified in CRC participants before treatment and compared to non-cancer controls. They were tested against the tumour's clinico-pathological features. The assays were repeated 3 and 6 months after surgery or, where applicable, before and after adjuvant therapy.

CECs and EPCs were highest in CRC and correlated to VEGF only. Both increased in a linear trend with CRC stage and differentiation. Angiogenin was diagnostic of CRC and vWf detected metastatic disease. All markers fell after surgery but varied after adjuvant treatment. Only lower CD34+CD45- cells identified responders to anti-angiogenic therapy. Mathematical models, incorporating pre-treatment CEC, EPC and angiogenin levels with CRC stage, predicted progression within 2 years.

In summary, the endotheliome and angiome are important to understanding the vascular biology of CRC staging and prognosis. Clinically, they are crucial determinants of outcomes and may therefore aid decisions on therapeutic strategies.

Dedication

I dedicate this thesis to my gorgeous and inimitable family, for within these covers lies the reasons for missed recitals, late pickups and cancelled holidays, but now completed, the week in the Caribbean we thoroughly deserve (sponsors welcomed)!

Acknowledgements

Research requires multidisciplinary support without which there would have been no final product. It involved the efforts of countless dedicated NHS and research staff, whom I wish to acknowledge (and for those I have forgotten, my sincerest apologies). First, I wish to thank the staff of the Dudley Group of Hospitals NHS trust (DGOH), Sandwell and City Hospital NHS Trust (SWBH), and Haemostasis, Thrombosis and Vascular Biology Unit of the University of Birmingham (HTVBU) who gifted their time and support during recruitment and beyond. I would like to particularly mention:

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Declaration

This thesis is a record of work conducted at the Haemostasis, Thrombosis and Vascular Biology Unit, Centre for Cardiovascular Sciences, University of Birmingham, City Hospital and the Department of Surgery, Russell's Hall Hospital, West Midlands.

I confirm its preparation and work performed is entirely my own and has not been submitted or accepted previously for a higher degree. The publications/presentations to learned national and international societies from this work are appended.

Sean Ramcharan

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Abbreviations & Definitions

AJCC-TNM	American Joint Committee on Cancer Tumour, Node and Metastasis classification
APC	Allophycocyanin conjugate for FACS (see below)
BD	Benign disease group
BM	Bone marrow
BMI	Body mass index [Kg/m^2]
CAD	Coronary artery disease
CD	Cluster of differentiation or classification determinant
CEC	Circulating endothelial cell
CI	Confidence interval
COSHH	Control of Substances Hazardous to Health
CRC	Colorectal cancer
CT	Computerised tomography scans
CV	Coefficient of variation
EC	Endothelial cell or endothelium
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
FACS	Fluorescence activated cell sorting
FBC	Full blood count
FC	Flow cytometry
FITC	Fluorescein isothiocyanate conjugate for FACS
FOLFOX	Regime of Folinic Acid, 5- fluorouracil and oxaliplatin
FOLFIRI	Regime of Folinic Acid, 5- fluorouracil and irinotecan
FSC	Forward scatter (flow cytometry)
G-CSF	Granulocyte- colony stimulating factor
HC	Healthy control
HPC	Haematopoeitic cells
HSC	Haematopoeitic stem cells
IMS	Immunomagnetic bead separation

IQR	Interquartile range
KDR	Kinase insert domain receptor or VEGFR-2
mAJCC	Modified American Joint Committee on Cancer (TMN)
mCRC	Metastatic colorectal cancer
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MVD	Microvessel density (tumour)
NO	Nitric oxide
NOS	Nitric oxide synthase
PB	Peripheral blood
PBS	Phosphate buffer saline
PE	Phycoerythrin conjugate for FACS
PerCP	Peridinin chlorophyll conjugate for FACS
PET scan	Positron emission tomography scan
PPV	Positive predictive value
ROC	Receiver operator characteristic
sE-sel	Soluble E-Selectin
SCAD	Stable coronary artery disease
SD	Standard deviation
SSC	Side scatter (flow cytometry)
TF	Tissue factor
TNM	Tumour Node Metastasis classification
USS	Ultrasound scan
U&E	Urea and electrolytes
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWf	von Willebrand factor
WCC	White cell count

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Chapter 1

Introduction

1.1 General introduction

After cardiovascular disease, cancer is the second greatest burden on human health. With approximately 13% of all deaths (7.4 million), it is the leading cause of mortality worldwide (1). In the UK, colorectal cancer (CRC) is responsible for 16,000 of the 150,000 cancer deaths annually, and is the second most common cause after lung cancer. It remains the third most frequently occurring with 38,000 new cases per year, the second most common in women after breast cancer and, likewise in men, third after prostate and lung cancer (2, 3).

With over a hundred new cases per day, CRC places a great demand on resources for diagnosis, counselling, specialist treatment (i.e. surgery, radiotherapy and chemotherapy), specialist services (e.g. stoma care, palliative services), and follow-up typically over five years. Furthermore, advances in treatment will eventually push cancer into the realms of chronic diseases, similar to diabetes or rheumatoid arthritis (4). A greater burden on health economics is likely from better therapies and, consequentially, the long-term surveillance required. Already in a relatively short period earlier diagnosis and improved treatment doubled the 5-year survival rate to over 50% within the last 30 years (3, 5).

However, CRC is characterised by a high mortality rate, mainly from diagnosis or presentation at an advanced stage, which justified screening policies (6). A number of strategies were available to screen average risk populations ranging from low cost faecal occult blood test (FOBT) to more invasive and costly flexible sigmoidoscopy, colonoscopy or virtual CT-colonoscopy (7). Currently the UK offers screening colonoscopy after a positive routine FOBT between the ages of 60 to 74 years (6, 8). Only 20 of 1000 FOBTs are positive, of which 2 will have cancer at colonoscopy. There are no diagnostic or prognostic biomarkers that are simple, easily accessible, and relatively inexpensive with good sensitivities and specificities. Although a number of potential markers existed, test performances before, during and after treatment were generally inconsistent and poorly defined. More expensive assays for genomics and, more recently, serum proteonomics are promising technologies. However antibody assays (e.g. ELISA) and flow cytometry (FC) were usually easier, faster, inexpensive, reproducible, accessible and minimally invasive (9).

Fundamentally, cancer is characterized by abnormal cell proliferation, invasion and metastasis, all of which are orchestrated by a complex interplay of genetic factors, cytokines and growth mediators. Crucially all depend on a good blood supply to convey growth mediators to cancer cells, facilitate invasion and metastasize. The endothelium (EC) is the gateway to these processes but disturbances of homeostatic functions, such as haemostasis, barrier to tumour invasion, vasomotor activity, to name a few, were known in CRC (10-13).

Perturbation of EC activity is established in CRC, as reflected in changes to biomarkers expressed by blood vessels in the tumour and those secreted into the peripheral circulation (14, 15). Some, for example plasma vWf, correlated to disease progression and survival (16). More cells with EC morphology and phenotype were found in the peripheral blood (PB) of CRC participants, and other solid organ tumours, than in healthy controls (17). Logically, research is aimed to expand treatment options for CRC that stopped EC activity in angiogenesis. For example, the antibody to vascular endothelial growth factor (VEGF), Bevacizumab, extended survival by at least 6 months in metastatic CRC (2, 18). A marker of its effectiveness would be useful, hence a number of EC surrogates were suggested (19). The origins of these cells are heavily debated. They may arise from existing normal and/ or tumour vasculature, (known as mature circulating endothelial cells or CECs) or represent endothelial progenitor cells (EPCs) derived from stem cells (probably from the bone marrow) that may contribute to neovascularisation (17, 20, 21).

Other EC derived markers were suggested for their clinical and prognostic value in CRC but to date no single test has translated to clinical care and many were altered in other tumours and non-cancer disorders (10, 22-24). I therefore theorised that measuring a combination of biomarkers reflective of the EC's many functions are more important determinants of stage, prognosis, and treatment outcomes than any single test. Therefore EC activity in CRC is worthy of further study and to prove its importance my thesis structure is outlined below.

Thesis Overview

Chapter 1 first considers an overview of vascular biology of the endothelium (EC). The primary importance of ECs in cancer is explored in section 1.2 and in angiogenesis in Section 1.3. From these, two neologisms of the assessment of EC activity are introduced, that is the endotheliome and angiome. In section 1.4, CRC is discussed, specifically the pathophysiology and clinical management. Section 1.5 supports the importance of the endotheliome and angiome in CRC. In the final introductory section (1.6), aspects of the cell biology of CRC, angiogenesis and the EC are outlined in 'what we know' and 'what we don't know'. From these, original hypotheses are formulated and the details of their investigations are provided.

In the second chapter, (a) patient and control recruitment, (b) laboratory and clinical materials and (c) methodology, and (d) statistical methods for analysis are described. In the third chapter the findings of each study on EC activity in CRC are presented. The cross-sectional findings tested whether EC activity is indeed abnormal in CRC when compared to the three proposed control groups. The EC markers relative to disease stage are then investigated. The longitudinal studies tested the changes of the markers after surgery with or without adjuvant therapy. All markers are then analysed for their predictive value of disease recurrence and survival at two years.

In the final chapter the results are discussed and the conclusions are formulated. Finally, studies beyond the remit of this thesis are proposed for future investigations.

1.2 Vascular biology and the endothelium (EC)

The role of the EC in human physiology (and, ultimately, pathophysiology) sparked much debate. Before electron microscopy, many thought that the innermost layer of the blood vessel, barely visible under light microscopy, was functionless. The observations of Mallory in 1898 (25) of endothelial-like cells in the blood of patients with typhoid fever prompted Sabin and Doan in 1926 (26) to conclude that:

".... desquamated endothelium is practically a constant constituent of blood, is not identical to monocytes, and numbers are increased in many pathological conditions"

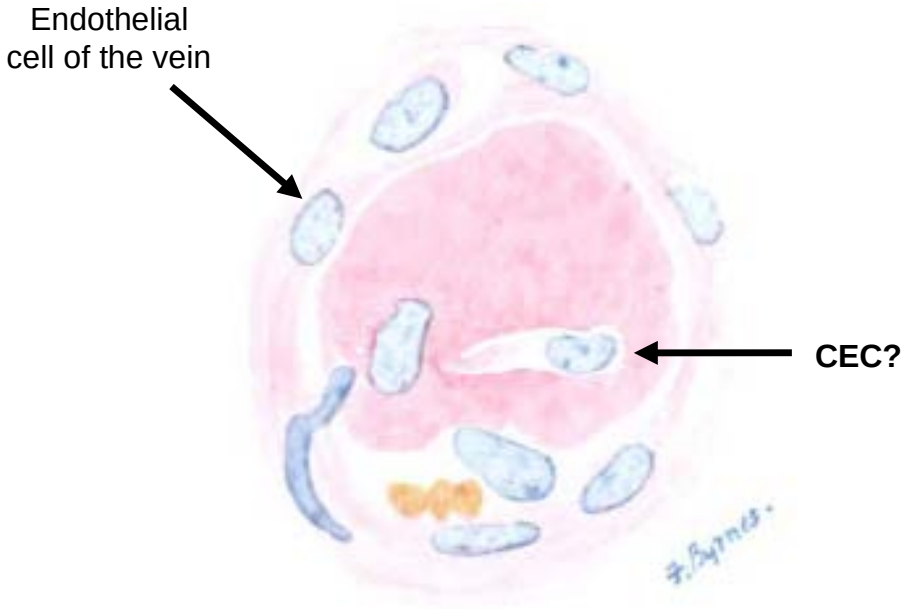


Figure 1: Plate from the Journal of Experimental Medicine, 1898.
Mallory described this as fibrin in a vein but was later suggested by Sabin in 1926 to be a CEC (26). The CEC was therefore proposed as a valuable tool to measure disease activity and prognosis.

This probably is the first recognition of circulating ECs in peripheral blood (PB) as markers of disease processes such as inflammation, infection and cancer. Over the last 3 decades much discovery on its physiology lent to the understanding these roles. Broadly, pathological EC activity either promoted both neo-vascular (and neo-lymphatic) formation (e.g. in cancer and rheumatoid arthritis) or restricted vessel growth (e.g. in atherosclerosis). While the tools to assess these activities, directly or indirectly, are under intense research, many were not translated to clinical use. They included circulating plasma and cellular markers, physiological studies and radiological techniques. Many EC-derived markers e.g. von Willibrand factor (vWf), nitric oxide (NO), were elevated in CRC but their clinical value was inconclusive. Recently CECs and EPCs were proposed for monitoring anti-angiogenic therapy (19, 28). However, clinical application was hindered by the lack of standardised methodology, inconsistent definitions, and inconclusive findings.

Therefore, assessment EC activity in CRC deserved further study. I proposed no single marker could yield useful information. This is probably why no EC marker of diagnosis or prognosis exists in mainstream practice. To support this concept, the current understanding of EC biology is reviewed. I aim to demonstrate their importance as crucial determinants of CRC staging, treatment outcomes and progression. I introduce the multifactorial assessment of EC activity, called the endotheliome and angiome. That is, the endothelium, appended to 'endotheliome' measures **endotheli**al function as a whole (**-ome**). This is parallel to other ‘-omes’, such as the cytome, which considers the total functions of a single cell and

proteomics for protein expression. The angiome, as a subset of the endotheliome, assesses angiogenic activity. The endotheliome and angiome are measures of EC activity that reflect the tumour microenvironment. I hypothesised they may add to or may be better than the current methods of assessing CRC prognosis and outcomes.

1.2.1 The physiological functions of the EC and changes in CRC

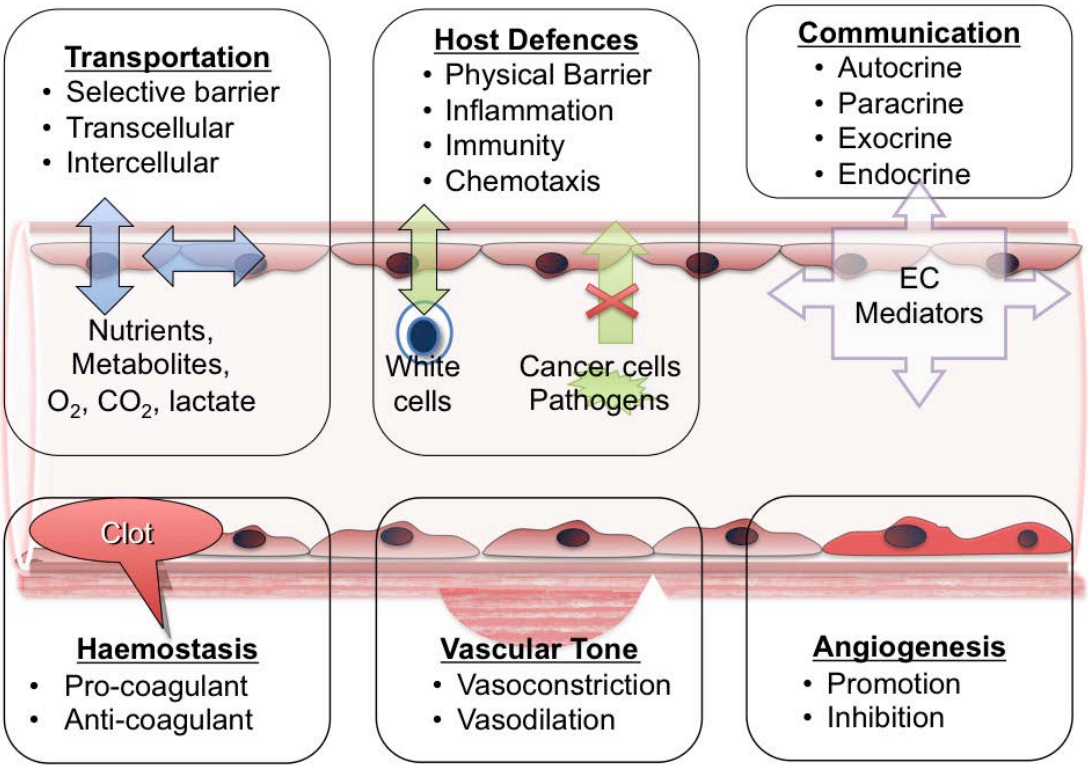
Multicellular organisms establish a circulating network to deliver nutrients, remove waste products and as a means for inter-organ communication (13). Human vasculature comprises of blood and lymphatic vessels all lined by ECs. Vascular ECs are ‘ubiquitous’ from the innermost lining of the heart to the smallest capillaries. It is visible on routine histology as a monolayer of flattened nuclei and is the largest organ in the body. In an adult it has 1-6x10^{13} cells, weighs approximately 1kg and covers a surface area of approximately 4000 to 7000 m^2 (29). Ultrastructurally, each cell is anchored to the underlying basal lamina and attached to each other by adhesion junctions, which prevents diffusion between cells (30).

The EC’s biological properties primarily are to simultaneously maintain tissue homeostasis (with oxygen and nutrients), vessel wall permeability, appropriate blood flow and lumen patency. These functions are regulated by intricate paracrine, endocrine and autocrine activities of a vast number of mediators some of which are directly secreted by the EC. It has anti-oxidant and anti-inflammatory properties,

trafficked leukocyte adhesion and migration, prevents smooth muscle proliferation and migration, and regulats platelet adhesion and aggregation (29).

Though a single layer, its position as the innermost lining of blood vessels requires the EC to respond to a number of stresses and signals. It itself produces a variety of signalling molecules that exert autocrine and paracrine effects which regulate vascular tone, cell to cell and cell to basement membrane adhesion, haemostasis, thrombo-resistance, smooth muscle proliferation and inflammation (31). These physiological roles are summarised in Figure 2.

Figure 2: The heterotrophic functions of the EC in vascular homeostasis.

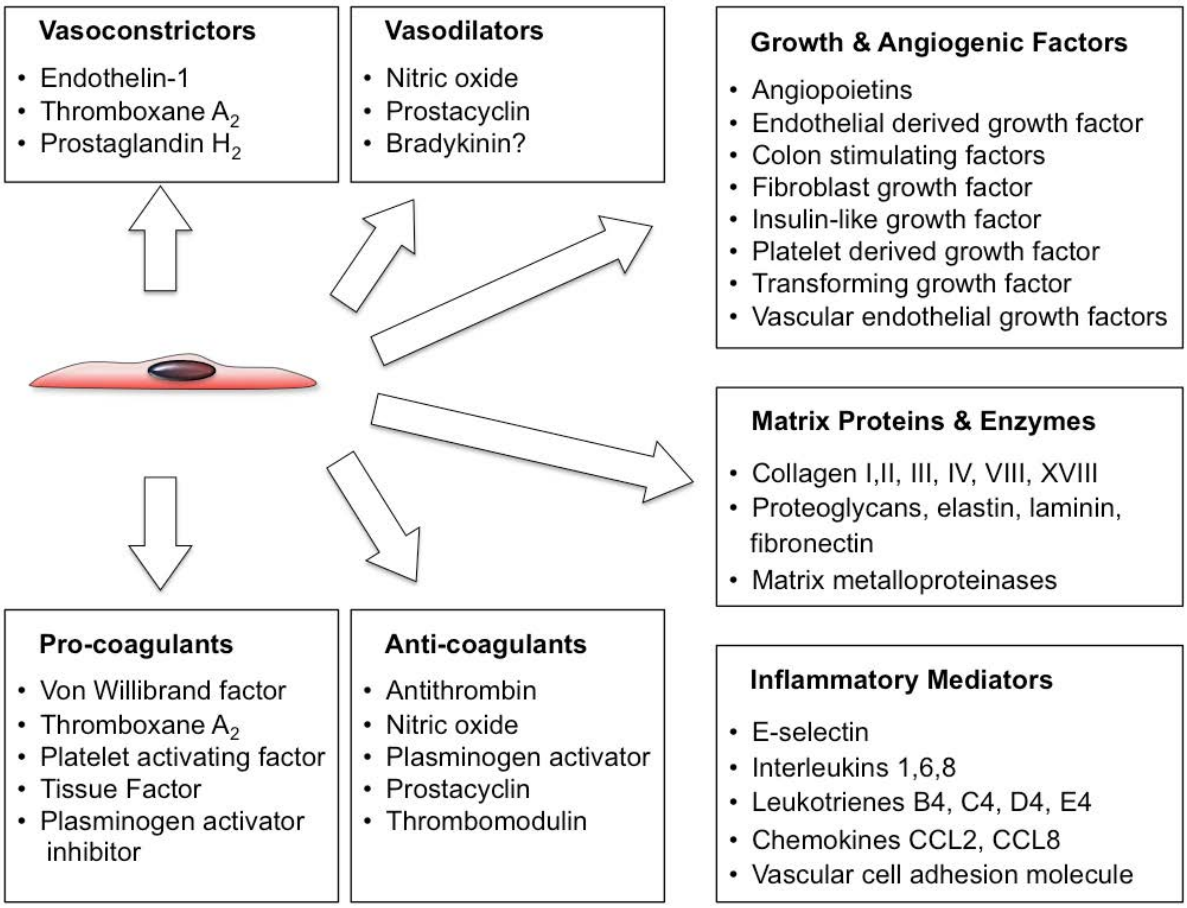


Overall, Sawada et al described the EC as a ‘gate’ e.g. to metastasis, and a ‘fence’ to the egress of molecules aided by transport proteins (32). Neovessels in cancers are derived by angiogenesis from pre-existing host microvessels, primarily venules, but heterogeneity within tumours differs significantly from the organised structure within normal tissues (33). They are microscopically distinguishable by their irregular thickness, aberrant basement membrane, deficient pericytes and loosely associated ECs (34). Movement across the normal EC barrier is usually highly controlled, mainly by caveolae invaginations of scaffolding proteins to form vesicles (transcellular transport) and selective transport of essential macromolecules across tight junctions (paracellular transport) (35). In tumours these activities are significantly increased and further facilitated by larger EC fenestrations varying from 10 to 1000nm (36). A higher proportion of ECs (from angiogenesis), pericyte deficiency and basement changes also enhances vascular permeability. Overlapping margins, excessive EC sprouting and overall loss of the barrier function superseded the normal monolayer with tight junctions(33). Tumour cells often filled the spaces by ‘mimicking’ ECs (37). The wide intercellular gaps leak blood, fibrin and fluid into the interstitial space, which raises the intra-tumour pressure and lowers the pH (38). The irregular EC lining impairs blood flow resulting in hypoxia and hypoperfusion (39). Overall ECs are unable to fulfil their duties to supply nutrients and remove waste products, thus creating the microenvironment for tumour cells to grow (40).

With fundamental structural changes to blood vessels, the EC becomes highly active or ‘dysregulated’ to its demands. It is not surprising therefore that a number of its

biomarkers, summarised in figure 4, were altered in CRC and spanned the range of its functions: haemostasis, vasomotor control, inflammation and angiogenesis.

Figure 4: EC-derived factors of homeostasis altered in CRC.



All factors listed above are elevated and correlated to poorer outcomes in CRC. Some, such as bradykinin and NO worked through mediators of inflammation (IL-6) to facilitate tumour growth.

These functions and their changes in CRC are examined next.

1.2.1.1 The role of EC-derived mediators in CRC

Vasoactive mediators in CRC

Normally, changes in shear stress, blood pressure and oxygen tension stimulate the release of EC-derived mediators of blood flow to improve organ perfusion by still unknown mechanisms (41). The equilibrium between vasodilatation and vasoconstriction to immediate local demands is controlled by the interaction between these mediators and the vascular smooth muscle, the latter often deficient in tumour blood vessels. A number of mediators interact with the EC but very few are produced by the EC directly. Normal pathways are summarily dependent or independent of the potent vasodilator nitric oxide (NO). In response to shear stress NO is released by the activity of nitric oxide synthase in ECs or eNOS on L-arginine and oxygen (42). NO diffuses into the blood vessel wall and increases cGMP (through the activation of guanylate cyclase), a potent mediator of smooth muscle relaxation.

In cancer, levels of eNOS are increased by chaotic blood flow, nutrient demands and VEGF, the key promotor of angiogenesis (43-45). Ziche et al showed that VEGF would be otherwise ineffective without an intact NO/cGMP pathway (46). There were no in vitro studies on vasoactivity of the heterogeneous vessels in tumours. However, the lack of muscular coats, poorer vasoconstrictive control and increased but sluggish blood flow were the more likely consequences. Also, other dichotomous effects on tumour growth were reported. That is, on one hand NO may modulate angiogenesis, cell cycle invasion and metastasis (47) but on the other, inhibit DNA

synthesis and regulate tumour cell apoptosis (48). Higher levels of NO (and VEGF) were linked to poorer disease free survival (DFS) in CRC (49). However, the assay's reliability is uncertain given the short half-life of NO and its concentration is estimated indirectly by measuring the breakdown product, nitrite, with the Griess method.

ECs also produce the vasodilator bradykinin, which is a potent vasodilator for long term tissue perfusion and vascular remodelling (50). Only recently in vivo studies revealed it increased IL-6 levels (linked to angiogenesis), contributed to invasion and migration of CRC cells (51). Similarly endothelin-1/endothelin A receptor (ETAR) axis promoted metastasis in SW480 and SW620 cell lines (52). However the clinical value in CRC prognosis of both IL-6 and endothelin require further evaluation.

Thromboxane A_2 and prostacyclin are both potent vasomodulators with short half-lives and plasma levels are therefore estimated indirectly by measuring metabolite levels. Both are highly expressed in CRC cells from increased COX-2 activity, an enzyme established as a key promoter of the adenoma-carcinoma transformation and angiogenesis (53). They also have antagonistic coagulation functions; this and other EC biomarkers of haemostasis are discussed next.

The role of haemostasis factors in CRC

The interplay of the coagulation cascade and its antagonists is complex; ECs express a variety of proteins involved in these pathways but the vast majority are produced in the liver (54). Coagulation proteins bind to their specific EC protease-activated receptors to transduce the expression of genes for coagulation, as well as angiogenesis, leucocyte adhesion, and regulation of the vascular tone. Tissue factor (TF) is a glycoprotein pro-coagulant receptor for factor VII expressed by sub-endothelial cells and inhibited by tissue factor pathway inhibitor synthesized by and bound to the surface of ECs. TF is typically not expressed on ECs themselves except in cancer and inflammatory disorders (55, 56).

Briefly, TF activated factor X, which then combines with factor Va to convert prothrombin to thrombin (57). Thrombin is both anticoagulant and pro-coagulant. Thrombomodulin expressed on EC surfaces is the major physiological buffer to thrombin. It blocks sites on thrombin that bind to fibrinogen, platelets and factor V. The thrombin-thrombomodulin complex activated protein C pathway that was augmented by the EC protein C receptor (EPCR). Activated protein C must dissociate from EPCR before binding to protein S, an anticoagulant to factor Va. (57, 58). TF expression in CRC tissue was certainly correlated to overall survival (OS) but plasma levels were immeasurable (55). Thrombomodulin was also higher in the tumour and plasma but were not correlated to disease progression (59).

The majority of vWf is derived from ECs in two forms: dimers that are secreted into the plasma and sub-endothelial matrix, and granular multimer stored in Weibel Palade bodies for rapid mobilization during clotting (60). Bound vWf stabilized factor VIII and was a cofactor for platelet binding to exposed extracellular matrix in injured vessel walls. Blann et al dubbed vWf as the 'gold standard' of EC activity when measured in plasma, as the contribution from sub-endothelial cells and ∝-granules of megakaryocytes was likely to be minimal (61). Significantly higher levels were correlated to CRC metastasis and survival (62). More details on vWf are provided section 1.5.2 along with the other biomarkers for my study.

Prostacyclin (a platelet aggregation inhibitor) is almost exclusively derived from ECs and highly expressed in tumour vessels but circulating levels are difficult to measure (63). Thromboxane A_2 was also expressed by COX-2 activity in CRC cells and therefore not a sensitive EC biomarker (64). ECs also produce plasminogen activator inhibitor (PAI), the main physiological antagonist to plasminogen activators (t-PA, also found on ECs, and urokinase-PA) involved in fibrinolysis (54). Disease recurrence and poor survival were correlated to high levels expressed in CRC tissue no reports were found on relationships with plasma concentrations (65). Hepatocytes and smooth muscle of blood vessels are other sources of plasma PAI (66). The EPICOR study (long-tErm follow-up of antithrombotic management Patterns In acute CORonary syndrome) found that elevated quartile levels were a common risk factor to both CRC and cardiovascular disease (67).

The role of ECs in inflammation and CRC

Inflammatory processes involving interactions between leukocytes, tumour cells and ECs are established in cancer (68). ECs are strategically located at the blood-tissue interface to produce and react to various low molecular weight proteins of inflammation generically called cytokines (69). While EC-derived products included interleukins (-1,-6,-8), colony stimulating factors (CSF), chemokines (CCL-2, -8), interferons and leukotrienes, the vast majority of these are from tumour, and associated inflammatory cells such as macrophages and T-cells.

Leucocyte passage regulated by ECs involves attachment, rolling, firm adhesion and transmigration (29, 70). Tumour cell migration is thought to mimic this activity but the in vitro evidence beyond tumour to EC adhesion was lacking (71). The evidence from mice models on colon, breast and lung cancers is that tumour cells stimulated EC activation via chemokines (e.g. CCL2) to express E-selection, intercellular adhesion molecule (ICAM1) and vascular cell adhesion molecule (VCAM) (72, 73). Further CAM expression occurred also with leucocyte interaction and contact with cancer integrins (74, 75). VEGF or TGF from the cancer cells signal disruption of VE-cadherin-b-catenin complex to induce opening of the EC junctions (76). E-selectin expression required de novo transcription, hence expressed many hours after activation and, in liver metastases, promoted trans-endothelial migration via sialyl Lewis ligands on colon cancer cells (77). Uner et al reported serum levels correlated to metastatic liver disease and poorer overall survival (78) but further evaluation with disease stage and progression is required.

Matrix proteins from ECs maintained or laid down the basement membrane of blood vessels and, along with the extracellular matrix (ECM), is degraded by proteases including MMPs, plasminogen activator and cathepsins (79). MMPs may be produced by ECs under the influence of VEGF to penetrate the basement membrane for angiogenesis, but most are derived from colon cancer cells (80). A poorer prognosis of CRC was correlated to over-expression of membrane-type-1-matrix MMP (81). Serum levels of MMP-7 were significantly higher than healthy participants and therefore a potential biomarker of the disease (79, 81, 82).

With the activities of the EC in vascular homeostasis and its changes in CRC outlined, the methods used to investigate its activity are explored next.

1.2.2 Assessing EC activity

Our understanding of vascular biology of EC activity emerged from studies on factors secreted into plasma, their physiological action on other cells, the presence of ECs within the vascular circulation and the anatomical relationship within tissues. Broadly investigations of EC activity took four forms as outlined in figure 4 and table 1. Physiological tests focused mainly on disorders causing disruption or dysfunction e.g. cardiovascular disease, rheumatoid arthritis, and obstructive sleep apnoea (22, 83). Hence, they were not applicable to cancer. Radiological tests will be discussed briefly in section 1.3. Plasma and cellular markers in cancer are discussed next.

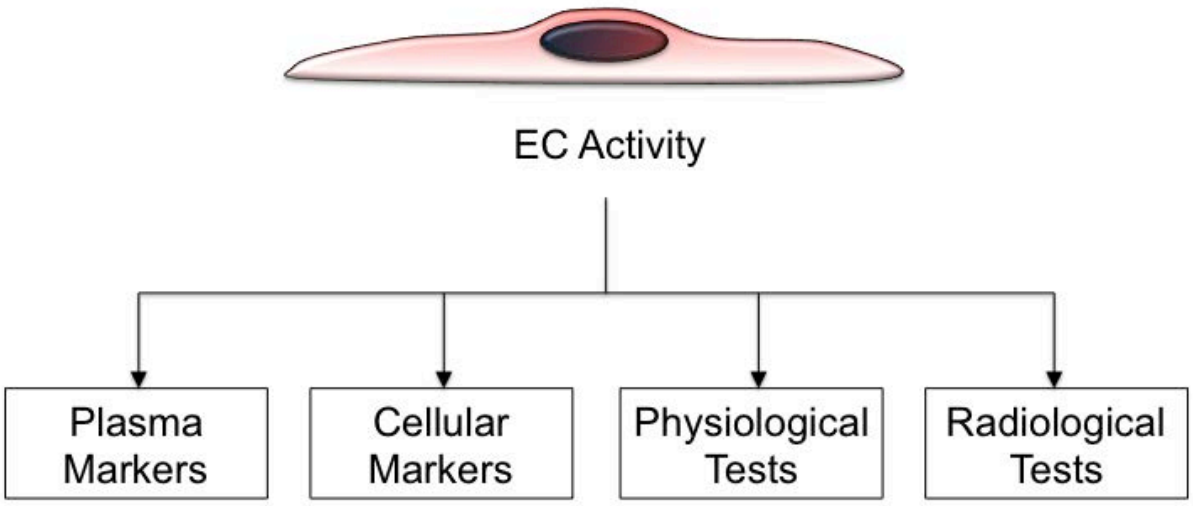


Figure 4: Summary of methods to assess EC Activity in disease.

Table 1: Summary of markers and tests used to assess EC activity in disease.

	Cancer	CVS[A]	Others[B]
1. Plasma markers of EC-specific activity			
▪ Nitric oxide (46, 64, 84)	+	+	+
▪ Prostacyclin (64)	+	+	+
▪ Endothelin-1 (52, 83)	+	+	+
▪ Bradykinin (85, 86)	-	+	+
▪ Thromboxane A_2 (87, 88)	+	+	+
▪ Platelet activating factor (54, 88)	+	+	+
▪ von Willibrand factor (89)	+	+	+
▪ Plasminogen activator inhibitor (54, 88)	+	+	+
▪ Soluble thrombomodulin (59, 83)	+	+	+
▪ Tissue Factor (55, 89)	+	+	+
▪ E-selectin (22, 90)	+	+	+
2. Circulating cellular markers			
▪ CECs (17, 91)	+	+	+
▪ CPCs (24)	+	+	+
▪ EPCs (19)	+	+	+
▪ EMPs (92)	+	+	+
3. Physiological tests			
▪ Coronary endothelial function (83)	-	+	+
▪ Flow mediated dilatation (83)	-	+	+
▪ Pulse wave analysis (83)	-	+	+
4. Radiological Tests			
▪ US Doppler & Duplex Scans (83),	+	+	+
▪ Computerised Tomography[C] (93)	+	+	+
▪ Magnetic Resonance Imaging[C] (93)	+	+	+

[A] CVS: Cardiovascular diseases. [B] Others: Rheumatoid arthritis, obstructive sleep apnoea or diabetes mellitus. [C] with contrast enhancers. US: Ultrasound. CPC- circulating progenitor cells. EMP: endothelial microparticles. CEC: circulating endothelial cells. EPC: endothelial progenitors.

1.2.3 Plasma markers of EC activity in CRC

Unfortunately many EC-derived markers are confined to clinical research, and often difficult and/or expensive to measure. They provided information on EC biology but their clinical value is limited by assay availability, conflicting results and under-powering of studies. Nevertheless, there is considerable interest in many cancers (61). Table 2 summarises those produced by the EC in CRC as discussed above. Angiogenic and growth factors are described in section 1.3 on angiogenesis.

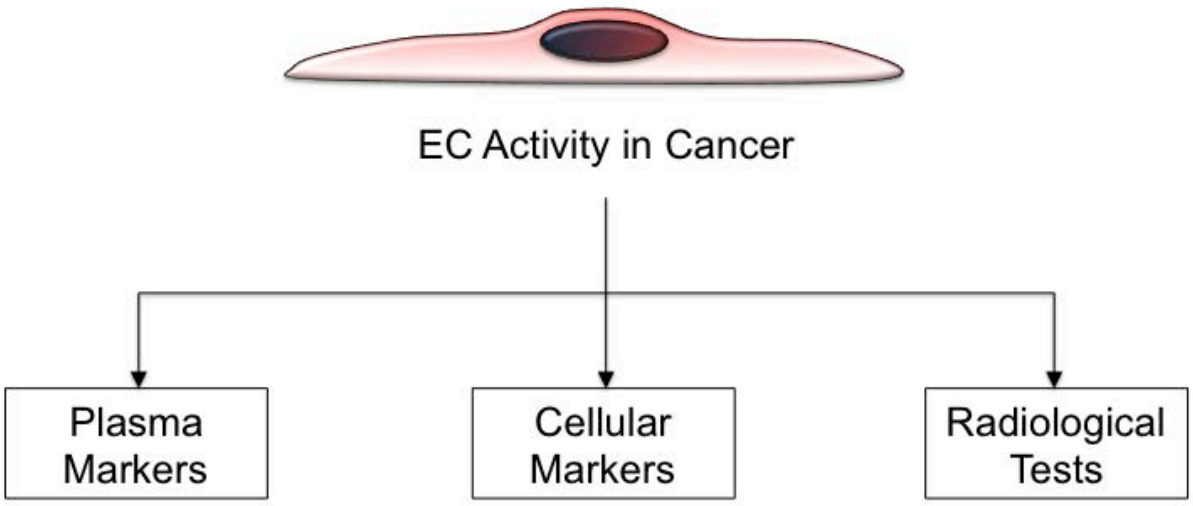


Figure 6: Markers to assess EC activity in cancer

Table 2: Potential roles of EC-derived biomarkers in CRC

Factor	Role in Colorectal Cancer	Ref
Vasodilators		
NO, eNOS	Roles include DNA damage, malignant transformation, angiogenesis, metastasis, cancer blood flow and immune surveillance. NO may enhance radio- and chemo- therapy.	(94) (43) (10)
Prostacyclin PGE_2	Both prostanoids produced by COX-2 (highly expressed by both CRC cells and ECs) promote tumour proliferation, angiogenesis, and metastasis but inhibit apoptosis.	(95) (62) (87)
Vasoconstrictors		
Thromboxane A_2	A product of COX-2 highly expressed in CRC cells; depletion of thromboxane synthetase inhibited tumour proliferation	(62) (87)
Endothelins	Endothelins-1, were also produced by tumour and stromal cells, and stimulated tumour growth and progression	(96)
Procoagulants		
vWf	Increased plasma levels occur in colorectal cancer patients though recently intracellular levels in ECs of CRC vessels were significantly less than those of normal blood vessels.	(11) (23)
PAI	High PAI levels were associated with poor prognosis but are mainly produced by tumour cells	(54)
Tissue Factor	A primary initiator of blood coagulation, TF was involved in hypercoagulability, tumour growth, angiogenesis, and metastasis. Elevated TF expression by cancer cells and tumour ECs were associated with the expression of mutant K- ras, EGFR or p53.	(97)
Anti-thrombotics		
Prostacyclin	Roles in cancer as above. Primarily it was a potent anti-thrombotic.	
Thrombomodulin tPA, heparin	Both were highly expressed on cancer tissue but only tPA correlated to disease progression. Exogenous heparin may promote growth.	(98)
Matrix Products		
MMPs	MMP 1, 3, 7 & 14 were expressed by CRC tissue, associated with poor prognosis independently (MMP) of Dukes' stage. As a proteolytic to ECM matrix they increase invasion & metastasis.	(99) (82)
Inflammatory mediators		
Interleukins CCLs	Abnormal levels of IL-1 & IL-6 were associated with increasing tumour size, stage, metastasis and poor prognosis. In tumour-liver models CCL2 levels predicted metastasis.	(100)
E-selectin & CAMs	Cellular Adhesion Molecule, E-selectin and $\alpha_1\beta_2$ Integrin produced by ECs are associated with a higher metastatic potential in CRC	(101)

NO- Nitric Oxide; eNOS- nitric oxide synthetase; PAI- Plasminogen Activator Inhibitor; COX-2- cyclooxygenase 2; tPA- tissue plasminogen activator; MMP – matrix metalloproteins; CCL- chemokine. CAM- cellular adhesion molecules.

1.2.4 Cellular markers of EC activity in CRC

Physiological loss of mature ECs is detectable in plasma as CECs (102). The review of Goon et al suggested that these mature mural cells, shed by senescence or disease processes, were replaced by angiogenesis from neighbouring cells and/or reparative EPCs derived mainly from bone marrow stem cells (92).

1.2.4.1 The CEC

Endothelial-like cells were described in peripheral smears for many years. In the 1970's their morphology was defined by light microscopy, May–Grünwald Giemsa staining, Ficoll density centrifugation and separation. In animal studies vascular damage by ovalbumin, trisodium citrate and shock from coliform endotoxemia increased the number of CECs (102, 103). In humans this was shown in smoking, cardiovascular disease, acute myocardial infarction, immunosuppression, hypertension and homocysteinaemia after methionine load (104-108). Hladovec et al suggested these cells were anucleate carcasses similar to those from trisodium citrate induced vascular damage (108).

The EC phenotypes of the CECs isolated from blood were later defined by indirect immunofluoresence by antibodies to intracellular vWf, the prototypical marker of ECs (109, 110). However poor specificity highlight the need for a reliable cell surface protein. Potential candidates were either non-specific (e.g. adhesion molecules, integrins) or were intra-cellular (e.g. tPA, vWf) and therefore relatively inaccessible to

monoclonal antibodies without cell permeabilization. In 1991 two groups reported antibodies to newly discovered cell surface antigens on ECs (HEC 19 and S-Endo 1) and used them to quantify CECs (111, 112). Dignat-George et al subsequently characterized their antibody to the CD146 molecule. Solovey et al used a similar antibody, P1H12, to detect CECs in sickle cell anaemia (61, 113, 114).

Over the last 20 years various techniques further characterised and isolated CECs. Not surprisingly, levels were higher in a number of conditions, including cancer, when compared to healthy controls (92). However, results were inconsistent probably from the variations in the methodology of isolation and detection. Immunotypes were not exclusively specific or sensitive to CECs as they shared surface receptors with other cells (see table 3). Recent strategies, theoretically, increased the accuracy of detection by the use of more than one monoclonal antibody and the application of cell sorters. Nevertheless the relatively small numbers would inevitably result in significant inter- and intra-observer variability (61). At publication of this thesis, ‘accurate’ detection and enumeration remained a challenge. CECs, usually absent in the blood of most healthy individuals, were elevated in cancer probably from vascular invasion and vessel heterogeneity. It was proposed they were driven from the intima and the consequence, not the initiator, of the pathology (61). However it is uncertain if they are exclusively from vessels at the source of insult or included those from distant sites under the influence of circulating mediators released from the source i.e. the entire endothelium is altered by cancer (17, 61).

Characterising CECs

In healthy adults there are between 0 to 20 cells per millilitre of PB (115). Given their low numbers technical errors in sampling, preparation and analysis were expected. Our unit demonstrated that traumatic venesection increased CEC counts, the cells themselves having arisen from the site of venepuncture (116). The phenotypic expression of CECs was the subject of much debate but despite recommendations of Woywodt et al on technical standardization, general consensus was lacking (117). Multiple phenotype descriptions, variations in assays and heterogeneity of donors accounted for the wide variations in CEC counts reported (See Table 4). As Blann et al noted, limitations prevented a meta-analysis of their role in cancer (61).

Alternatively the multiplicity in phenotypes described may reflect sub-populations of CECs and therefore the dynamic status of ECs in fulfilling their various functions at any given time (17). This argument may justify assays that used more than one immunophenotype but highlighted the need for further studies to characterize the subsets and, assuming a correlation existed, their corresponding function(s).

Nevertheless, the ideal marker specific to and constantly expressed by all CECs remained elusive. A number of phenotypes expressed by ECs were identified and many were co-expressed by other cells e.g. leucocytes and platelets, (see table 3).

Table 3: Summary of markers used in assays to identify ECs.

Marker	Description/ Expression	CEC	Co expression
CD31[a]	Leucocyte adhesion molecule	+	Platelets, leucocytes (118, 119)
CD34	Cell to cell adhesion factor/ glycoprotein	+	HPCs, blood vessel ECs, dendritic cells, mast cells (120)
CD36[b]	Scavenger receptor	+	Platelets, monocytes and dendritic cells (121, 122)
CD45	Signalling molecule*	-	Pan-leucocyte (123)
CD54[c]	I-CAM	+	Lymphocytes, monocytes (124)
CD62-E	E-selectin- CAM	+	None (125)
CD62-P	P-selectin- CAM	+	Platelets (125)
CD90	Thymus cell antigen	(+)[1]	Neurons, stem cells, T-cells, melanomas, fibroblasts (126)
CD105[d]	Mediated angiogenesis and oncogenesis	+	HSCs, monocytes (127)
CD106[e]	Adhesion molecule for leucocytes (VCAM)	(+)[2]	Lymphocytes, monocytes, eosinophils, basophils (128)
CD117[f]	Stem cell factor receptor	(+)[1]	HSCs (129)
CD137	Tumour necrosis factor receptor	+	ECs, DCs, T cells, NK cells, granulocytes (130)
CD144	VE-cadherin- EC adhesion glycoprotein	+	Pan-ECs only (131)
CD146[g]	EC adhesion molecule	+	Pericytes, melanomas, gliomas, fibroblasts, T cells (132)
AcLDL[j]	Probe	+	None (133)
vWf	Von Willibrand factor	+	Platelets (89)
UEA-1	Ulex europaeus agglutinin 1 probe	+	None (134)

HSC Haematopoietic Stem Cells; j - [DiI-AcLDL] 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; a -PECAM; b- CR1- collagen receptor 1; c- ICAM-1; d –Endoglin; e - VCAM-1; f -C-Kitt; g -MelCAM; i -B7-H3. (+)[1] Some ECs. (+)[2] Upregulated after EC activation.
* For cell growth, differentiation, mitosis & oncogenesis

The majority of assays defined the CEC as bearing the CD146 antigen and not haematopoietic (i.e. CD45) or progenitor markers e.g. CD133 (see table 4). Of the multitude of surface markers described, ECs were unique in their uptake of Ulex Eurpaeus Lectin-1 (UEA-1) and acetylated low-density lipoproteins (Dil Ac-LDL) (132, 134-136). Hence Woywodt et al advocated their use for CEC validation but lengthy and costly multistage assays are significantly practical limitations (117).

Immunomagnetic separation (IMS) with flourochrome labelled CD146 antibodies bound to magnetic beads involved identification and/or enumeration by light microscopy, electron microscopy, and/or flow cytometry (tables 5 and 7). Lengthy batch processing may further compromise the results from cell loss by enrichment, apoptosis, lysis of red blood cells, concentration by centrifugation and preparation of a mononuclear cell suspension by density centrifugation e.g. Ficoll (117, 137).

Recently, real time PCR identified CD146 genes of human microvascular lung ECs (HMVEC-L) and CD144 genes in rectal cancer ECs (138, 139). However, the authors reported a higher sensitivity but lower specificity when compared to FC.

Table 4: CECs – assay protocols, phenotypes and relationship with cancers

Tumour(s)	Protocol	Phenotype	CEC levels (median & IQR cells/ml of blood) Cancer	Control	P< 0.05	Study
Prostate Cancer n=31	L, W, FAb & FC	$CD45^-CD146^+$ $CD34^+$	25 [a] (12-52)	28 [a] (11-43)	**n**	(140)
Breast Cancer n=160	L, W, FAb & FC	$CD45^-CD146^+$ $CD34^+$	9.4 [a] (5-12.7)	7.7 [a] (6-10)	**y**	(24)
Breast Cancer n=41	FHDGC, CD45 DC & FC	$CD45^-$ $CD146^+$	61 [b] (11-2335)	7 [b] (2-54)	**y**	(141)
CLL n=20	FAb & FC	$CD45^-$ $CD34^+$ $CD146^+$	26.5 [a] (7-89)	18.5 [a] (4-66)	**y**	(142)
Various metastatic carcinomas n=206	IMS-CD 146 & CellTrack®	$DAPI^+$ $CD45^-$ $CD146^+$ $CD105^+$	111 +/-255	21 +/-18	**y**	(143)
GIST n=16	CFr/T, FAb, L,W & FC	$CD45^-$ $CD31^+$ $P1H12^+$ $CD133^-$	1090 [d]	540 [d]	**y**	(144)
MDS n=128	L, W & FC	$CD45^-$ $CD34^+$ $CD146^+$ $CD133^-$	5120 [d]	1530 [d]	**y**	(145)
Various cancers[c] n=112	IMS-CD146 & LM	$CD146^+$ $CD31^+$ vWf^+ $VEGRF2^+$	399+/-36 [d]	121+/-16 [d]	**y**	(146)
Lymphoma (n=30) & Breast Cancer (n=46)	FAb L, No Wash FC	Active CEC $CD45^-$ $P1H12^+$ $CD31^+$ $CD105^+$ $CD106^+$ $CD133^+$	6.8[e] (5-8.6)	1.2 [e] (0.1-2.3)	**y**	(17)
		Resting CEC $CD45^-$ $P1H12^+$ $CD31^+$ $CD105^-$ $CD106^-$ $CD133^-$	39.1 [e] (16.8-61.4)	7.9 [e] (4.7-11)	**y**	

CFU-EC: colony forming unit-endothelial cell; EOC: endothelial outgrowth cells; CFr/T: controlled freezing & thaw procedure; FAb: Labelling with flourochromes; L: lysis procedure; W: wash procedure; LM: light microscopy; FC; flow cytometry; ICS: immunocytostaining; GIST: Gastrointestinal Stromal Tumour; FHDGC: Ficoll-Hypaque density gradient centrifugation; CD45 DC: RosetteSep®Human CD45 Depletion cocktail (StemCell Technologies, Vancouver BC); MDS-Myledysplastic Syndrome; CellTracks ®system (Immunicon Corp): automated system of cell identification and enumeration. [a] Median + IQR. [b] Median + IQR number of positive events on flow cytometry analysis every 600 seconds. [c] Breast (n=10), ovarian (n=5), prostate (n=25), colon (n=13),head & neck (n=10), renal cell (n=6) cancers. [d] Events only. [e] Median & IQR cells per µL.

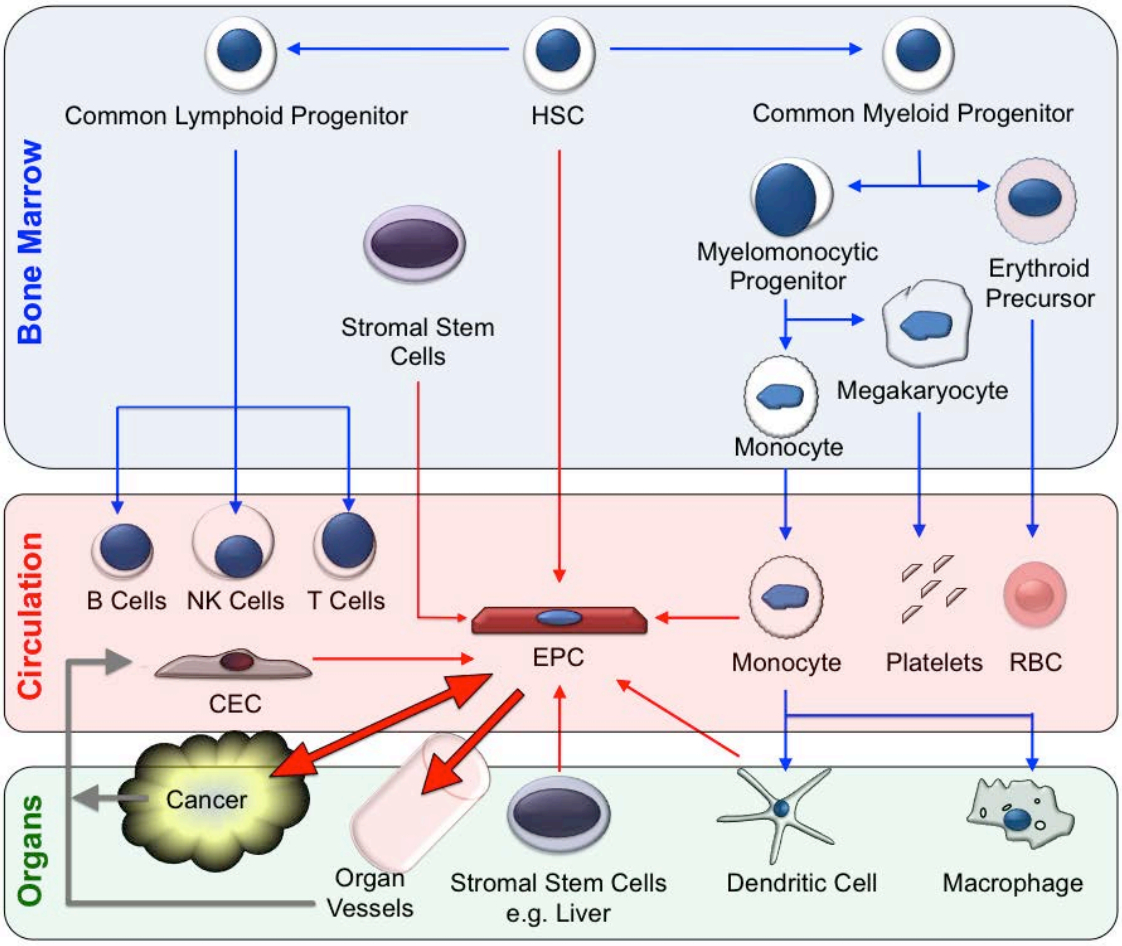
1.2.4.2 The EPC

In 1997 Asahara et al described cells co-expressing VEGR-2 and CD34 with both EC and stem cell characteristics (147). He deduced that bone marrow derived progenitors might have a role in neovascularisation by developing into ECs. These EPCs have since been characterised by a number of phenotypes (See table 5). EC progenitors were originally defined as VEGF receptor 2-expressing (VEGFR-2) cells that were mobilized from the BM by VEGF (148-150). However, co-expression by subsets of other blood cells, mainly progenitor cells and mature CECs, raised doubt on their origin and function. Alternatively, a spectrum of cells may exist between immature haematopoietic stem cells (HSC) and fully differentiated EC (151).

The most compelling role of EPCs was vascular development or vasculogenesis (147). Mesodermal precursors differentiated into ECs to form a primary plexus of neovessels. This process, thought to be restricted to prenatal life, was challenged when human CD34+ cells from PB, umbilical cord blood and bone marrow of both in vitro studies and in vivo mice models differentiated into ECs and blood vessels (147, 150). These EC precursors were thought to arise from common myeloid progenitors and granulocyte/macrophage progenitors and were an intrinsic component of myeloid differentiation (152, 153). This discovery added to the theory of vasculogenesis in human embryonic development (as no clear evidence existed previously) and challenged the traditional concept that ECs proliferated, migrated, and remodelled from pre-existing blood vessels at the site of angiogenesis only.

EPCs are primarily derived from the haematopoietic stem cell (HSC) pool in bone marrow, which also gave rise to myeloid and lymphoid cell lines (figure 7).

Figure 7: Summary of the proposed origins of EPCs.



RBC- Red blood cells. NK- natural killer cells. HSC- haematopoietic stem cell

EPCs were cultured from monocytes (154), dendritic cells (155) and CECs (156). Other origins reported were stromal (mesenchymal) stem cell recruited to tumours and cancer stem cells, as both differentiated into vessels with EC phenotype(s) (152, 157, 158). However there were no reports of this potential from CECs specifically shed from heterogeneous vessels of the tumour vasculature (92).

EPCs were shown to incorporate in blood vessels of tumours, healing wounds, hind limb ischemia, myocardial infarcts and those denuded of ECs (159-161). Likewise, bone marrow stem cell recipients had ECs of both donor and recipient phenotypes (162). In 2001 Vasa et al suggested EPCs were a ‘backup pool’ from the inverse correlation between their numbers and an adverse cardiovascular risk score. Remarkably, the functional properties such as cell adherence, migration, invasion and vessel formation appear to be attenuated in cardiovascular disease [CVD] (163). EPCs were suggested as biomarkers of CVD outcomes, specifically to monitor prevention strategies (164). EPC transplantation offered a promising approach to the revascularisation of tissues e.g. myocardium, after ischemic events (165). They were also proposed as either therapeutic vectors of anti-angiogenic drug delivery and targets against angiogenesis-mediated cancer progression (166).

Characterising and Enumerating EPCs

At publication of this thesis and like CECs, no clear phenotype of EPCs existed. The strategy of designating multiple FC detected surface markers was widely accepted but not without its criticisms. Co-expression by other cells (table 5) cast considerable doubt on their origins and their proposed EC destiny.

Table 5: Markers used in assays for EPCs, HSCs and CECs

Marker	Description	EPC	HSC	CEC	Co expression
CD31[a]	Cellular adhesion molecule	+	+	+	Platelets, leucocytes (118, 119)
CD34	Cell to cell adhesion factor/ glycoprotein	+	+	+	Vascular ECs, dendritic cells, mast cells (120)
CD36[b]	Scavenger receptor	?	+	+	Platelets, monocytes, DCs (121)
CD44	Cell adhesion and migration receptor	+	+	?	Stromal stem cells (167)
CD45	Signalling molecule	-	+	-	Pan-leucocyte (123)
CD54[c]	Leucocyte adhesion molecule	+	+	+	Lymphocytes, monocytes (124)
CD62-E	E-selectin- CAM	+	-	+	None (125)
CD62-P	P-selectin- CAM	+	-	+	Platelets (125)
CD90	Thymus cell antigen	+	+	(+)[1]	Neurons, stem cells, T-cells, melanomas, fibroblasts (126)
CD105[d]	Mediates angiogenesis and oncogenesis	+	+	+	HPCs, monocytes (127)
CD106[e]	Adhesion molecule for leucocytes	(+)[2]	-	(+)[2]	Lymphocytes, monocytes, eosinophils, basophils (128)
CD117[f]	Stem cell factor receptor	+	+	(+)[1]	HSCs (129)
CD133	HCs and nervous system marker	+	+	-	CRC, glioblastomas, neuronal & glial stem cells (164)
CD137	Tumour necrosis factor receptor	+	+	+	ECs, DCs, T cells, NK cells, granulocytes (130)
CD144	VE-cadherin- EC adhesion glycoprotein	+	-	+	Pan-ECs only (131)
CD146[g]	Melanoma associated adhesion molecule	+	-	+	Pericytes, melanomas, gliomas, fibroblasts, T cells (132)
CD164	Adhesion glycoprotein	+	+	-	HSC precursors (168)
CD309	VEGFR-2 in angiogenesis	+[3]	(+)[5]	-	Some HSCs (169)
AcLDL[j]	Probe	+	-	+	None (133)
vWf	Von Willibrand factor	+	-	+	Platelets (89)
UEA-1	Ulex europaeus agglutinin 1	+	-	+	None (134)

HSC Haematopoeitic Stem Cells; Plts- platelets; KCs- Kupffer cells; DC- dendritic cells; [j] - [DiI-AcLDL] 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; [a] PECAM; [b] CR1- collagen receptor 1; [c] ICAM-1; [d] Endoglin; [e] VCAM-1; [f] C-Kit; [g] MelCAM; [h] Tie-2 receptor; [i] B7-H3; (+)[1] Some ECs; (+)[2] Upregulated after EC activation; (+)[3] VEGFR-2 positive EPCs shown in vitro by flow cytometry. (-)[4] Not yet demonstrated. (+)[5] Some haematopoietic stem cell populations.

Identifying multiple surface markers produced a complicated list of putative immunophenotypes (table 6). Furthermore the identity in many studies was based on unselected peripheral blood mononuclear cells (MNC) or small numbers of EPCs within the MNC population rather than pure high count specimens. Initial studies on cells purified from umbilical cord blood, bone marrow and adult PB revealed cells with both $CD34^+$ and VEGFR-2^+ surface markers (147, 149). These generated into mature ECs in vitro and were the basis of most FC studies. The once widely accepted subset $CD133^+/CD34^+/VEGFR2^+$ cells [markers also expressed by colon cancer stem cells (170, 171)] were not directly shown to form ECs in vitro or in vivo, a phenomenon considered a pre-requisite to the phenotype validation (61).

There was also uncertainty that the conditions for in vitro differentiation of EPCs into ECs occurred naturally. A critical but artificial step of was the introduction of high concentrations of the cocktail of angiogenic factors in mice models (172). Furthermore, $CD133^+/CD34^+/VEGFR2^+$ cells were possibly primitive hematopoietic progenitors expressing CD45 (pan-leucocytic marker), which lost CD133 as they assumed phenotypes typical of mature ECs (173). Instead of EC replacement, they may contribute to repair through cytokines to regulate homeostasis, and signal the angiogenic switch of native ECs to restore continuity of the monolayer (164). Though relatively low, reported levels in PB in healthy participants were inconsistent, ranging from 70-210 cells/mL to upwards of 3000–5000 cells/mL, reflecting once again the variations in techniques of cell isolation and enumeration. Typically, lower counts were found in FC protocols compared to cultured monocytes (151, 174).

Table 6: The immunophenotypes of EPCs studied in human disease.

Reference	Subjects	Phenotypes			
Peichev 2000 (175)	Fetal liver, CB, PB	$CD34^+$	$CD133^+$	$CD309^+$	
Gill 2001 (176)	PB in trauma	$CD133^+$	$CD309^+$		
Burger 2002 (177)	CB, BM, PB in CVD	$CD34^+$	$FGFR1^+$		
Pelosi 2002 (178)	PBMNC culture	$CD34^+$	$CD309^+$		
Cogle 2004 (179)	BM	$CD34^+$	$CD45^+$		
Hildbrand 2004 (180)	CB	$CD11b^+$	$CD34^+$		
Kondo 2004 (181)	PB of smokers	$CD34^+$	$CD45^+$	$CD133^+$	$CD309^+$
Elsheikh 2005 (182)	PBMNC	$CD14^+$	$CD309^+$		
Romagnani 2005 (183)	PBMNC	$CD14^+$	$CD34^+$		
Delorme 2005 (184)	CBMNC	$CD34^+$	$CD45^+$	$CD146^+$	
Furstenberger 2006 (139)	PB in breast cancers	$CD34^+$	$CD45^{Low}$	$CD309^+$	
Friedrich 2006 (185)	Carotid artery plaques	$CD34^-$	$CD133^+$	$CD309^+$	
Westenbrink 2007 (186)	PB in CVD	$CD34^+$	$CD45^-$		
Yip 2007 (187)	PB in Stroke	$CD31^+$	$CD34^+$	$CD62L^+$	
Allanore 2007 (188)	PB in MS	$CD34^+$	$CD133^+$		
Martin 2008 (189)	PB in OSA	$CD34^+$	$CD45^+$	$CD133^+$	
Pircher 2008 (190)	PB in cancer	$CD34^+$	$CD45^-$	$CD133^+$	$CD309^+$
Goon 2009 (24)	PB in breast cancer	$CD34^+$	$CD45^-$	$CD133^+$	
Blann 2011 (140)	PB in Prostate cancer	$CD34^+$	$CD45^-$	$CD309^+$	

CD309=VEGFR-2; PB- peripheral blood; CVD – cardiovascular disease; CB- cord blood; BM- bone marrow; PBMNC – peripheral blood mononuclear cells; OSA-Obstructive sleep opnoea; CBMNC cord blood mononuclear cells.

Asahara et al postulated that vasculogenesis was regulated by a number of mediators between the site of neovascularisation and the bone marrow (147). VEGF was key to this process, a rise in EPC found as early as 24 hours after exogenous injection although the contribution of other mediators was vague. Granulocyte macrophage colony-stimulating factor (GM-CSF) also induced a rise, and enhanced vascularisation of CRC cell lines in animal models (191-193). Elevated levels were reported after recombinant human erythropoietin for anaemia in renal failure (194).

In vivo evidence for EPC activity in cancer

Lyden at al reported that EPCs contributed about 90% to the vascularisation of lymphomas in angiogenesis defective mutant mice (195). Transplanting wild-type murine bone marrow (BM) reversed tumour regression in mice with poor angiogenic potential. EPC incorporation was described in other tumour models, although the type of tumour and differences in choice of markers may have contributed in part to variations between studies (table 7). Tumours following BM transplantation had a small percentage of BM-derived ECs, typically 10-14 days after transplantation. Multicolour fluorescent in situ hybridisation (FISH) and fluorescent probes for vWf and CD45 levels ranged from 1% in sarcomas to 12% in lymphomas (196). Estimates were that EPCs could constitute as much as 38 to 50% of all ECs in tumour neovessels (see table 5), but more recent reports showed very low averages of 4.9%, similar to normal tissues (151, 196-198). In other solid tumour mice models, no EPC incorporation was reported at any time point (172, 199).

Recently the HSC origin of EPCs was questioned when in vitro breast cancer stem cells had characteristics of ECs (200). Alternatively false positives were from stromal stem cells found within 'normal' tissues rather than co-expression from tumour associated cells (157). EPCs may intuitively distinguish between well- and poorly-differentiated breast cancers (201). Surprisingly robust EPC mobilisation occurred after the maximum tolerated dose of chemotherapy was administered but consistently fell after anti-angiogenic chemotherapy (166, 202). Gao et al reported tumour growth was impaired by blocking EPC mobilisation and postulated roles in neovascularisation other than vasculogenesis (166).

The accuracy of conventional histological analyses was also questioned. Recent 3D high-resolution multichannel (sequential) confocal scanning of whole mounts blood vessels showed BM-derived cells to be perivascular and not in the EC monolayer (203). The authors concluded that earlier studies suffered co-localization i.e. false positives, from monocytes, lymphocytes and macrophages (tables 5 and 6). Purhohen et al suggested that while there was the capacity of adult BM stem cells to selectively differentiate into vascular ECs in experimental models, it was not a typical in vivo function and therefore an extremely rare event (172).

A number of enumeration techniques were reported over two decades for EPCs in cancer (see table 8). The initial methods employed Ficoll-Hypaque density gradient centrifugation followed by cell culture (211-213). Significant colony growth was seen in breast, liver and non-small lung cancer when compared to healthy controls. Less EPCs were found in breast cancer by FC, but the cells were better described as

circulating progenitors (CPC) not EPCs (24). Probably the most accurate definition of EPCs is (CD45-CD34+KDR+ by Blann et al, though the protocol may not have differentiated them from CECs, as numbers of both were very similar (140).

Table 7: EPC incorporation into the vasculature of tumours in animals models transplanted with bone marrow (BM) derived cells.

Reference	Tumour	Marker	Detection Technique	Donor Cell transplanted	% uptake
(195)	Lymphoma	vWf	IHC	Mononuclear cells	90
(204)	Neuroblastoma cell	CD31, CD34	IF	Modified BM cells, no RBCs	5
(205)	Uterine Carcinoma	CD31	IHC	Mononuclear cells	16
(206)	Breast cancer	CD31	IHC	BM cells	1.3
(199)	Lung cancer, Lymphoma	CD31	IF	BM cells	0
(196)	Various human tumours	vWf	FISH	na	1 to 12
(208)	Lung Cancer, Melanoma, Breast cancer	CD31	IF, FC	BM cells	<1
(209)	Colon cancer	CD31, vWf	FC	na	40
(210)	Lung cancer, melanoma, breast cancer	CD31, VCAM, CD144, LP	FC, 3D microscopy	BM cells	2 to 20
(166)	Lung cancer, breast cancer	CD31	IF, FC	BM cells	12

LP- Lectin perfusion; IHC- Immunohistochemistry; IF- immunoflourescence; FISH- fluorescence in situ hybridization; BM cells- non-selected bone marrow cells; MAPC- multipotent adult progenitor cells; GFP- green fluorescent protein. FLK-1- KDR or CD309. Tie-2 is angiopoeitin receptor.

Table 8: EPCs- Techniques of enumeration and relationship with cancer

			EPC levels [median + IQR cells/ml]			
Cancer(s)	**Protocol**	**Markers**	**Cancer**	**Control**	**P< 0.05**	**Study**
Prostate n=31	FC	CD45-CD309+ CD34+	38 [15-74]	32 [18-82]	**n**	(140)
Breast n=160	FC	CD45-CD133+ CD34+	121 [81-186]	169 [106-241]	**y**	(24)
Non-small cell lung cancer n=10	FHDGC, IMS- CD34, FAb & ICS	CD34+ CD133+	1.2%[A] (0.8-1.6)	0.8% [A] (0.4-1.3)	**y**	(190)
		CD45- CD34+ CD133+	90% [A] (57-95)	42%[A] (6-51)	**y**	
		CD45- CD34+ CD133+ VEGFR2+	0.18% [A]	0.01%[A]	**y**	
Breast n=25	FHDGC, FAb & FC	CD14+ VEGFR2+ CD133+	↑EPCs with cancer stage	None	**-**	(214)
Breast n=47	FAb & FC	CD34+ VEGFR2+	0.4% +/- 0.28[A]	0.18%+/- 0.13[A]	**y**	(215)
	FHDGC, EC culture, LM & FC	vWf+ Dil-ac-LDL+ CD34+ VEGFR2+	EOC to confirm EC origin only			
Liver n=64	Culture System, separation & CFU-EC; FC	CD45- CD34+ CD133+	CFU-EC: 2 times > controls		**y**	(211)
			FC: 0.82%[A] in cancer vs 0.26% for controls		**y**	
Non-small cell lung cancer n=53	FAb & FC	CD34+ VEGFR2+ CD133+	1162	345	**y**	(212)
Liver n=80	FHDGC, Dil-ac-LDL & UEA-1 isolation, Culture, FAb & FC	CFU-ECs CD133+ VEGFR2+ & CD34+ VEGFR2+	CFU scores 10 fold higher than controls & correlated to ↑EPCs		**y**	(216)
Breast (n=19) & Gastric (n=52)	FHDGC, Culture & ICS	Dil-a-LDL, UEA-1, CFU-ECs	37.6 per unit area	40.2 per unit area	**n**	(213)

CFU-EC colony forming unit-endothelial cell; EOC endothelial outgrowth cells; CFr/T controlled freezing & thaw procedure; FAb Labelling with flourochromes; LM light microscopy; FC flow cytometry; ICS immunocytostaining; Dil-ac-LDL Dil-acetylated low-density lipoprotein; UEA-1 Ulex europaeus agglutinin I;CLL Chronic Lymphocytic Leukemia; FHDGC: Ficoll-Hypaque density gradient centrifugation. [A] Percentage of monoculear cells in PB.

1.2.5 Summary of the EC in CRC- why is the endotheliome important?

Our current understanding of EC biology in cancer emerged from studies on factors secreted into plasma (e.g. vWf), physiological action on other cells (e.g. NO on VEGF production and angiogenesis), and the presence of endothelial cells (CECs, EPCs) within the circulation. There is ample evidence of EC perturbation in cancer, e.g. increased secretion and release of EC specific markers such as, NO, vWf and soluble E-selectin. These reflect activation of the relatively quiescent endothelium. Similarly, circulating ECs may result from the loss of endothelial integrity (CECs) and increased EPCs to angiogenic or bone marrow stimulators, like VEGF.

While newer methods for assessing vascular biology await translation into clinical practice, additional tools are needed to monitor treatments that target the EC (e.g. anti-VEGF). However, the various subsets of endothelial form, function, dysfunction and its involvement in cancer were often studied in isolation. I therefore propose that EC activity incorporating histological, genetic, coagulation, inflammatory or adhesion markers in normal homeostasis and cancer warranted evaluation within a unifying concept called 'the endotheliome'. However the endotheliome aims to measure more than EC expression but also the effects on the cancer microenvironment, staging, prognosis and the long-term outcomes of treatment.

It follows therefore that, as a clinical tool, the practical and cost implications of the endotheliome are challenging. Furthermore the EC has different properties at various anatomical sites (e.g. lung for gas transfer, aorta for cardiac output) and studies often

primarily focus on arterial and capillary but less on venous outcomes. For CRC, a key requirement would be to synthesise an array of vascular modifications that occur within the EC environment at clinically relevant time points (e.g. before and after surgery, with or without chemotherapy) and to understand how those changes informed outcomes. The study of the endotheliome is not an attempt at a new field but to unify the vast data already in existence on the EC in CRC, to aid future studies and developments. Our publication of the concept of the endotheliome with 'Thrombosis Research' (217) is shown in appendix 10.

Given the practical and cost limitations, I hypothesise that the endotheliome in CRC is assessable by a combination of three groups of circulating markers: EC-specific plasma markers, cellular markers and angiogenic markers. It is therefore a question of selecting 'best' in each group. Whilst the choice was based on sensitivity and specificity, it must also consider patient factors. Ideally, the method must be accessible to all cancer patients, transferable to the clinical setting, inform treatment strategies, and predict prognosis both of disease progression and survival.

Having discussed EC-specific and circulating cellular markers of the endotheliome, the most important role of the EC, that of angiogenesis and the subset of its assessment, the angiome, is explored next.

1.3 The Angiome

The angiome, a subset of the endotheliome, is defined as the assessment of angiogenic activity. It was Judah Folkman's pioneering premise in 1971 that angiogenesis was the crucial step in tumour growth and the basis of vascular biology research for almost four decades (13, 219). Angiogenesis is explored next.

1.3.1 Angiogenesis

Angiogenesis commences in utero and continues throughout adult life to ensure the primary directive of the vasculature is maintained. The macro- and micro- scopic tissue and organ structure of vertebrates requires the efficient simultaneous transport of gases, liquids, nutrients, signalling-molecules and circulating cells. Anatomically, the delivery of blood via the circulation (first described by William Harvey in 1628) proceeded from the heart through arteries into smaller arterioles and, finally, into extensive networks of capillary beds that allow for exchange of the gases and metabolic products (220). To increase transport to growing tissues during embryogenesis, arteries and veins expand through circumferential growth and remodelling processes, whereas capillaries sprout and branch into larger, more complex networks or primary capillary plexi (218). Similar remodelling processes were thought to be important for postnatal growth, development and healing, as well as tumour growth. While many theories exist, two basic models were widely accepted but not mutually exclusive: angiogenesis or the development of new vessels from

pre-existing ones, and vasculogenesis in which de novo vessels involve bone marrow derived EC and pericyte progenitors (147, 219, 221). However angiogenesis often encompassed all processes of blood vessel development. The formation of new blood vessels from pre-existing ones involved either sprouting or lumen splitting into two conduits by the intussusception of tissue bridges [figure 8] (222). Vasculogenesis and the role of EPCs also continued in postnatal life (147).

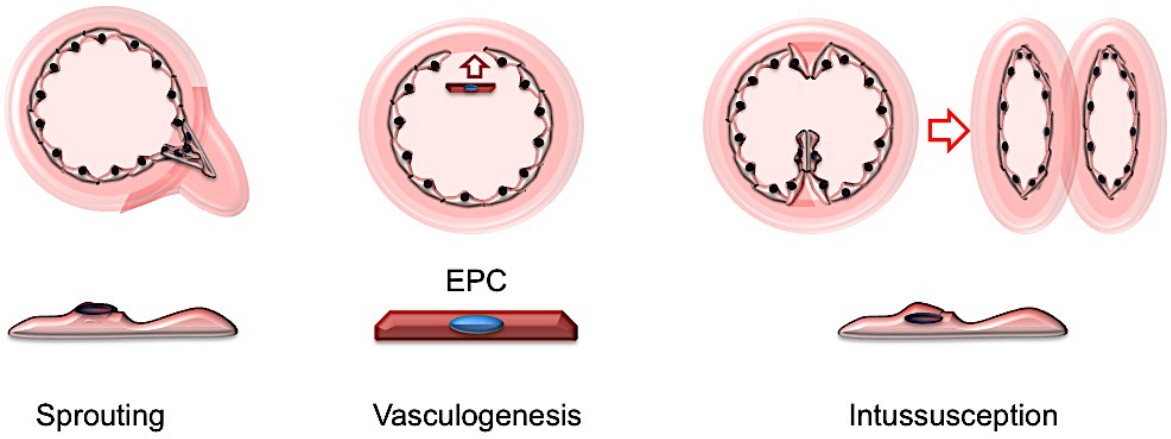


Figure 8: The central role of ECs in angiogenesis

ECs multiply through sprouting or added to the endothelium by EPCs. Neovessels form by sprouting and /or intussception in areas of high angiogenic activity.

Ausprunk and Folkman (223) and later modified by Paku and Paweletz (224) described several well-characterised steps of sprouting (223). Angiogenic growth factors via receptors on ECs stimulate protease release against the basement membrane of the blood vessel. Intact ECs proliferate in parallel to the basal-luminal polarity towards the denuded site and surrounding matrix to form a slit like lumen. Basement membrane is deposited continuously by ECs except at the tip. Pericytes migrate along the basement membrane for complete coverage of the vessel. The solid sprouts so formed connect to neighbouring vessels and ECs migrate in tandem towards the angiogenic stimulus. The sprouts restructure into loops that developed a lumen whilst simultaneously connecting to and infiltrating surrounding blood vessels.

The four phases of intussusception described by Burri et al have been demonstrated in both rat and human postnatal lung tissue (225). The opposing capillary walls grew into the lumen and established contact. At this zone, the EC junctions reconfigured and the bilayer of capillary walls perforated to allow the permeation of vascular growth factors. This zone filled with stromal cells, including bone marrow derived pericytes and myofibroblasts. These cells produced extracellular matrix (ECM), specifically collagen fibres, which organised ECs to form the lumen. Capillaries were increased efficiently without a corresponding rise in ECs (222, 225).

To form mature, functional vessels, angiogenesis must be regulated by the sequential interplay of a number of ligand–receptor interactions on ECs, pericytes and other supportive stromal cells (like monocytes/macrophages).

1.3.1.1 The angiogenic switch

The angiogenic switch was cleverly summarised by Hanahan and Folkman (226) as the activation of the normally quiescent EC by the balance tipped in favour of its activators over endogenous inhibitors (figure 9). The healthy body controls angiogenesis through a balance of on and off switches i.e. pro-angiogenesis factors and angiogenesis inhibitors. Broadly, deregulation of these processes in disease may cause either excessive new vessel formation, as in cancer, macular degeneration, psoriasis and rheumatoid arthritis or insufficient angiogenesis as in coronary artery disease, stroke, ulcers, infertility and scleroderma (61).

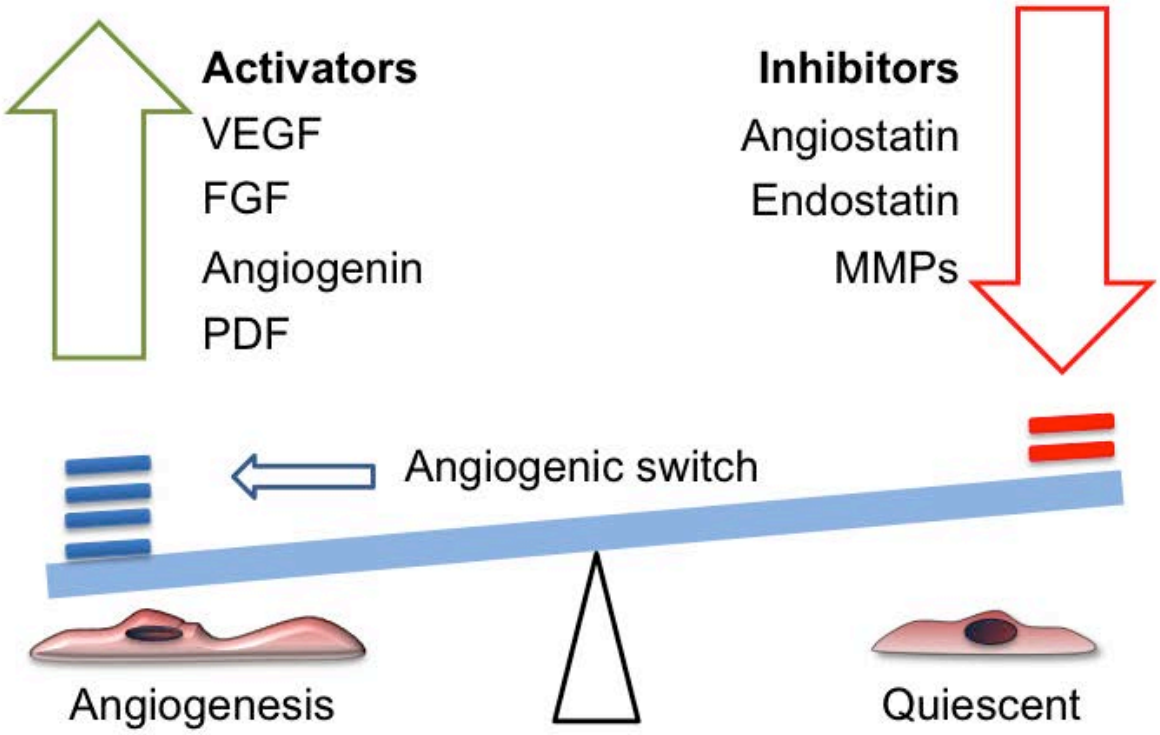


Figure 9: The angiogenic switch in which activators 'outweigh' inhibitors and promote sprouting of ECs. Adapted from Hanahan and Folkman 1996 (226).

The most important activator is vascular endothelial growth factor A (VEGFA), a member of a large family of potent angiogenic regulators including placental growth factor (PIGF), VEGFB, VEGFC and VEGFD (227). It also induces vascular permeability and vasodilatation (when injected intravenously), and probably promotes a strong survival stimulus on resident ECs. VEGF, on binding to VEGFR-1 and VEGFR-2, induced EC proliferation, tightly regulated c-ligand binding to various receptors and spliced other subtypes such as the anti-angiogenic b-isoform of VEGFA. When bound to the tyrosine kinase receptor VEGFR-2 (KDR or FLK1) it promotes differentiation, proliferation and sprouting of ECs. However, VEGFR, a weak tyrosine kinase, counteracts this activity by ‘trapping’ VEGF (228, 229).

Sprouting is controlled by a number of pro-angiogenic and inhibitory signals (see figure 10). Proteolysis of the ECM increases the bioavailability of VEGF-A (isoform 121) whereas contact with other ECs and pericytes induces EC quiescence. The tips ECs promote capillary growth without compromise to tissue perfusion (230, 231). Differential growth of ECs by VEGFA increased the expression of DLL4 in tip cells and up regulated notch receptors in neighbouring quiescent ECs. DLL4 suppressed VEGFR-2 expression of notch positive neighbouring quiescent ECs, preventing their conversion to the tip cell phenotype. The unguided proliferation of ECs is promoted by freely diffusible VEGF A isoform 121 in humans whilst the elongating filopodia of the tip cell maintains its awareness of direction and polarity by the spatial concentration gradient of matrix anchored VEGF (232).

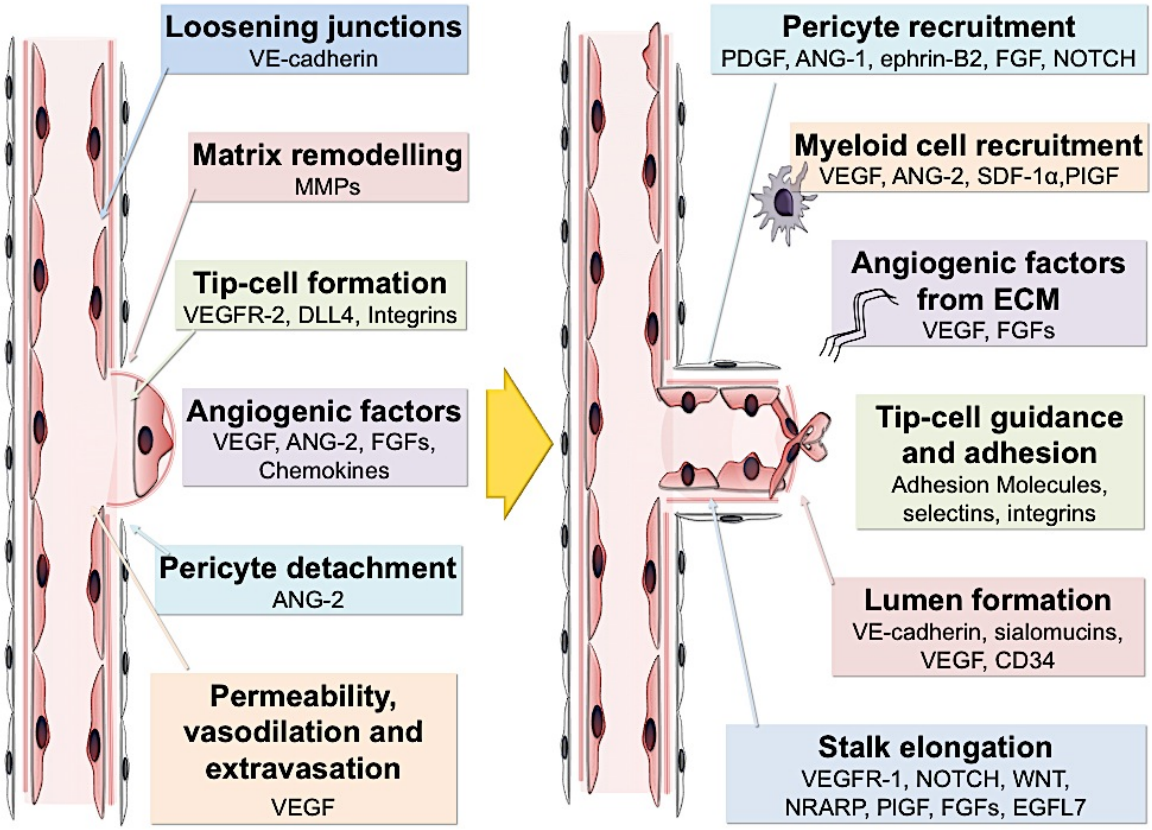


Figure 10: Angiogenic factors in sprouting and stalk elongation

The extension of the sprout, which assumes a cone-like morphology, may involve EC migration within the stalk (demonstrated in mouse retina) or proliferation within the tip. Filopodia formation was observed in some stalk ECs with elevated expression of DLL4 and PDGF (233). In order for vessels to connect with each other the tip cells must halt their motility when encountering their target, other sprouts or capillaries. Adhesive contacts between EC junctions are established when compatible vessels meet, whilst repulsive signals prevent abnormal connections like arteriovenous shunts. Lumen formation is controlled by EGF-like domain-7 (EGFL7), an ECM-associated protein, expressed on ECs that suppress matrix adhesion but not EC proliferation. The established lumen and improved oxygen delivery stabilises the

tubes by suppressing VEGFA expression (234, 235). Pericytes are recruited to promote vessel maturation and inhibit angiogenesis resulting in the elimination of unwanted connections (or pruning) by EC apoptosis or retraction (232). The vascular network may also extend by intussusception (222). Despite strong morphological evidence to support this process little was known about its physiological regulation and pathological contribution to angiogenesis.

1.3.1.2 Vasculogenesis and EPCs

While the majority of vessels grow from the in situ EC, there was much interest in the role of embryological mechanisms first proposed by Ashahara et al (147). BM derived progenitors contribute to the regenerative and pathological growth of blood vessel in postnatal life. EPCs may aid the circumferential enlargement of the vessels and incorporate into EC monolayer lining during sprouting and possibly intussusception (236). Their contribution was thought to be quite small and further characterisation of the steps was needed (172). VEGF mobilises EPCs and non-EC progenitor monocytes (called bone-marrow-derived circulating cells [RBCCs]) that express chemokine receptor 4 (CXCR4). RBCCs, in response to VEGFA, expressed the CXCR4 ligand or stromal-derived factor-1 (SDF1), which aided the cells' retention in the perivascular space (237). Details on EPCs were discussed in section 1.2.4.2.

The next section addresses angiogenesis in cancer.

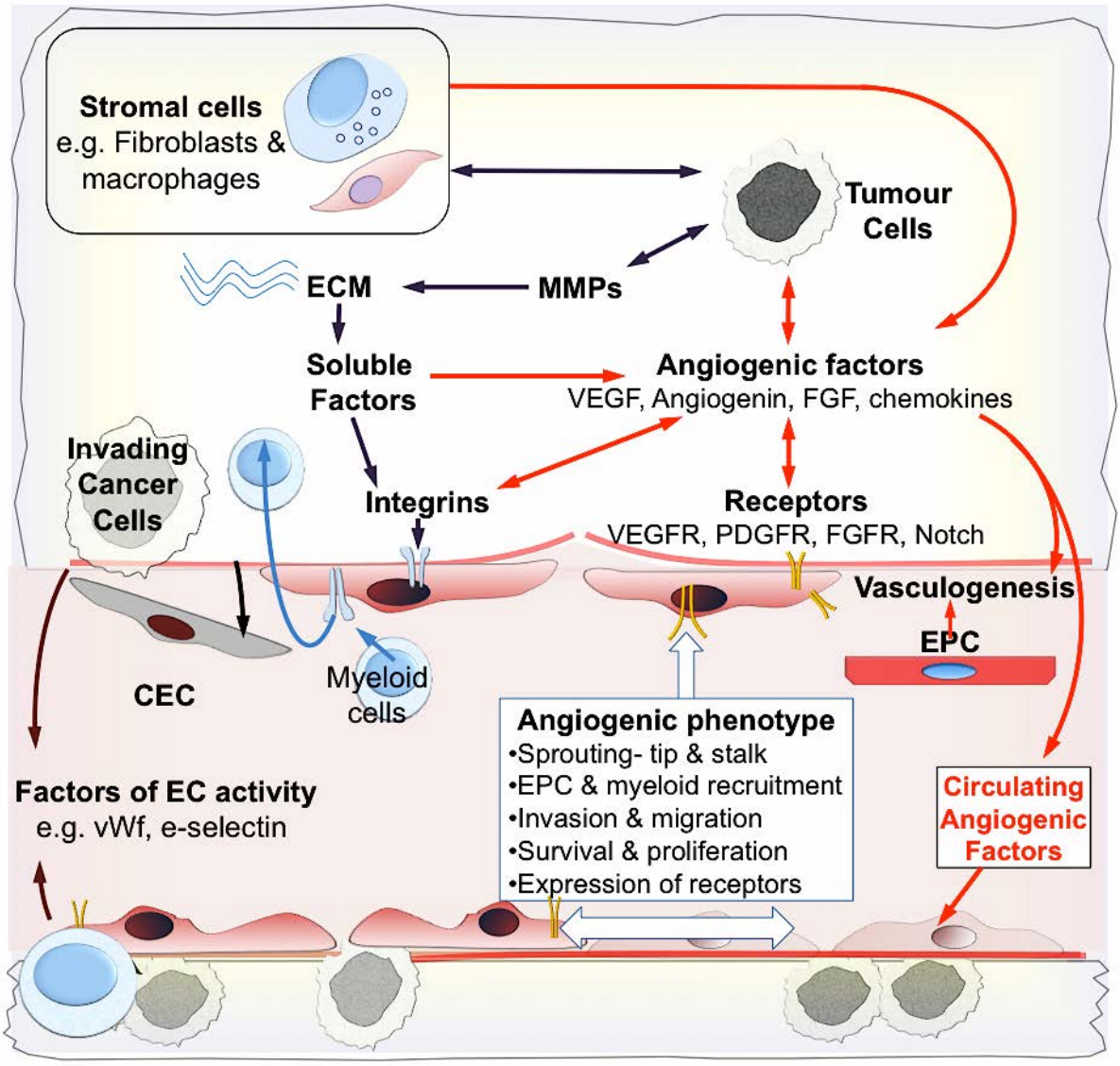
1.3.2 Tumour angiogenesis

The need for tumours to form neovessels was first postulated 65 years ago and later developed by Folkman who theorised that anti-angiogenesis was an effective strategy for cancer treatment (13). The mechanisms for neovessel formation involve: co-option [sprouting from surrounding native vessels] (238), vasculogenic mimicry [cancer cells that mimic activities of ECs] (239), mosaicism [cancer cells in the luminal surface] (240), and vasculogenesis (165). The angiogenic switch is the dominance of stimulators over inhibitors to EC proliferation (226). ECs, stromal cells, and ECM also produce many of these promoters along with the cancer cells. Within the tumour's microenvironment promotors signal sprouting and migration of EPCs for vasculogenesis; there was no in vivo evidence for intussusception (68). The main trigger is the VEGF-A pathway up-regulated mainly by tissue hypoxia and or cell death (241). The mechanisms for angiogenesis in the CRC are explored next.

Mechanism of angiogenesis in CRC

A variety of cell types promote angiogenesis or the 'angiogenic switch' primarily in response to tumour growth of a few millimetres, hypoxia and nutritional deprivation (221). The heterogeneous vessels carry nutrients irregularly via the tortuous tumour vasculature, which continuously remodels to cope with the growing tumour's demand for a 'better' blood supply (242). The mechanisms of ECs in angiogenesis and in the CRC microenvironment are summarised in figure 11.

Figure 11: Angiogenesis and the EC in CRC.



In summary basal membrane is degraded by MMPs of cancer cells and ECs; the exposed membrane attracts platelets, which carry angiogenic and permeability factors e.g. PDGF, an activator of ECs and perivascular cells (243). Tumour-associated fibroblasts deposit ECM and cancer cells release stimulators such as VEGF, angiogenin and FGF (244, 245). MMPs cleave ECM fragments, expose

hidden epitopes of endogenous inhibitors of angiogenesis e.g. endostatin, but attract inflammatory cells that also secrete angiogenic modulators (246). Promoters exceed inhibitors for the angiogenic switch (tables 9 and 10). ECs express pro-angiogenic receptors, which stimulate intracellular pathways for sprouting and the exposed basement membranes attract EPCs (165). The roles of ECs are shown in figure 12.

Figure 12: Summary of the roles of ECs in CRC

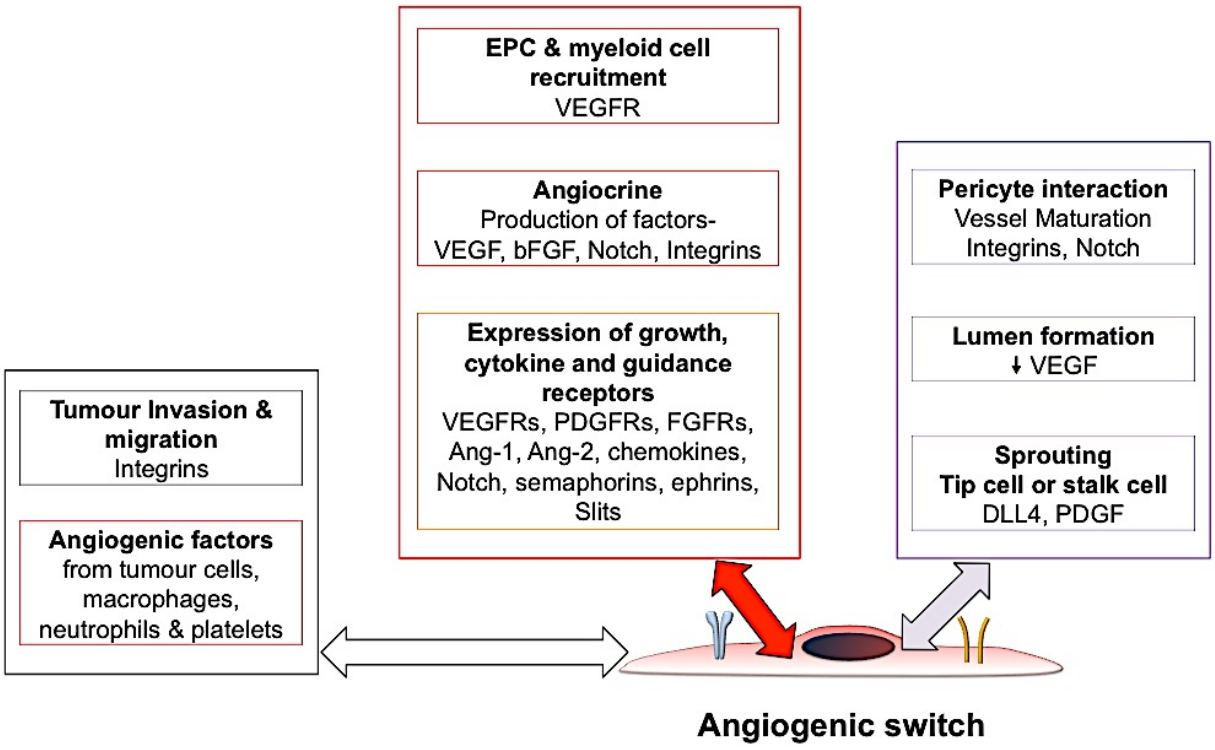


Table 9: Summary of important endogenous activators of angiogenesis in CRC

Activator	Function	Ref
VEGF -A, -B, -C, -D PIGF	Stimulates EC activity in angiogenesis and vasculogenesis. Increases vessel permeability. Stimulates leucocyte adhesion, and monocyte/ macrophage migration	(229)
VEGFR-2	Tyrosine Kinase receptors that integrate angiogenic and survival signals mediated by VEGF	(229)
Angiopoetins-1, -2	Promote EC sprouting, stabilised neovessels and reduced vessel permeability	(247)
Platelet derived growth factor (PDGF)	Recruits smooth muscle cells and cells of mesenchymal origin	(248)
TGF-β1, endoglin and other TGF-β receptors	TGFs stimulated ECM production and vascular remodelling; endoglin on ECs activate macrophages and smooth muscle cells	(249)
FGF, HGF/MET	Growth factors that also stimulate angiogenesis	(250, 251)
Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$	Enzymes against and receptors for matrix proteins	(252)
VE-cadherin, PECAM	Endothelial cell junction molecules	(101)
Plasminogen activators, MMPs	Remodels matrix and releases growth factors	(54, 82)
PAI-1	Stabilises nascent blood vessels	(67)
NOS; COX-2	Stimulates angiogenesis and vasodilation	(47, 95)
Cytokines/ Chemokines (e.g. IL-6, TNF- α)	Pleiotropic roles- tumour growth & survival; myeloid cell recruitment; T-cell activation.	(253)

VEGF-vascular endothelial growth factor; VEGFR- vascular endothelial growth factor receptor; HGF- hepatocyte growth factor; MET- HGF receptor; PDGF- platelet derived growth factor; TGF- transforming growth factor; FGF- fibroblast growth factor; PAI- plasminogen activator inhibitor; NOS- nitric oxide synthase; COX- cyclooxygenase.

Table 10: Summary of endogenous inhibitors of angiogenesis in CRC

Inhibitor	Function	Ref
VEGFR-1; NRP-1	A 'trap' for angiogenic promoters VEGF-A, VEGF-B, and PGIF	(229)
Angiopoetin-2	Mixed antagonist to Angiopoetin -1	(247)
Thrombospondin-1	Inhibits EC migration, growth, adhesion to cells & ECM, and survival.	(254)
Endostatin (from ECM-degraded collagen XVIII)	Inhibits EC proliferation, migration and survival; reduces tumour growth	(255)
Other ECM degradation-derived inhibitors	Arresten, tumstatin and canstatin activity similar to endostatin.	(256)
Platelet factor 4	Inhibits binding of bFGF and VEGF to receptor	(257)
Tissue inhibitors of MMPs	Chelates MMPs and suppresses pathological angiogenesis	(258)
Chemokines (CXCL-4, -9,-10, -11)	Inhibits EC chemotaxis and proliferation; inhibits activities of FGF and VEGF	(253)
NO	Controversial role as inhibitor to EC sprouting	(48)

NRP- neuropelin; MMPs- matrix metallopreteinases.

The basement membrane is cleaved, exposing fragments that alter integrin activity and facilitate sequestration of myeloid cells. These cells transform into tumour associated macrophages (TAMs) and neutrophils that also promote angiogenesis (246). EC surface receptors factors also initiate intracellular pathways to promote migration, invasion, survival and proliferation. ECs interact with perivascular cells and TAMs to determine their tip or stalk prototype (259).

Lyden et al showed that blocking VEGFR1 and VEGFR2 prevented the recruitment of EPCs, amongst other BM derived haematopoietic precursors, and eventually impaired tumour angiogenesis and growth (195). The pre-clinical studies of Kobayashi et al suggest ECs actively attract progenitor cells by ‘angiocrine’ factors such as Notch ligands (260). De Palma et al also found that angiopoietin-2 released by tumour ECs recruit Tie2-expressing monocytes, and the resultant TAMs signalled ECs to release more pro-angiogenic factors, including VEGF (261).

In summary, angiogenesis is an essential requirement of tumour growth and metastasis. In this context, measuring its activity may potentially give further insight into CRC progression particularly for EC targeted treatment. In the next section, the current literature on assessing angiogenesis is discussed including potential surrogates for future clinical and research application.

1.3.3 Assessing the angiome

Having established angiogenesis as critical to tumour growth and metastasis, it follows that its assessment may inform cancer treatment and prognosis. Specifically, the angiome may inform the tissue distribution of anticancer drugs and therefore a determinant of tumour response. Methods include measuring circulating plasma factors, radiological imaging, immunohistochemistry and circulating cellular markers such as EPCs. These methods however remain within the realms of laboratory research and are yet to be translated to the clinical setting. These parameters provide ‘‘snap-shots’’ that do not necessarily reflect the dynamic status of the tumour’s microvasculature or relate to the molecular factors that regulate its growth. There is a need for markers of angiogenesis that can be monitored non-invasively in vivo at repeated intervals in large number of patients. In the next section I explore the various markers that may potentially be used to assess the angiome in CRC.

1.3.3.1 Angiogenic growth factors and their receptors

The concentration of circulating angiogenic factors can be measured easily by enzyme-linked immunosorbent assay (ELISA). The first meta-analysis on VEGF in cancer by Kut et al suggest a prognostic value of its pre-treatment levels in CRC patients, specifically VEGF-A (262). A number of other potential biomarkers include bFGF, platelet-derived growth factor (PDGF), transforming growth factor-beta (TGFβ) and angiogenin (263-267). Their clinical significance is less conclusive as they were

also detected in some healthy controls. VEGF continues to spark interest more so for its correlation with other emerging biomarkers of CRC (268).

Dirix et al first showed that serum VEGF and bFGF levels were higher in progressive (unresponsive) disease when compared to chemo-sensitive metatstatic CRC [mCRC] (269). Much of the circulating VEGF is found largely stored in platelets and serum levels (which contains more VEGF after platelet release from coagulation) may not truly reflect tumour expression (270, 271). However, Peterson et al showed that the platelet load of VEGF correlated to disease stage of CRC (272). Clinical trials on anti-angiogenic (anti-VEGF) therapy measured VEGF to monitor treatment response (273). Hyodo et al reported low pre-treatment plasma VEGF levels of mCRC were correlated to a significantly better response rate and prognosis with chemotherapy (274). However plasma VEGF varied widely, often overlapping with that of healthy controls (274, 275). With no clinical cut off point, translation to bedside application remains elusive. The angiogenic effects are dependent on the internalization of surface bound VEGF and plasma levels, having no correlation with intracellular levels, may not necessarily reflect its activity (276). Soluble serum VEGFR-1 and VEGFR-2 were detected in the CRC patients but not healthy individuals (277). The ratio of VEGF to VEGFR1 was shown to provide better prognostic value than serum VEGF, VEGFR1 or VEGFR2 alone (277, 278).

Proteomics and genomics may offer other surrogates of angiogenic assessment. Circulating mRNA and DNA indirectly measure angiogenic factors by quantitative PCR and may provide a holistic pattern of gene, protein and metabolite expression

(279). Miniaturised ELISA assay (Luminex) quantifies hundreds of proteins in small volumes of plasma/serum and offers a promising ‘single-stop’ angiogenic status evaluation (280, 281). The key question remains, what do we measure?

1.3.3.2 Circulating cellular markers

The roles of EPCs and CECs are discussed in section 1.2.2. Recently other BM-derived cells were described in angiogenesis. Inflammatory infiltrates contain $CD34^{+}$-, $Tie2^{+}$- and $VEGFR2^{+}$- expressing monocytes which may regulate angiogenesis and tumour growth by paracrine mechanisms (203). Goede et al cast doubt when Ties2-expressing monocyte (TEMs) levels in CRC were similar to healthy controls (282). DePalma et al suggested that TEMs were a diverse fraction of monocytes that require further exploration for cancer staging and prognosis (203, 261).

1.3.3.3 Immunohistochemistry

A crude but inconsistent histological measure of angiogenesis is tumour volume. Microvessel density (MVD), first described by Weidner et al in 1991 correlated with breast cancer survival and/or recurrence (12). Immunohistochemistry (IHC) with EC markers (CD31, CD34, and vWf) stained microvessels, not seen on conventional histology, identifies ‘hot spots’ (areas of the highest vascularity) in the tumour section on low power (x 40) field. Individual microvessels were counted under a high power

(x 200) field in a defined area and the average vessel count in five hot spots was taken as the MVD. However ‘vasculogenic mimicry’ (the formation of a well-structured microcirculation with tumour cells) devoid of ECs and independent of angiogenesis, lacked the phenotypes used in IHC analyses (283). A meta-analysis of CRC showed a very high MVD correlated with recurrence [RR = 2.84; 95% CI: 1.95-4.16] and overall survival (OS) [RR=1.65; 95% CI: 1.27-2.14] (284). However, inconsistent methods of microvessels selection and restrictions to post-resection quantification prevent repeated measures and no better than routine histology.

1.3.3.4 Radiological tests

Imaging of tumour angiogenesis or vascularity was not routinely used in CRCs as they are expensive, experimental and of unknown clinical value. Tumour growth and metastasis is routinely evaluated by several non-invasive modalities: computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET-CT), single photon tomography (SPECT), and ultrasound (285). Only MRI of the rectum may detect extramural vascular invasion [sensitivity 66%, specificity 88%], a poor prognostic indicator of rectal cancers (286).

Potentially CT and/or MRI may image angiogenesis and vascularity by a variety of contrast mechanisms e.g. blood flow, microvessel permeability and diameter, water diffusion, tissue oxygenation and metabolism. Dynamic MRI of tumour vascularity i.e. MRI with contrast media, may identify more aggressive disease (280, 287).

1.3.4 Summary of the angiome

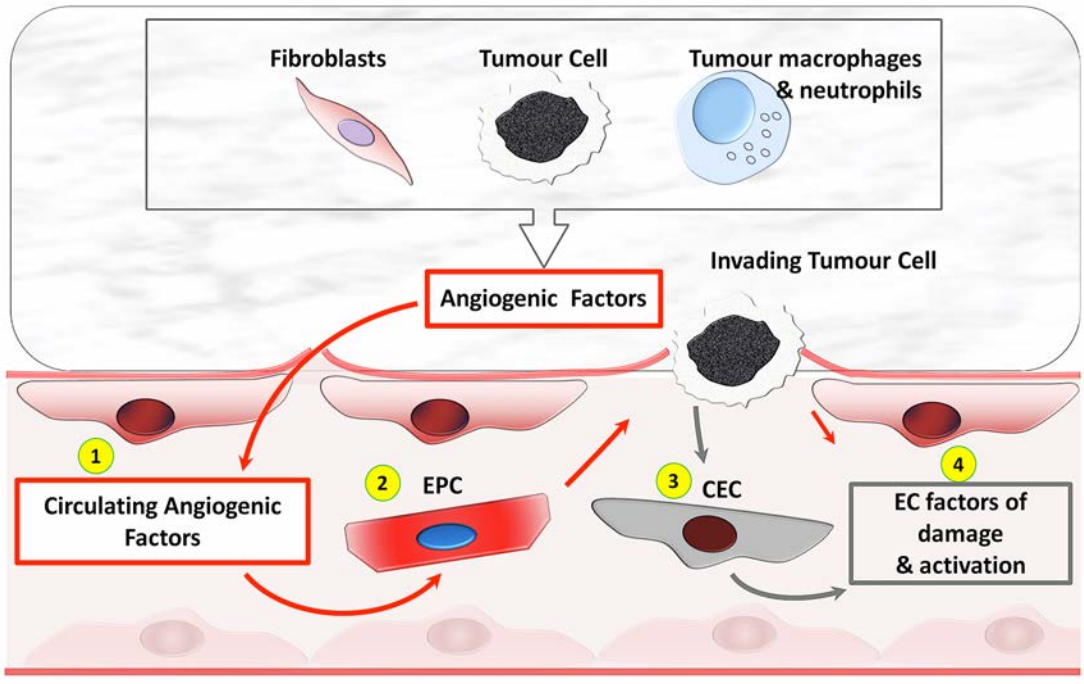
In summary the angiome is an important tool for assessing the angiogenic activity of a tumour. It requires further development by incorporating plasma, cellular, histological and radiological markers in CRC. Studies on the relationship of cellular with EC-specific plasma markers in CRC were lacking and therefore worthy of further investigation. Our publication of the concept of the angiome with the Journal of Clinical Pathology (268) is appended.

Analysing factors of the endothelial-tumour interaction may offer further clinical information of tumour activity and/or response to treatment. In the next section I will discuss and give justification for the factors that I have chosen for the endotheliome and angiome in CRC staging, treatment and prognosis.

1.4 The Endotheliome and Angiome in CRC

To recapitulate, various cells in CRC ‘activate’ ECs via the angiogenic switch to promote neovascularisation for growth and metastasis. Angiogenesis is a complex multistep process: breakdown of the EC basement membrane, digestion of the extracellular matrix, proliferation and migration of ECs towards the angiogenic stimuli and the formation of functioning capillaries (333). CECs are probably released from the vessel wall by invading tumours and EPCs contribute to vasculogenesis. These processes are regulated by factors released from tumour cells, stromal cells and ECs within the tumour-EC microenvironment (see figure 13).

Figure 13: The proposed endotheliome and angiome in CRC



The Endotheliome and Angiome will be determined by measuring (1) angiogenic factors, (2 & 3) cellular markers and (4) EC factors of activation/damage.

The concept of a multifactorial assessment model of EC activity and angiogenesis is not new. Studies of the role of plasma and cellular markers in cancer are shown in table 11. Despite their heterogeneity, the results were inconsistent as correlations seen in hepatocellular and lung cancers (190, 211) were not demonstrated in lymphomas, breast cancers or gastrointestinal stromal tumours (17, 146). Goon et al investigated their value in disease stage and concluded that cellular, not plasma markers, correlated with the Nottingham Prognostic Index (NPI) in breast cancer (24).

As expected the most quantified factor of angiogenesis is VEGF. It however did not correlate to the increased levels of CECs reported. Higher VEGF and its receptor, VEGFR-2, were positively correlated to EPCs only in GIST tumours, non-small cell carcinomas and hepatocellular cancers (17, 144, 190, 211). However, this was not seen in breast and prostate cancer.

Exactly why correlations of cellular with plasma markers were inconsistent is probably multifactorial. They may be from the variations in techniques used to assess cellular markers and the lack of consensus on clear definitions of CECs and EPCs. Most studies were small; other confounders were less focused study criteria (i.e. the inclusion of different cancer), undefined disease stages and/or inclusion of patients with concurrent disorders e.g. cardiovascular disease, that may alter these markers.

Table 11: The relationship of cellular and plasma markers in cancer

Study	Tumour Type(s)	Cancer vs Controls		Correlations with Plasma Markers
		CEC	EPC	
(140)	Prostate Cancer n=84	None	None	↑ vWf & ↑ E-selectin. No correlations.
(24)	Breast Cancer (n=160)	↑ x 1.3	↑ x 1.3	↑ VEGF, ↑ Angiogenin, ↑ vWf. No correlations.
(190)	Lung cancer (n=10)	NA	↑ x 2.1	↑ VEGF correlated to ↑ EPC.
(142)	CLL (n=20)	↑ x 1.4	NA	↑ FGF-2, ↓TSP-1 and equivocal VEGF No correlations.
(144)	GIST (n=16)	↑ x 2	NA	↑ VEGF and sVEGFR-2. no correlation.
(215)	Breast Cancer (n=47)	NA	↑ x 2.2	↑VEGF, ↑b-FGF. No correlations.
(334)	Gliomas (n=39)	NA	↑ x 13	↑ eNOS. No correlation.
(211)	Hepatocellular cancer (n=64)	NA	↑ x 2	↑ VEGF, ↑ PDG-BB. No correlations.
(335)	Multiple myeloma (n=31)	↑ x 6	Not given	↑ M protein, ↑ β_2 microglobulin. All Correlated to ↑ CECs.
(139)	Breast Cancer (n=16)	↑ x 4.3	↑ x 2.6	↑ VCAM-1, ↑ VEGF but normal EPO, Ang-2, soluble endoglin levels. Correlations not given.
(216)	Hepatocellular Cancer (n=80)	NA	↑ x 3.4	↑VEGF & ↑IL-8 correlated with ↑EPC.
(146)	Various Cancers[a] (n=112)	↑ x 3.3	NA	↑VEGF,↑PIGF,↑SDF-1α,↑SCF No correlations.
(213)	Breast & Gastric cancers (n=71)	NA	↓ x 0.03	↑VEGF- no correlation.
(17)	Lymphoma & Breast (n=76)	↑ x 5.3	NA	↑ VCAM-1 correlated with ↑ CEC.

SDF-1α stromal cell derived factor 1α ; SCF stem cell factor ; EPO erythropoietin; Ang-2 angiopoietin-2; TSP-1 Thrombospondin 1; FGF-2 Fibroblast Growth Factor 2; eNOS endothelial nitric oxide synthetase; GIST- gastrointestinal stromal tumour [a] Breast (n=10), ovarian (n=5), prostate (n=25), colon (n=13),head & neck (n=10), renal cell (n=6) cancers. CLL: Chronic lymphocytic leukemia

The markers that I have chosen for this study are:

1. **Cellular markers**
    a. **CECs** marker of tumour invasion
    b. **EPCs** marker of angiogenesis

2. **Plasma Markers**
    a. **vWf** Marker of EC damage (from vascular invasion and displacement from blood vessels in cancer)
    b. **E-selectin** Marker of EC activation, tumour invasion and angiogenesis.
    c. **Angiogenin** Marker of angiogenesis
    d. **VEGF** Marker of angiogenesis

I next look at the cellular markers chosen for this study in colorectal cancer.

1.4.1 Cellular Markers

As outlined already, CECs and EPCs are promising cellular markers of EC activity in CRC and may have a role in monitoring chemotherapy. However, what is not clear is how these cells vary with cancer stage and surgical treatment. To date no study has addressed this specifically in CRC and their prognostic significance.

CECs

Circulating endothelial cell analysis has been revolutionised by the use of multiple phenotypic markers and flow cytometry. They include CD31, CD34, CD144, CD62E, CD105, CD106, CD146 and KDR (see table 3).

Using 4 or more colour-cytometry, researchers have shown that the vast majority of mature CECs are $CD45^-/34^+/31^+/146^+/133^-$ and come from the $CD34^+$ fraction of mononuclear cells in blood. To quantify CECs I have used the definition of $CD45^-/CD34^+/CD146^+$ i.e. CD45 excludes leukocytes and progenitors, CD146 is heavily expressed on mature ECs (not on progenitor cells) and CD34 found on most ECs in vivo (17, 184, 197, 336). Non-endothelial $CD34^+$ cells include a small population of progenitor and stem cells (both of which also heavily express CD133). CD 31 was excluded as its antibodies cross-react with the majority of leukocytes, platelets and haematopoietic progenitors (337). Also, in cancer endothelial cell lines a small number do not express CD31 and CD105 (338, 339).

EPCs

As already described in section 1.2.2.2, EPCs probably represent a heterogeneous group of cells. Markers that have been described include CD34, CD105, CD133, CD309, CD144, CD 146, and CD45 (dim). Controversially a minority are CD146 positive, counter to the argument of its expression exclusively on mature ECs (169, 197, 340). Unfortunately CD133 expression is lost with maturation. It was also found on haematopoietic and other progenitors, thus raising the possibility that those positive cells were non-EC stem cell derivatives (169, 178).

The most important studies to date showed the growth of $CD45^{+}/CD34^{+}$ progenitor/stem cells into ECs with Weibel-Palade bodies and bearing the mature EC phenotype $CD45^{-}/CD31^{+}/CD105^{+}/KDR^{+}/CD34^{+}$, i.e. a true transformation of progenitor into mature ECs (341, 342). Though KDR is co-expressed in other cell types, I have taken the more popular definition of the EPC as $CD45^{-}/ CD34^{+} /KDR^{+}$ since KDR, though initially low in $CD45^{-}/CD133^{+}/CD34^{+}$ cells, is up-regulated and CD133 down-regulated as the cell matures. CD133 positive cells were therefore best described as forerunners to the EPC [or circulating progenitor cell, CPC] (343-345).

Table 12 summarised the findings of studies on that measured both CECs and EPCs in various cancers against normal controls and phenotypes used to identify the cells.

Table 12: EPCs & CECs - markers of identification and relationship with Cancer

		CEC levels				EPC levels			
Study	**Cancer**	Markers	Cancer	Control	<0.05	Markers	Cancer	Control	<0.05
(140)	Prostate Cancer	$CD45^{-}$ $CD146^{+}$ $CD34^{+}$	28 [11-43] cells/ml	21 (14-45) cells/ml	n	$CD45^{-}$ $CD309^{+}$ $CD34^{+}$	32 [18-82] cells/ml	38 [15-74] cells/ml	n
(24)	Breast Cancer n=160	$CD45^{-}$ $CD146^{+}$ $CD34^{+}$	14 [A] [8-22] cells/ml	8 [A] [6-10] cells/ml	y	$CD45^{-}$ $CD133^{+}$ $CD34^{+}$	120 [A] [72-150] cells/ml	169 [A] [106-241] cells/ml	y
(346)	Head & Neck Cancers n=22	$CD45^{-}$ $CD146^{+}$ $7AAD^{-}$ $CD31^{+}$	2 [A] [0-5] /5x10^{5} events	2 [A] [0-7] /5x10^{5} events	n	$CD45^{-}$ $CD133^{+}$ $CD309^{+}$ $CD3^{-}$ $CD19^{-}$ $CD33^{-}$ $7AAD^{-}$	5 [A] [1-41] /5x10^{5} events	2 [A] [0-7] /5x10^{5} events	n
(334)	Gliomas n=39	$CD34^{+}$ $CD146^{+}$ KDR^{-}	Not given			$CD34^{+}$ KDR^{+} $CD133^{+}$ AND $CD34^{+}$ KDR^{+} $CD105^{+}$	0.18% [B] [0-3.6]	0.013% [B] [0-0.04]	y
(335)	Myeloma n=31	$CD34^{+}$ $CD146^{+}$ $CD105^{+}$ $CD11b^{-}$	6 times higher than controls [C]		y	CFU-ECs KDR^{+} $CD133^{+}$	Not Stated		
(139)	Breast Cancer n=16	$CD31^{+}$ $CD34^{+}$ $CD45^{-}$ $CD146^{+}$	5700 [C]	1300 [C]	y	$CD34^{+}$ $CD45^{low}$ KDR^{+}	370 [C]	140 [C]	y
(347)	AML n=48	$CD31^{+}$ $CD34^{+}$ $CD45^{-}$ $CD133^{-}$ $CD146^{+}$	36,700 [C]	3200 [C]	y	$CD45^{-}$ $CD133^{+}$ $CD31^{+}$ $CD34^{+}$ $CD146^{-}$	700 [C]	100 [C]	y

Results presented as median values and interquartile ranges. Sig= significance of $p<0.05$. [A] Events by flow cytometry. [B] Percentage of monoculear cells in peripheral blood. [C] CFU- colony forming units. CFU-ECs- colony forming units for endothelial cells.

CECs and EPCs have been proposed as markers for oncological treatment. Changes in their levels, particularly with bevacuximab, have been positively correlated to overall survival (OS) and progression free survival (PFS) (see table 13).

Table 13: Role of CECs and EPCs in colorectal cancer follow-up and survival.

Study	n	Marker(s)	Method	Treatment	Findings
(348)	24 mCRC	CECs Apo-CECs	FC (4-colour)	1st line Chemotherapy + Bevacuximab	Longer PFS when both markers were high after the 6th cycle (p=0.002; r=0.83)
(349)	99 mCRC	CEC	FC (4-colour)	1st line Chemotherapy + Bevacuximab	Poor OS (p=0.025) & PFS (p=0.002) when CECs were high before and after 1cycle.
(350)	69 mCRC	EPC (CEP) $CXCR4^+$ CEC	FC (4-colour)	1st line Chemotherapy + Bevacuximab	EPC <0.04% or CEC<20% on day 4 of treatment: longer PFS and OS (p<0.001)
(28)	64 mCRC	CEC	*CellSearch System*™	FOLFOX4 *VS* FOLFOX4 + Bevacuximab	Longer PFS (p=0.003) and OS (p=0.027) when CECs where <65 cells/ml before Bevacuximab
(201)	40 mCRC	EPC (CEP) tCEC rCEC	FC (MP)	1st line Chemotherapy + Bevacuximab	Longer PFS if tCECs were <40 cells/ml. EPCs- no correlations with survival
(351)	33 CRC- All stages	CD 133 mRNA monocytes	rt-PCR	NA	CD133>4.79 correlated to higher recurrence rates OR 14.6 (1.7-126) independent of stage.

mCRC: metastatic colorectal cancer. Apo-CEC: apoptotic circulating endothelial cells. CEP: circulating endothelial progenitors. tCEC: total circulating endothelial cells. rCEC: resting circulating endothelial cells. FC: flow cytometry. PFS: progression-free survival. OS: Overall survival. MP: Multiparametric FC. rt-PCR: Semiquantitative reverse transcriptase real-time PCR. First-line or fluorouracil (5-FU)-based therapy. Bevacuximab- Anti-VEGF-A monoclonal antibody. FOLFOX4- Folinic Acid + 5- fluorouracil + oxaliplatin

Next I give justification for the plasma markers chosen for my thesis.

1.4.2 Plasma Markers

As outlined already various plasma cellular markers of EC activity may have some value in disease staging. However, to my knowledge, this has not been evaluated alongside CECs and EPCs in the prognosis and treatment of colorectal cancer.

Angiogenin

Angiogenin was first isolated in cultured HT-29 human colon adenocarcinoma, is encoded on chromosome 14q11 and is a single chain ribonuclease with 123 amino acids and weighs 14400 Da. (352, 353). It is similar to bovine pancreatic ribonuclease A though 10^5-10^6 weaker than human RNase A and, when inhibited, blocked angiogenesis in embryonic development (354-357).

Angiogenin may bind to cell surface actin on ECs and the complex that dissociates from the cell surfaces trigger tPA generated plasmin from plasminogen. Plasmin degrades basement membrane and extracellular matrix to allow ECs to penetrate perivascular tissues towards neovascularisation. Angiogenin adheres more rapidly to HT-29 human colon cancer cells compared to other ECM proteins. It is postulated to therefore aid metastasis by EC attachment prior to trans-vessel migration (358, 359).

There is still a lack of understanding of the mechanisms behind cellular proliferation observed in angiogenin treated HUVECs and smooth muscles. It may involve

extracellular signal-related kinase phosphorylation of stress-associated proteins (360, 361). A putative 170-kDa angiogenin receptor has been proposed that triggers proliferation of ECs migrated into the perivascular space but down-regulate at a critical cell density and with the formation of a capillary network (359, 362). The mechanisms of nuclear translocation of angiogenin were unknown but occur rapidly in ECs, enhance ribosomal RNA transcription, and may involve the regulation of some steps in EC proliferation by other angiogenic factors e.g. b FGF, VEGF (363).

Shimoyama et al showed serum levels of angiogenin were reflected in the distribution of angiogenin and its genetic expression in CRC tissues. The mean serum angiogenin was significantly higher in cancer patients versus healthy controls and significantly higher in mCRC. Though a small study it showed a correlation with high sera angiogenin and worse disease-specific survival [p=0.03] (364).

It is therefore my view that there is a paucity of clinical data on angiogenin as a biomarker. This probably reflected the difficulties in elucidating its exact role in the mechanisms of angiogenesis. Furthermore, there was little related data with other endothelial biomarkers and to date; none examined its relationship with circulating endothelioid cells in colorectal cancer.

VEGF

To recapitulate, VEGF is a heparin-binding peptide and potent angiogenic factor with specific mitogenic activity in epithelial cells. It is a key player in tumour angiogenesis because it induces EC proliferation, increased vascular permeability, promoted the extravasation of proteins from tumour vessels, and mediates the formation of the fibrin matrix through which stromal cells invade. The rapid growth of cells, hypoxia and apoptosis within the solid tumour stimulates VEGF production, which in turn leads to further angiogenesis in areas demanding more nutrients (365, 366).

VEGF is probably the most studied of all biomarkers in cancer. Numerous reports consistently showed its expression in the circulation and tumour-tissue interface correlated to disease stage and hold prognostic value in CRC. VEGF expression has an inverse relationship to prognosis and serum VEGF levels were significantly higher when a rapid disease progression occurred (269). The first meta-analysis of the VEGF expression in resected colorectal cancer in predicting survival analysed 2050 patients in 18 independent studies. Trials using circulating VEGF levels were excluded to avoid the theoretical contribution from platelets, monocytes, and granulocytes in peripheral blood. Higher VEGF expression in tumour tissue indicated a shorter relapse-free survival [RR, 2.84; 95% CI, 1.95-4.16; $P < .001$] and poorer overall survival [RR, 1.65; 95% CI, 1.27-2.14; $P < .001$] (284). However this does not negate the consistent findings that circulating VEGF, as a minimally invasive biomarker, is of predictive value in disease progression (366).

VEGF levels have been shown to correlate with CECs and EPCs in some cancers but to date there are no studies of this relationship in CRC stage, surgical treatment and survival. However there is emerging support for their role in predicting the response of CRC to chemotherapy, specifically in the use of anti-VEGF, though results between studies are not consistent (table 13).

It is now well established that VEGF is one of the major player in the angiogenic switch in colorectal cancer except in tumours with DNA microsatellite instability. The capacity to express high levels of VEGF is acquired in pre-malignant phases of colorectal cancer development. The regulation of VEGF expression is under the control of p53 as well as ras and src pathways. Serum or tumour VEGF levels may have prognostic relevance in specific subgroups of patients, and more likely so when combined with other markers of angiogenesis, like KDR receptor, TF, MVD, MMP2.

The demonstration that neutralizing anti-VEGF antibodies are efficacious in somewhat prolonging survival in metastatic colon cancer has been the tour de force of antiangiogenic drug development. Measurable therapeutic and biological effects are also seen in breast, renal and rectal carcinoma. Willett et al. specifically demonstrated that following a single infusion of antibody, reductions are seen in tumour perfusion, vascular volume, microvascular density, interstitial fluid pressure and the number of EPCs, while vessel maturity had increased.

E-Selectin

E-selectin (E-Sel) is an adhesion molecule expressed by cytokine activated ECs to mediate the migration of neutrophils and monocytes in inflammation. It is often considered a marker of activation of the normally quiescent ECs with growing evidence that it aids the attachment of malignant cells during metastasis (90, 367, 368). Small studies of circulating E-Sel suggest a diagnostic and prognostic value in CRC but s controversial on the link to life expectancy and site of metastasis (78, 369). High levels of E-Sel have been linked to VEGF and the coagulation cascade in non-CRC tumours (370). Blann et al did not find a correlation with CECs and plasma levels of e-selectin despite high levels of both in prostate cancer (140). To date this relationship has not been reported CRC in the published literature.

vWf

vWf is a multimeric glycoprotein synthesised in ECs and megakaryocytes, stored in Weibel-Pallade bodies, released in EC damage and under the effects of inflammatory cytokines (27,28). High vWf plasma levels have been seen in CRC patients when compared to controls as with many cancers, the highest seen metastatic disease (11, 16). Non-significant correlations with CEA levels are reported, but those of tumour characteristics such as lymph node spread and venous invasion are inconclusive (23). Blood group O has significantly lower plasma levels than other phenotypes, though conflicting reports have emerged (371, 372). Additionally many factors such

as myocardial infarction, diabetes mellitus, liver disease and acute infections influence vWF plasma levels. Schellerer et al concluded that on its own vWf was not a useful biomarker in CRC (23, 24). Goon et al and Blann et al found no relationship with vWf, CECs and EPCs in breast and prostate cancer respectively (29, 139). However, to date this relationship has not been explored in CRC.

In the next section I summarise the introduction, generate questions for the endotheliome and angiome in CRC, and propose my study hypotheses.

1.5 Summary of the Introduction

Our understanding of endothelial activity and angiogenesis in diseases continues to evolve. Given that many questions are yet to be answered from the above observations, the vascular infrastructure of CRC, inferred by circulating plasma markers, is worthy of further study. Certainly, when compared to healthy individuals, various markers of EC activity are disturbed. However, the clinical ramifications of these assessors remain unclear and such changes may merely reflect the broad disturbance in many physiological systems that may occur in disease in general. Furthermore, as newer therapies emerge more accurate assessment tools will be required. There are a number of limitations to current assessors most notably a lack of standardized methodology. Further studies towards improving the accuracy and the assessment of the results of current tests are required before translation to clinical application. Therefore, larger disease specific studies with more stringent exclusion criteria are necessary.

Most notably, the benefits seen from recent clinical data on the efficacy of anti-VEGF therapy for metastatic cancer will continue to drive research into other modulators of angiogenesis. The promise lies in more tailored, tumour-specific treatment and will probably be determined by the tumour's genetic make-up including its angiogenic potential e.g. kras and p53 status. With the expected rise in clinically validated angiogenic modifiers, better surrogate markers, other than the maximum tolerated dose, are needed. To date there is no single marker to monitor anti-angiogenesis but recent reports support of circulating cellular markers (CECs, EPCs), despite the limitations to accurately enumerate them. However, the importance of the multiple

assessment strategy of any therapy (involving histological, circulating and imaging markers) is its 'predictive' potential of the tumour's response to treatment. Furthermore targeting angiogenesis will require more accurate measurements of EC activity. In summary the status of the endotheliome and angiome are crucial determinants in CRC treatment and prognosis.

The project I describe aims to clarify fundamental questions of EC activity in CRC as measure in PB. Building on existing publications and the expertise of my colleagues, I have identified the following key facts (1.5.1) and questions for my study (1.5.2):

1.5.1 What was known of the endotheliome and angiome in CRC:

1. Endothelial cells and angiogenesis are fundamentally essential to CRC.
2. Flow-cytometry defined CECs and EPCs were partially investigated in CRC.
3. CRC demonstrate a spectrum of EC and angiogenic activity.
4. Well vascularised CRC are likely to be more advanced but relatively chemosensitive versus less advanced and 'under-vascularised' ones.
5. Plasma markers of EC activity and angiogenesis are often high in mCRC.
6. Surgery, chemotherapy and/or radiotherapy (and/or combined therapy) alter levels of circulating plasma markers of EC and angiogenic activity.
7. Plasma markers of EC and angiogenic activity may predict outcomes before and during treatment of CRC.

1.5.2 What was NOT known of the endotheliome and angiome in CRC:

1. What are the relative components of the endotheliome and angiome for CRC?
2. Are the endotheliome and angiome generic or specific to CRC and stage?
3. How might radiological, histological, circulating cellular and plasma markers define the endotheliome and angiome in CRC?
4. How does the measured endotheliome and angiome alter with intervention(s)?
5. Does the measured endotheliome and angiome before treatment predict outcome and/or response to the treatment?
6. Does the measured endotheliome and angiome reflect CRC vascular activity and could this predict treatment response?

From these questions I next formulated the five hypotheses for my study.

1.5.3 Hypotheses

1. There are altered numbers of CECs and EPCs (and their ratio) in colorectal cancer when compared with healthy and non-cancer disease controls. The latter will be non-malignant lower gastrointestinal polyps (‘pre-cancerous’ disease) and stable coronary artery disease (positive control). A sub-hypothesis is that numbers of EPCs and CECs correlate significantly with each other.

2. Numbers/levels of CECs and/or EPCs vary with CRC disease stage.

3. Numbers/levels of CECs and/or EPCs correlate positively/negatively with plasma markers of the endotheliome and angiome, specifically vWf, angiogenin, sE-selectin and VEGF in CRC versus the controls.

4. Altered numbers/levels of CECs, EPCs and/or plasma markers of the endotheliome and angiome normalise after treatment by surgery, conventional chemotherapy or anti-VEGF antibody therapy.

5. Altered numbers/levels of CECs, EPCs and/or plasma markers predict disease progression and recurrence within 2 years.

1.5.4 Plan of Investigation

In order to test hypothesis 1, research indices were measured in the blood of patients with colorectal cancer and compared to three control groups: benign disease, fully healthy, with stable coronary artery disease [SCAD]. The SCAD group was included for two reasons: (i) to provide a 'positive control' for altered EPCs and CECs (22, 122) and (ii) to determine whether or not any changes in CRC are of a similar magnitude to those in SCAD. Subjects were recruited from patients and there relatives attending colorectal, cardiology and oncology clinics.

Hypothesis 2 within the colorectal cancer group, levels of cellular markers and growth factors should associate with disease stage as defined by Dukes' classification (and/or AJCC). The disease stage was determined in combination by routine radiology (CT scan) assessment and histological examination of the excised tumour.

Hypothesis 3 was tested by the correlation of ELISA-defined plasma markers of the endotheliome (vWf and soluble E selectin), and the angiome (VEGF,angiogenin) with that of CECs and EPCs from the participants recruited to hypotheses 1. This was determined by analysing these plasma markers, against data generated in hypothesis 1.

Hypothesis 4 was tested in the blood (for markers as in hypothesis 1 and 2) of patients before and on two occasions (3 and 6 months) after surgery for early disease with no adjuvant therapy. Similarly blood in subjects with advanced

colorectal cancer disease (with or without neoadjuvant therapy) was tested before and after standard and/or experimental chemotherapy.

Hypothesis 5 tested the predictive value of hypotheses 2 & 3 on outcomes at 2 years.

In the next section I detail the methodology of my study.

Chapter 2

Subjects and Methodology

2.1. Subjects

2.1.1 Patients with CRC

Participants with colorectal disease were identified from hospital referrals, outpatient clinics, endoscopy sessions, emergency presentation and colorectal cancer multidisciplinary meetings. They were recruited from colorectal surgery, endoscopy and oncology clinics, and acute ward admissions.

2.1.2 Controls

The general hypotheses called for several age-matched control groups.

a. Participants with benign colonic polyps (but free of any inflammatory bowel disease) as polyps are recognised ‘pre-cursors’ to colorectal cancer. Participants referred for lower gastrointestinal symptoms were approached after consultation at colorectal clinic and recommended for colonoscopy. Samples were drawn before bowel preparation began and participants were only included if found to have one or more tubllovillous adenomas of any size

with moderate to high-grade dysplasia. Polyps with high-grade dysplasia and uncertain to be cancerous were excluded.

b. Healthy controls free of any apparent disease were recruited. These participants were defined by careful clinical history, routine observations and blood tests. They were all relatives of patients attending the colorectal, cardiology, rehabilitation and oncology clinics.

c. Stable cardiovascular disease i.e. patients with known stable coronary artery disease for more than 3 months, and had no acute cardiovascular events during this time. Stable CAD were categorized on the basis of a history of stable angina with or without previous revascularisation procedure(s), left ventricular ejection fraction ≥ 45% and evidence of flow-limiting CAD, identified by either a positive exercise stress test or on coronary angiography (i.e. 70% stenosis in 1 major coronary artery). EC dysfunction is well established in CAD; hence this group was used as the positive control.

All participants gave a 20 ml blood sample, with their medical history and routine observations (blood pressure, temperature, heart rate and BMI) fully recorded.

2.1.3 Exclusion Criteria:

- Previous chemotherapy, immunotherapy or radiotherapy (for either colorectal or non-colorectal cancers)
- Received any investigational drug or agent/procedure (i.e. participated in a treatment trial) within 4 weeks of recruitment.

- Moderate or severe renal impairment [creatinine clearance <30ml/min calculated according to Cockroft-Gault formula]
- Any of the following laboratory values (within 2 weeks of recruitment):

  1. Absolute neutrophil count <1.5 x 10^9/L
  2. Hb < 10 g/dl
  3. Platelet count < 100 x 10^9/L
  4. Total bilirubin > 1.5 above the upper limit of normal

- Patients on anticoagulants, high dose aspirin (>325mg/day), anti-platelet drugs or known bleeding diathesis e.g. factor VIII or IX deficiency; low dose aspirin was accepted once used in excess of 6 months.
- Known coagulopathy e.g. deficiencies of proteins C & S, and factor V leiden.
- Clinically significant cardiovascular disease diagnosed within the last 12 months i.e. cerebrovascular accident, myocardial infarction, unstable angina, congestive heart failure, serious cardiac arrhythmia requiring medication or uncontrolled hypertension.
- Pregnant or lactating women.
- Concurrent malignancies unless there was a disease free interval of at least 10 years.
- Inflammatory bowel disease and/or peptic ulcer, either of which have been active or required medication in the last 2 years.
- History of uncontrolled seizures, central nervous system disorders, dementia or psychiatric disability.

- Concurrent inflammatory connective tissue disease (e.g. rheumatoid arthritis, systemic lupus erythematosis, arteritides)
- Participants were not recruited whilst on antibiotics and hormone replacement.

2.1.4 Ethical considerations

All participants were fully informed, gave signed consent according to the approved local ethics and research protocol in keeping with the Helsinki agreement (Registered with Black Country Research & Ethics Committee, Reference 08/H1202/178).

2.1.5 Research material

Venous blood was transported in EDTA vacutainers (stored in ice packs) to the Haemostasis, Thrombosis and Vascular Biology Unit (HTVBU) at City Hospital for preparation and analysis. The excised tumour was analysed by conventional histological techniques and reported in accordance with the minimum dataset guidelines of the Royal College of Pathologists on CRC [see appendix 2, page] (285, 373). Radiology reports were also guided by the standards of the Royal College of Radiologists, first edition (374). The Stage was obtained from the routine reports of the hospital's histopathology and radiology departments. All results, along with the therapeutic strategies were confirmed at the multi-disciplinary meetings.

2.2 Assays

2.2.1 CEC and EPC Quantification

The major equipments were the Becton Dickinson FACSCalibur™ flow cytometer and Bayer Advia Haematology Analyser. The assay development is outlined next followed by the detailed flow cytometry standard operating procedure (SOP).

Assay Development

This assay was initially developed to simultaneously detect CECs and CPCs (circulating progenitor cells expressing $CD45^{-}/CD133^{+}/CD34^{+}$) in breast cancer (24), adapted to detect EPCs in prostate cancer (140), and further developed by myself to improve discrimination between CECs and EPCs. The initial work provided details on FC settings for 4-colour detection and in accordance with the manufacturer's instructions. The main steps were maintained with no changes to volumes of the samples analysed and reagents used:

a. Quantification of WCC (Bayer Advia Haematology Analyser ™)

b. Quantification of CEC and EPC (by BD FACSCalibur™)

The steps for sample preparation were as follows:

I. Flourochrome Incubation

II. Lysis/fixation, centrifugation and decanting of supernatant

III. Re-suspension, centrifugation and decanting of supernatant

IV. Re-suspension in PBS for FC enumeration.

a. Quantification of WCC

From the fresh venous sample a WCC (full blood count) was obtained using the Bayer Advia Haematology Analyser ™. Start-up procedures, calibration and general maintenance of the Bayer Advia Machine was carried out as per the manufacturer's guidance. Calibrations were performed daily in accordance with the laboratory's protocol and an electronic copy stored on the hard drive of the operating computer.

b. Quantification of CEC and EPC

The venous sample was prepared for analysis by flow cytometry. Start-up procedures, calibration and general maintenance of the Becton Dickinson FACSCalibur™ flow cytometer were carried out as per the manufacturer's operation guide. Calibrations were performed daily in accordance with the laboratory's protocol and an electronic copy stored on the hard drive of the operating computer.

I. Flourochrome Incubation

To 0.2mL of blood 10µL each of the fluorochrome-labelled monoclonal anti-human mouse antibodies were added and the solution incubated in the dark at room temperature for 20 minutes. These antibodies were FITC-CD45, PE-CD146, PE-Cy 5-CD34 (Becton Dickinson, Oxford, UK) and VEGF R2 (KDR)-APC (R&D Systems).

II. Lysis/fixation, centrifugation and Decanting of supernatant

This removed red blood cells that may 'scatter' the lasers used by the FC machine and reduce false positives of the assay. The fixative reduced cell loss from apoptosis during storage and incubation. Three millilitres of 1 in 10 diluted FACS lysing/fixing solution was then added and the solution incubated for 15 minutes again in the dark at room temperature. The solution was centrifuged at 200g for 5 minutes and the supernatant containing lysed red blood cells was decanted.

III. Re-suspension, centrifugation and Decanting of supernatant

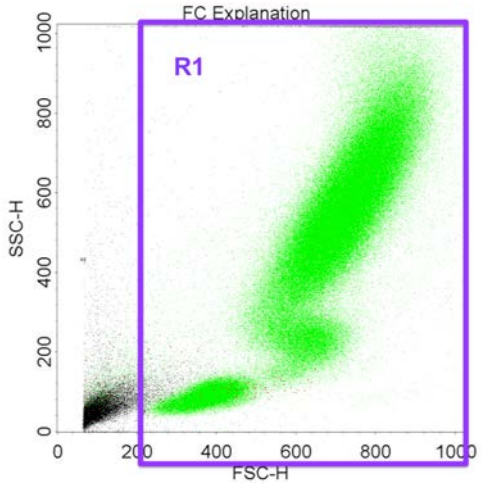
This wash step aimed to remove residual erythrocyte products. Three millilitres of buffer solution (PBS Gibco) was added and vortexed gently to resuspend the pellet. The suspension was centrifuged once again at 200g for 5 minutes, the supernatant decanted and the cells resuspended in 0.5 ml of PBS solution ready for analysis.

IV. FC Protocol Modifications

Each sample was analysed by FC with a 4-colour BD FACSCalibur™ machine (Becton Dickinson, Oxford, UK). Events were plotted according to forward scatter (FSC) and side scatter (SSC) profile i.e. measures of size and granularity respectively. The flow rate was maintained at 1500 or fewer events per second (less than 35 µl/min). The CellQuest Pro software on an Apple G4 computer was used to determine cell counts. It ran to achieve a target of 1,000,000 events or, if not, until the entire volume if the sample was analysed.

The first gating strategy morphologically included only mononuclear events (figure 14) whilst excluding cell doublets, platelets, dead cells/carcuses, cell debris, microparticles and high SSC events (highly granular dead and/or polynuclear cells).

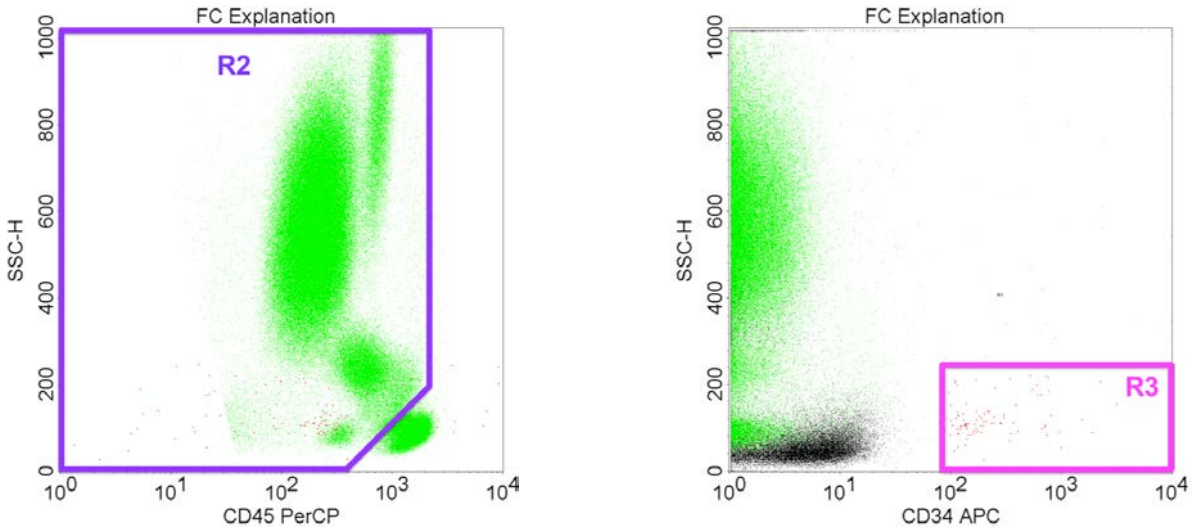
Figure 14: Flow cytometry Inclusion of mononuclear events in R1 (purple).



R1 gates for mononuclear events whilst excluding cell doublets, platelets, dead cells/carcuses, cell debris, microparticles, and highly granular dead and/or polynuclear cells.

The second gate identified events negative/dim for FITC-CD45 (the pan-leukocyte marker). A 'dump' channel also excluded all CD45 positive events (including $CD45^{dim}$ ones) and low to medium side scatter events including singlets (figure 15). From these events a third gate identified CD34 (PE-Cy 5) events in R3. Only high intensity doubly fluorescent events for CD146 and CD34 were defined as CECs; similarly EPCs were CD34 and KDR positive.

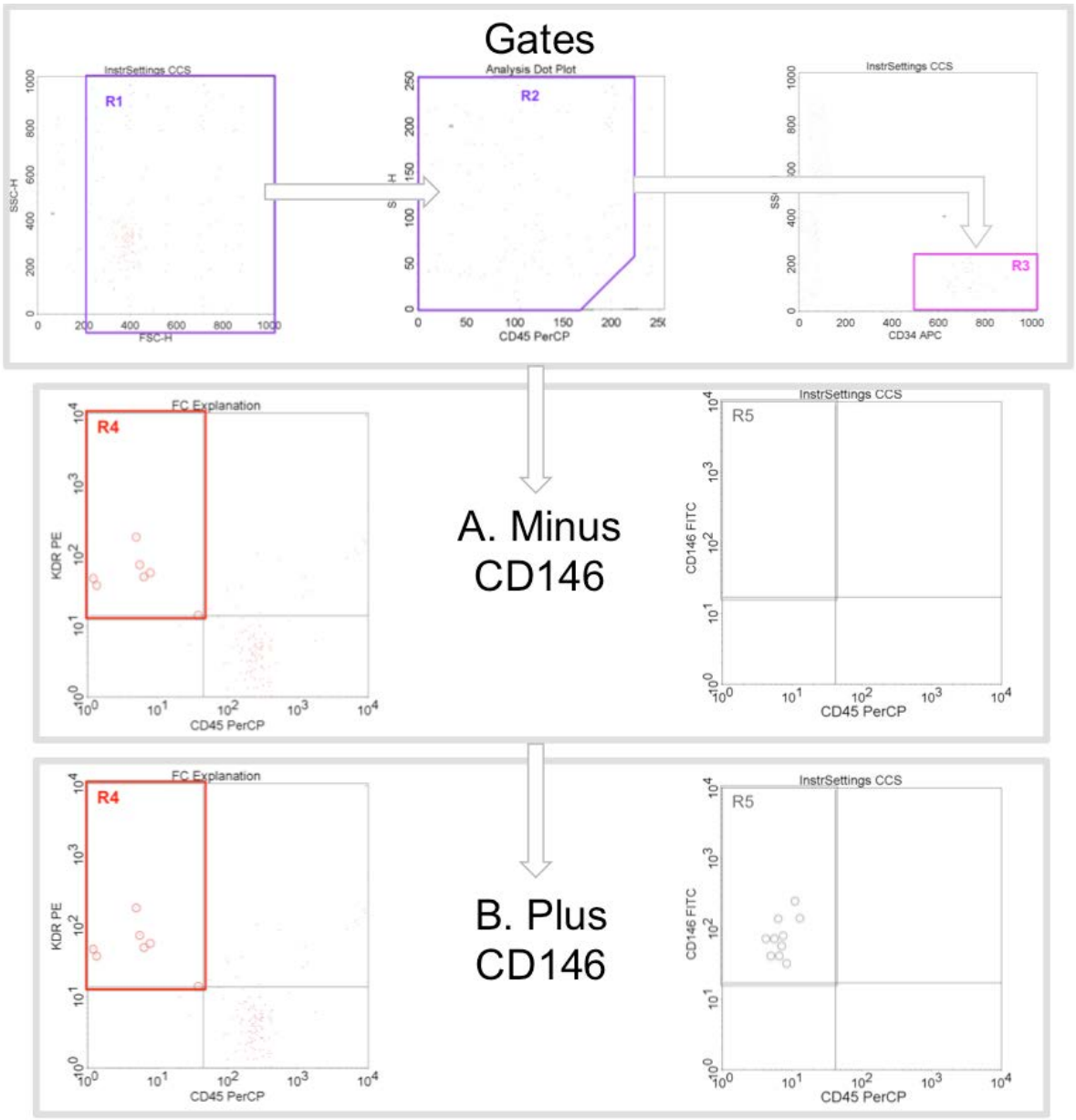
Figure 15: FC: events negative for CD45 (R2, outlined in purple) and positive for CD34 (R3, magenta rectangle).



To improve assay discrimination between both cells, HUVECs (angiogenesis activated expressing VEGR-2 from Cell Applications Inc.) were used. This was diluted to obtain one EPC per 10 µL of BD buffer solution (total of 20 events). Similarly, CECs were derived from cultured mature ECs (Cell Applications Inc.) and diluted to 1 in 20 µL of buffer. Both were added to make up a 200 µL suspension for

FC analysis. A flourochrome minus one (FMO) control assay was applied to optimise the gate settings and detect all CD146+ and KDR+ events (figure 16).

Figure 16: Assay improvement: flourochrome minus 1 with HUVECs and ECs.

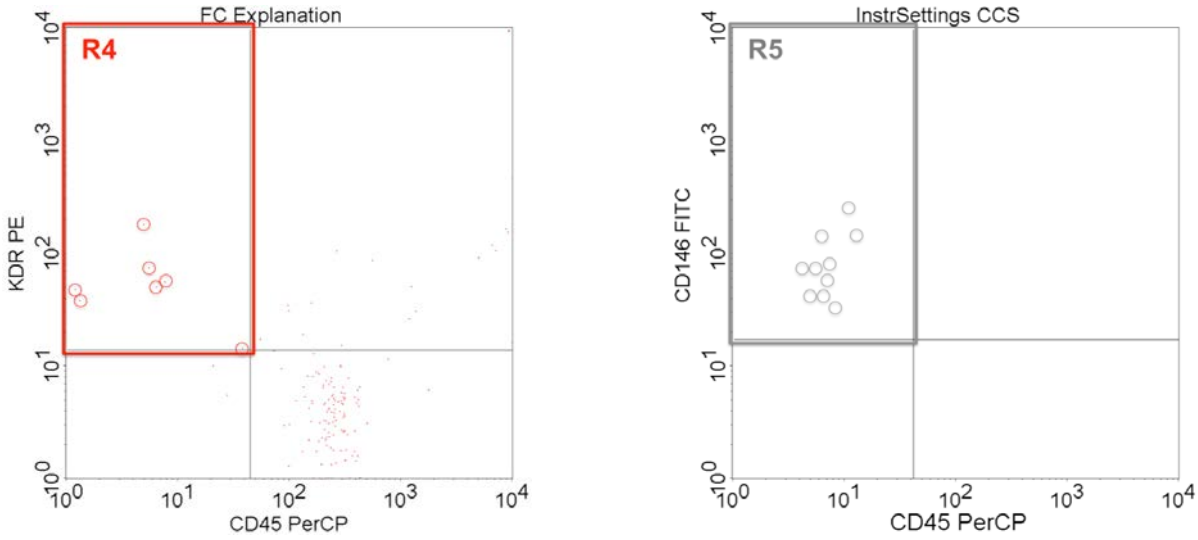


Using a flourochrome minus 1 technique:

A. All flourochromes minus CD146 detected all EPCs (R4 red circles) only, not CECs (R5)
B. All flourochromes discriminated between all EPCs (R4) and all CECs (R5).

When applied to a patient with metastatic CRC the following results were obtained with better discrimination between both cells (red box for EPCs and CECs in grey).

Figure 17: CECs & EPCs from CD34 positive events (gating not shown).



The assay improved the discrimination between EPCs and CECs in the protocol used for detecting the cells in breast cancer. Prior to this change, the 10 events in the CEC box (grey) would have been counted as EPC events (red box).

Determining EPC and CEC counts

The following calculation determined the CEC and EPC counts:

$$\text{CEC counts (cells/mL)} = \frac{\text{Number of EPC events} \times \text{White Blood Cell Count} \times 10^6}{\text{Number of white cell events}}$$

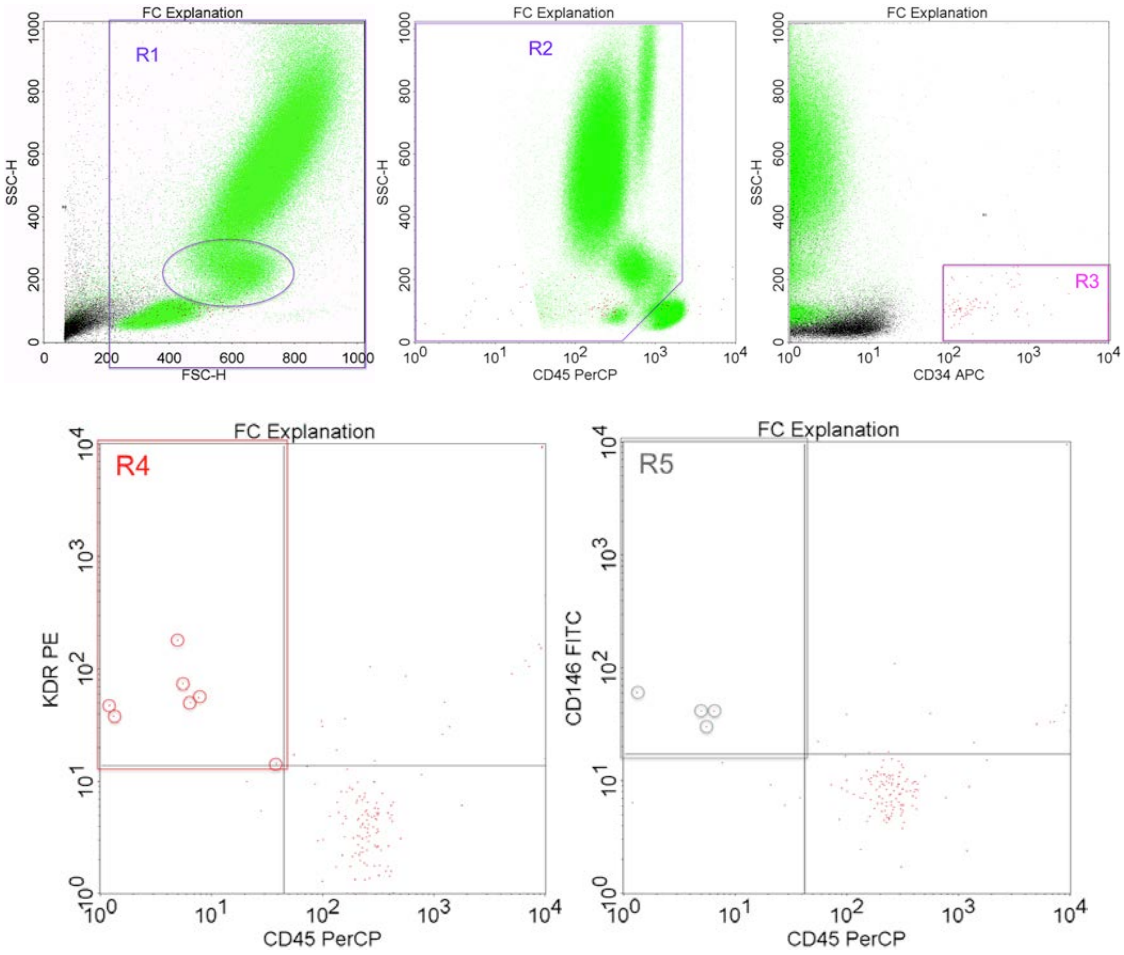
$$\text{EPC counts (cells/mL)} = \frac{\text{Number of EPC events} \times \text{White Blood Cell Count} \times 10^6}{\text{Number of white cell events}}$$

The number of EPC events, number of CEC events and number of white cell (WBC) events were determined by flow cytometry and the White Cell Count (WCC) from the Bayer Advia® full blood count output.

Interpretation with example (figure 18)

1. The WCC count by Bayer Advia® was determined e.g. 4.71 x10^6/mL.
2. The plot showed the FSC/SSC plot (in green), which gated nearly all cells, gate statistics out of 753078 total WBC events on FC.
3. The SSC to CD34 plot with the R3 box detected highly positive CD34 events. e.g. n=176 in the gate statistics out of 753078 total WBC events on FC.

Figure 18: Flow Cytometry example of results from a CRC participant.



Results: R1=753078, R3=127, R4=8 & R5=4 events.

4. The non-WCC events was confirmed in the R3 box of SSC/CD45 by collecting 'CD34+ve cells', i.e. n=127. Hence 127/753078 = 1.686 x 10^{-4} WBCs were CD34 positive. As a fraction of the WCC of the Bayer Advia® i.e. 4.71 x 10^{6} cells/ml, there were 794 CD34+ve cells/ml in the venous blood. These cells will be referred to CD34+CD45- cells, which include EPCs (8 events), CECs (4 events) and other cells/events of unknown classification that also express these phenotypes (782 events).

5. On the plot of KDR PE vs CD45 PerCP the CD34+ve events were scanned for KDR and CD45. The events, e.g. 8, in the upper left quadrant (R4) were KDR+ve but CD45-ve and therefore were EPCs. Thus 8/127 of the CD34+ve events were EPCs, so the EPC count was $8/127^{th}$ of 794 = 50 EPCs/mL.

6. Similarly, on the plot of CD146FITC vs CD45PerCP, CECs were low for CD45 and high for CD146 (R5), i.e. 4 events. Since all the cells in this analysis were CD34+ve, and there were 99 CD34+ events, 4/127 of the CD34+ve cells were CECs. That is $4/127^{th}$ of 794 CD34+ cells/mL, or 25 CECs/mL.

Appendix 3 contains the details of the Standard Operating Procedure to enumerate CECs and EPCs, along with the gating strategy.

2.2.2 ELISA for vWf Assay

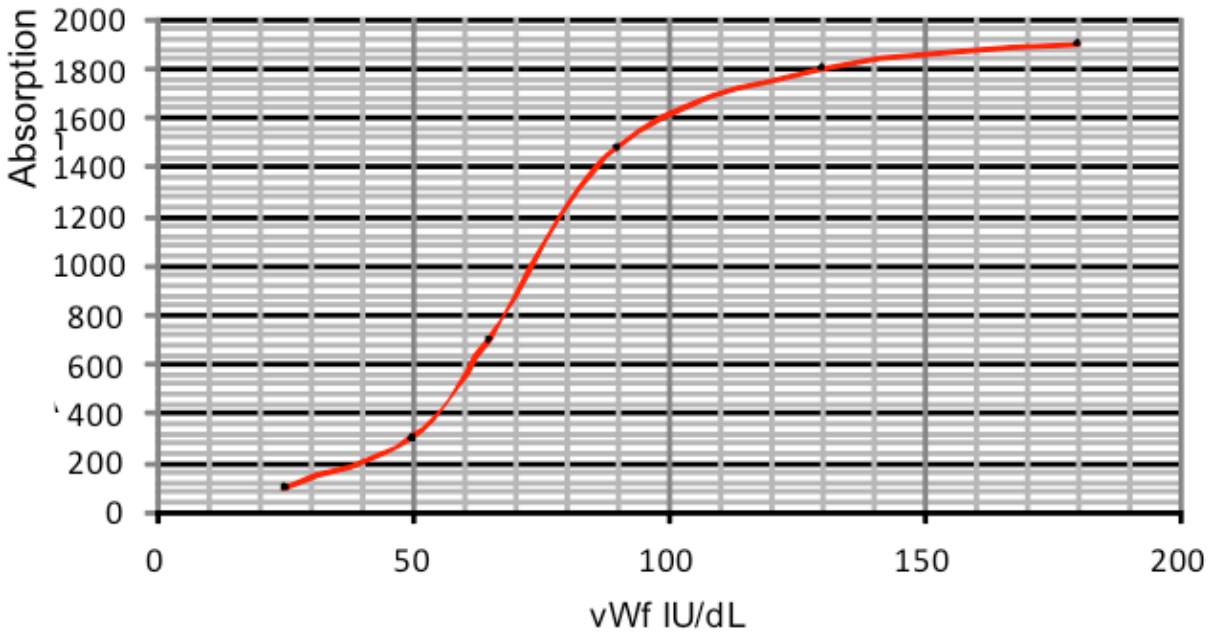
The method was modified from Short et al using commercial antisera from the Danish company, Dako, and recognised worldwide by most major Journals (60, 375-377). The full SOP is detailed in appendix 4.

Brief Method

1. The microtitre plate was coated with 100 µl of diluted primary antiserum (30 µl in 20.5 ml coating buffer pH 9.6) at room temperature for >60 minutes or overnight at 4^0C.
2. The plate was washed 3 times in PBS/tween, 100 µl of 1/40 serum or plasma in pbs/tween was added along with the standards and incubated for >60 minutes at room temperature.
3. The wash was repeated 3 times and 100 µl secondary antiserum was added i.e. the peroxidase-labelled conjugate (30 µl in 20.5 ml PBS/tween), and incubated for >45 minutes at room temperature.
4. The plate was washed and 100 µl substrate (OPD, hydrogen peroxide, pH 5 citrate buffer) was added. The colour developed almost immediately.
5. This was stopped with 50 µl acid, read at 492 nm
6. Plot against standard curve.

Expected values: in citrated normal plasma are in the region of a mean of 100 with a standard deviation 30 IU/dL. Typical values in stable atherosclerosis are 130 IU/dL and acute coronary syndromes often 150 IU/dL. The data is normally distributed.

Figure 19: Curve of the standards for vWf versus optical absorption.



This curve was generated from the standards of an ELISA performed in this study.

2.2.3 ELISA for soluble E-Selectin

This was the standard ELISA for measurement of soluble E-Selectin\CD62E (sE-Sel) using the duoset from R&D. My colleagues in cardiovascular disease and breast cancer studies have used it extensively (24, 116).

The brief method is described below and the full SOP is detailed in appendix 5.

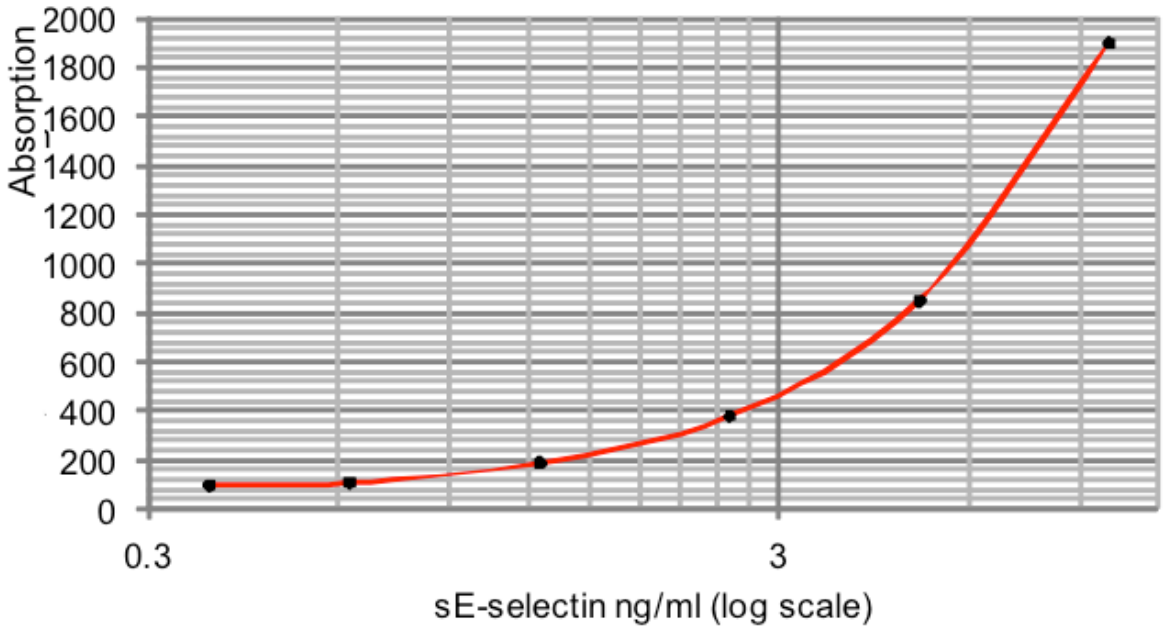
Brief method

1. The microtitre plates were coated with 112ul of capture primary antibody in PBS buffer and incubated in the fridge ($4^{o}C$) overnight or 1.5 hours at room temperature.
2. The plates were washed, 100 ul serum/plasma (diluted 1/5 i.e. 20 plasma plus 80 blue or pbs-tween buffer) with standards (top 'prepared' at 50 ng/mL) were added and incubated for 1.5 hours at room temperature. The 'Universal' plasma was also prepared.
3. The plates were washed again, 112 ul detection antibody (one vial in 20 mls 1% BSA PBS for two plates) was added and incubated for 1.5 hours at room temperature
4. Again the plates were washed, 100 ul Streptavidin-HRP conjugate (diluted 1/200 in PBS, i.e. 100 ul plus 20 mls) was added and incubated for a minimum of 20 minutes at room temperature in the dark**.**
5. The plate was washed again and 100ul of substrate (made up from equal volumes of reagents A and B) was added. This went blue after 3 – 5 minutes. The key definition was a clear gradation of blue colour from the top to the blank.

6. The reaction was stopped with 75ul Acid (a yellow reaction) and the optical density was read at 450 nm. A curve of standards was generated and used to determine the concentrations.

Expected values: Between 20-40ng/mL and the Universal around 40 ng/mL.

Figure 20: Curve of the standards for sE-sel ELISA vs optical absorption.



This curve was generated from the standards of an ELISA performed during this study.

2.2.4 ELISA for VEGF

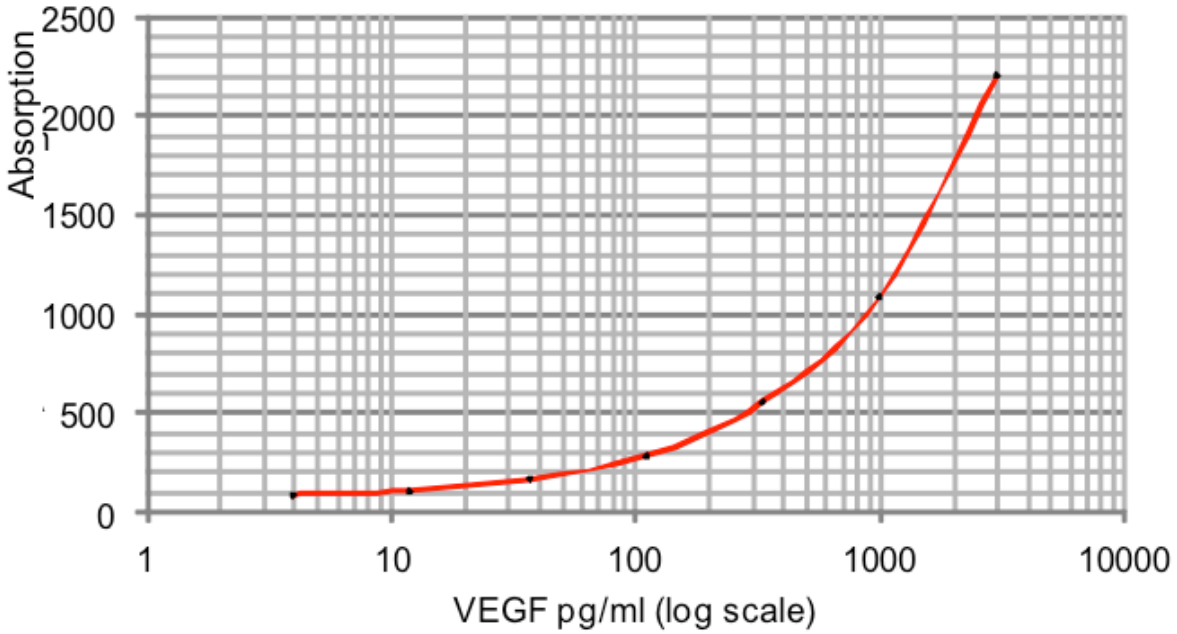
This ELISA used commercial antibodies from R&D and was performed by my colleagues in previous studies of cardiovascular disease and cancer (24, 91). The brief method is outlined below and the full SOP is detailed in appendix 6.

Brief Method:

1. Microtitre plates were coated with 100µl of primary antisera (40µl of 40µg/ml in 10ml coating buffer for 1 plate) and stored overnight in the fridge.
2. Plates were washed and blocked with 100µl /well of 5% Marvel (1g in 20mls PBS-T for 2 plates) for 1 hour at room temperature.
3. Plates were washed, 100µl of neat plasma and recombinant standards added and incubated for 2 hours at room temperature.. Standards were diluted tenfold with fresh tips for each sample.
4. Plates were washed; 100µl of 500ng/ml of biotinylated anti-human VEGF antibody (100µl of 5 µg/ml in 10ml PBS) was added and incubated for 2 hours at room temperature.
5. Plates were washed; extravidin peroxidase (100µl/well) was added and incubated for 45 minutes at room temperature.
6. tes were washed; 100µl substrate (Solutions A and B) was added and incubated for 30 minutes at room temperature. The blue colour developed.
7. Reaction was stopped with 50µl/well acid and read at 450 nm.

Expected values: Data was usually non-parametrically distributed. Controls generally had median values of about 30-50pg/ml (but wide IQRs, at times exceeding 200pg/ml). Patients' median values generally were between100 to 200 pg/ml.

Figure 21: Curve of standards for the VEGF ELISA vs optical absorption.



This curve was generated from the standards of an ELISA performed during this study.

2.2.5 ELISA for angiogenin

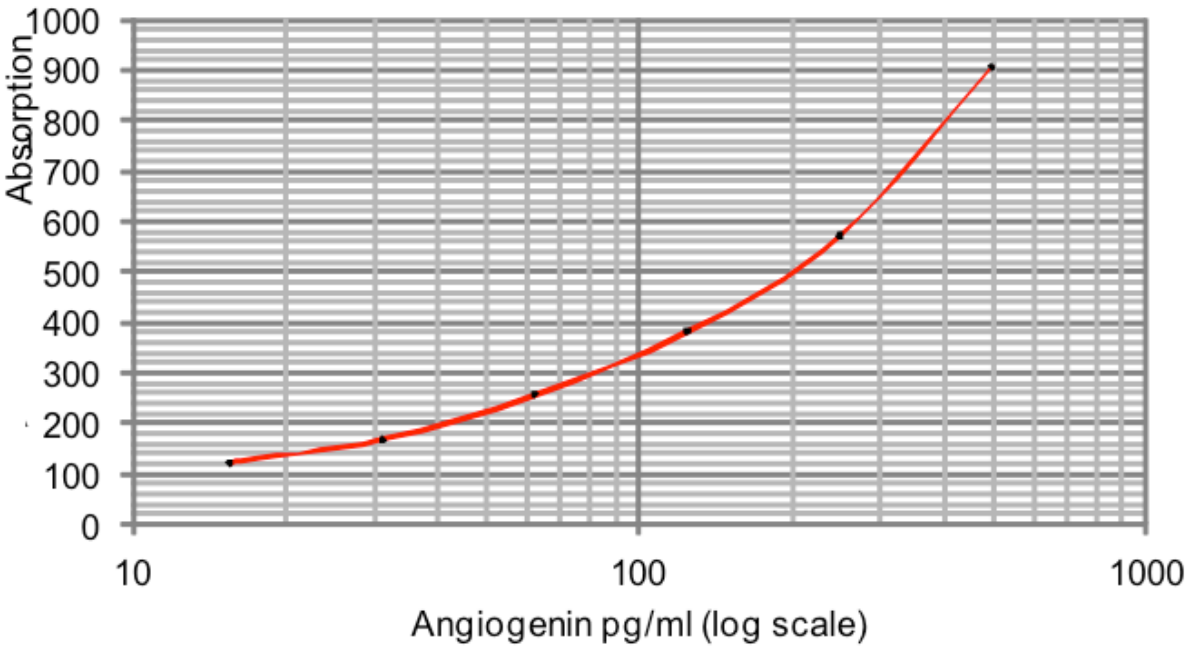
This ELISA uses commercial antibodies from R&D systems and performed by my colleagues in previous studies of breast cancer and cardiovascular disease (24, 378). The brief method is outlined below and the full SOP is detailed in appendix 7.

Brief Method:

1. Microtitre plate wells were coated with 100µl of primary antiserum at room temperature for 90 minutes or at 4^{o}C overnight.
2. Plates were washed, plasma added and recombinant standards diluted in PBS/tween for 90 minutes at room temperature.
3. Plates were washed again, 100µl of biotinylated anti-human angiogenin antibody added to each well for 90 minutes at room temperature.
4. Plates were washed again; 100µl/well of streptavidin-HRP (50µl strep-HRP in 10mls of PBS-T for 1 plate) was added and incubated for at least 20 minutes at room temperature avoiding direct light.
5. Plates were washed again, 100µl warm substrate solution (5mls A + 5mls B for 1 plate) were added. The colour developed in less than 5 minutes.
6. The reaction was stopped with 50µl/well of acid and read at 450 nm.

Expected values: parametric data was obtained and the controls generally had median values of about 5 pg/ml.

Figure 22: Curve of standards for Angiogenin ELISA vs absorption.



This curve was generated from the standards of an ELISA performed during this study.

2.2.6 Assay Variability

The coefficients of variation (CV) of the assays are shown below. They were determined from processing samples of 7 healthy volunteers on 5 and 3 different occasions for the FC protocol and ELISAs respectively. The angiogenin ELISA was re-performed another 3 ocassions as, of all the protocols, stopping the very swift colour change required additional practice. The CV was calculated as follows:

$$CV = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100\%$$

Assay	Inter-observer CV (%)	Intra-observer CV (%)
CEC	39 (24-60)	24 (13-47)
EPC	28 (16-40)	17 (11-24)
vWf	9 (6-9)	4 (4-5)
E-Sel	6 (5-9)	4 (4-5)
VEGF	8 (8-10)	5 (4-5)
Angiogenin	9 (7-10)	5 (4-5)

CV: cumulative variability. Values expressed as means and ranges.

2.3 Statistics and power calculation

As the Department had experience in measuring CECs in breast cancer, rheumatoid disease, and in cardiovascular disease (24, 91, 379), the indexed results were used as the test statistic for power calculations.

2.3.1 Cross-sectional Studies

Hypothesis 1

I predicted that CECs will be raised in colorectal cancer (CRC) compared to both healthy controls (HC) and patients with benign colorectal disease (BD), and that levels in CRC will be comparable to those with stable coronary artery disease (SCAD). A model in statistical package Minitab 16 was developed of a median (interquartile range) count of 3 (1.5-5.0) CECs/mL in CRC and SCAD compared to 1 (0-3) cells/mL on HCs and BD [CECs were known to have a non-normal distribution]. For $p<0.05$ between CRC and SCAD versus the other two groups, and $p<0.01$ overall (Kruskall-Wallis test), 25 subjects were required in each of the four groups.

I regarded n=20 as an absolute minimum and aimed to recruit an excess to provide improved confidence. A sample size of n=20 provided the two-sided p value of <0.05 and the 1-beta power of 0.8 for a correlation coefficient (r) of 0.55. My co-investigators generated published data comparing CECs (median 8, IQR 4-12) and

EPCs (median 78, IQR 42-128) in 19 women with breast cancer and no correlation was found (Spearman r= -0.086, p=0.726).

Hypothesis 2

Cellular markers were predicted to associate with CRC stage as defined by Dukes' classification. This is effectively the same sample size estimation as in hypothesis 1 with four distinct groups i.e. Dukes stages A, B, C and D. Therefore I recruited n=25 in each staging group giving 100 patients overall. However, a participant's tumour stage could not be pre-determined and accordingly, I aimed to recruit to excess, i.e. to circa 150, and ultimately towards correct staging ratios. Hence, far more CRC patients in hypothesis 1 were recruited, giving more power and confidence. It followed that with n=150, I would have the power to detect a correlation coefficient of 0.25, at $2p<0.05$ and 1-beta = 0.8. By definition, the disease stage progressed in a linear manner i.e. from good to bad. This therefore allowed further analysis by Altman's test of linear trend of ordered groups.

Hypothesis 3

This is a correlation matrix to determine the relationship of the plasma makers with CECs and EPCs of hypotheses 1 and 2 above.

Cross-sectional Analyses

Results were expressed as numbers and percentages, mean and standard deviation (SD) or median and interquartile range (IQR). Analyses between groups were performed using ANOVA, Kruskal Wallis, Mann-Whitney, t-test and Chi Square as appropriate. Correlations were by derived by Spearman's rank method or stepwise multiple regression. Analysis of variance (ANOVA) was inappropriate for changes with CRC stages as each were dependent on the previous in moving from one stage to the next. Altman's test of linear trend of ordered groups was applicable (380). A two-tailed p value < 0.05 was considered significant. Data was analysed by IBM SPSS Statistics software (2014) and figures drawn using Prism 6 package (2013).

2.3.2 Longitudinal Studies

Hypothesis 4

I predicted that treatments would reduce the CEC and EPC counts by a third. That is from a median (IQR) of 3 (1.5 – 5.0) cells/mL to 2 (0.5 – 3.5) cells/ml after treatment. If numbers of CECs fell in 21 patients, but rose in 4 patients (25 participants in total), then the calculated paired t test would give p=0.003 (verified by my supervisor, Dr Blann, a statistician with the University of Birmingham).

Hypothesis 5

To predict disease progression (death and/or recurrence and/or increase in disease size) all markers measured in the 150 CRC participants would have the power to detect a correlation coefficient of 0.25, at $2p<0.05$ and 1-beta = 0.8.

Data on outcomes was prospectively collected over the 2-year period after surgery. Details were obtained from hospital notes, clinic reviews, radiology reports, multidisciplinary outcomes and the Somerset Cancer Database. Follow-up was determined by local hospital policy and based on the guidelines of the ACPGBI (2007) (285). All patients were routinely discussed at the MDT meeting. Follow-up and further treatment were determined by histological prognostic factors and/or radiological evidence of disease spread (section 1.4). It was offered to all patients with primary CRC undergoing treatment with or without curative intent.

Follow-up after curative surgery typically began at 4–6 weeks after discharge from their operation. Patients undertook regular surveillance with (1) a minimum of two CTs of the chest, abdomen, and pelvis in the first 3 years, (2) regular serum carcinoembryonic antigen (CEA) tests at least 6 monthly in the first 3 years, and (3) surveillance colonoscopy was performed at 1 year after initial treatment. Investigations were restarted if there were clinical (e.g. weight loss, bowel obstruction masses, lymphadenopathy or hepatomegaly) or biochemical (rise in CEA) suspicion of recurrence. Contrast enhanced CT scan of the chest, abdomen and pelvis achieved confirmation of disease recurrence and/or metastases, if any. Indeterminate

lesions were further characterised by ultrasound +/- MRI +/- PET scans where appropriate. Biopsies were not routinely performed unless features on radiology were atypical or suspicious of primary tumours in other organs.

In considering the need for adjuvant treatment after potentially curative resection (i.e. no evidence of metastases), all patients were discussed at MDT. A follow-up enhanced CT scan was routinely performed 2-4 weeks after completing therapy. CT scans were performed during treatment if there was clinical or biological suspicion of disease progression, as described above.

After potentially curative surgery, indeterminate lesions on radiology i.e. of the abdomen (lymph nodes), liver or lungs on CT or MRI were typically less than 10 mm on size criteria. Patients were routinely followed-up at 3 months with further radiology (CT scan +/- MRI for liver or rectum) unless requested earlier by the MDT. Patients may or may not have received adjuvant therapy during this time. PET scans were not routinely performed unless the MDT decided further characterisation was necessary. For this study, lesions reported as indeterminate on repeat imaging (unchanged or absent) were not classed as ‘recurrent or metastatic’ disease.

For bowel resection (curative) and potentially resectable metastases to the liver +/- lung, and palliative surgery with non-resectable metastases, there was no set protocol for follow-up. They typically underwent chemotherapy (+/-radiotherapy), unless they declined and were followed-up at the discretion of the Oncologist.

Patients were monitored for disease progression/regression with regular clinic visits and definitively by contrast enhanced CT of chest, abdomen and pelvis.

Progression for this study was defined according to the widely accepted criteria of Eisenhauer et al (381, 382). It used ‘measurable’ evidence (X-rays, USS, CT, MRI, PET-CT or biopsy) of one of the following:

- Local recurrence or metastases after surgery with curative intent and/or
- For Dukes’ D, increasing tumour size of involved organs and/or new metastases, specifically of at least a 20% increase in the sum of the diameters of all lesions.

The temporal relationship with disease progression and survival was further analysed. The definitions accepted internationally on reporting outcome measures in cancer treatment (383-385) were:

Time to Progression **(TTP)**	Patients with evidence of disease spread to other organs and/or local recurrence (also known as disease free survival, DFS).
Progression Free Survival **(PFS)**	Patients that either had disease spread to other organs and/or local recurrence and/or or died.
Overall Survival **(OS)**	All cause mortality during or after treatment.

Longitudinal Analyses

Results were expressed as numbers and percentages, mean and standard deviation or median and interquartile range. Analyses between groups were performed using ANOVA, Kruskal-Wallis, Mann-Whitney, t-test and Chi-squared as appropriate. For hypothesis 4, data over time was analysed by repeated measures (two-way) analysis of variance (for parametric data) or by Friedman's method (for non-parametric data). For hypothesis 5, data was analysed by binary regression and Cox proportional hazards were estimated, and outcomes over time presented as Kaplan-Meier plots. All analyses were performed on IBM SPSS Software (2014) and figures produced by Prism 6 package (2013). A $p<0.05$ was taken to assume statistical significance.

Recapitulation

The introduction has set the scene and justified the project. The materials and methods have outlined the tools and plan of investigation. The section that follows will show the results and discussion of each of the study hypotheses.

Chapter 3

Clinical Studies

3.1 Cross-sectional Studies

3.1.1 Cellular markers in CRC versus controls

Abstract

Background: CECs and EPCs were elevated in metastatic CRC and other disorders, including cardiovascular disease. Both markers were hypothesised to be higher in CRC at all stages than in participants with benign colonic polyps (BD), stable coronary artery disease (SCAD) and healthy controls (HC).

Methods: Prospective study of 154 CRC participants, were compared with 26 BD, 33 SCAD, and 29 healthy age-matched controls. CECs (CD45-CD146+CD34+) and EPCs (CD45-CD34+KDR+) were enumerated by an improved 4-colour flow cytometry protocol.

Findings: The CEC and white cell counts (WCC) were higher in CRC versus BD and HC groups ($p<0.001$) but comparable to those with SCAD. EPCs ($p<0.001$) were elevated in CRC only and correlated to CECs (Spearman's $r=0.646$, $p<0.001$). On multivariate regression analysis only BMI influenced EPC levels in CRC ($p=0.03$). EPCs were higher in and hence predictive ($p<0.001$) of CRC compared to the controls, while CD34+CD45- cells were highest in SCAD ($p<0.0001$).

Conclusion: EPCs and CECs were important to the vascular biology of CRC. EPCs and CD34+CD45- cells, for unrecognised coronary artery disease, may be useful markers for clinical decisions in CRC management.

Background

CECs and EPCs were proposed as biomarkers to monitor response to treatment of metastatic CRC (28, 146, 201, 351). They were also altered in many diseases including cardiovascular disorders and other cancers (22, 92, 140, 386). For CRC, their value across all stages, performance against cardiovascular disease and differences with pre-cancerous disease, i.e. colonic polyps, were unknown. I hypothesised that levels were higher in CRC than healthy (HC) and benign polyp (BD) controls, but comparable to those with stable coronary artery disease (SCAD). The later was also used as a positive disease control.

Method

Participants were recruited as detailed in section 2.1 (page 94) and after applying the exclusion criteria of section 2.1.3 (page 94). CECs and EPCs were measured in peripheral blood by an improved 4-colour flow cytometry protocol (section 2.2.1, page 98). Pre-treatment levels were quantified in those with CRC (n=150) and compared to the 25 BD, 25 HC and 25 SCAD control groups.

Statistics

Results were expressed as numbers and percentages, mean and standard deviation (SD) or median and interquartile range (IQR). Analyses between groups were performed using ANOVA, Kruskal Wallis, Mann-Whitney, t-test and Chi Square as appropriate. Correlations were obtained by Spearman's rank method, and for the clinical variables, by stepwise multiple regression analyses. A two-tailed p value of <0.05 was considered statistically significant.

During the study period there were 335 patients diagnosed with CRC of which 10 declined to participate and 181 did not meet the inclusion/exclusion criteria. To ensure a target of 20 for each of the 4 Dukes' stages, I recruited 154 participants. Fewer were recruited for stage A (19) as compared to stages B, C and D (54, 49, 32 respectively). To obtain the 20 with stage A (equivalent to 1 in every 8 cancer recruited), at least 16 more participants were needed (as only 1 in 2.1 met the criteria) and therefore not achievable within the study's timeframe. For the controls, 109 candidates were approached, 5 declined and 16 did not meet the inclusion/exclusion criteria (12 of which were in the SCAD group). Furthermore, to ensure they were age-matched, I recruited 26 to benign disease, 29 to healthy and 33 to the SCAD control groups, exceeding the intended targets outlined above.

Results

The demographics of all participants are listed in Table 14, and unsurprisingly there were differences of the co-morbidities between groups. However altogether, they were matched for sex, age, blood pressure, family history and BMI.

Numbers of white blood cells, EPCs, CECs and the EPC/CEC ratio are shown in Table 15. CEC and EPC data was not normally distributed; hence non-parametric statistical tests (Kruskal-Wallis) were used to calculate the differences in median values. Other cell indices were normal distributed. Participants with CRC and SCAD had a higher white blood cell count (WCC) than other groups.

Patients with CRC and SCAD had comparable CEC counts though both were higher than in the other two groups. However, there were more EPCS in CRC than all other groups. It follows mathematically that the ratio of EPCs to CECs was highest in CRC.

Table 14: Demographics of patients with CRC versus control groups

	Colorectal Cancer [CRC]	Healthy Controls [HC]	Benign Controls* [BD]	Coronary Disease‡ [SCAD]	p
Number	154	29	26	33	-
Age (years)	73 (10)	70 (6)	70 (6)	71 (6)	0.683
Male (%)	86 (56)	15 (52)	13 (50)	18 (55)	0.616
Female (%)	78 (44)	14 (48)	13 (50)	15 (45)	
BMI (Kg/m^2)	27.4 (7)	25.9 (2)	25.8 (2)	27.1 (6)	0.434
Systolic BP (mmHg)	135 (16)	133 (9)	131 (9)	138 (11)	0.286
Diastolic BP (mmHg)	75 (10)	73 (8)	71 (7)	80 (9)	0.086
Family History (%)	26 (17)	3 (11)	2 (7)	5 (15)	0.145
Smokers (n (%))	49 (32)	5 (19)	7 (24)	0 (0)	**<0.001**[†]
Hypertension	83 (54)	0 (0)	0 (0)	16 (84)	**<0.001**
Previous MI/IHD	30 (19)	0 (0)	0 (0)	33 (100)	**<0.001**
Previous CVA	19 (12)	0 (0)	0 (0)	3 (9)	**<0.001**[c]
Hyperlipidemia	47 (31)	0 (0)	0 (0)	17 (52)	**<0.05**
Diabetes Mellitus	24 (16)	0 (0)	0 (0)	7 (21)	**<0.001**[d]
Heart Failure	10 (6)	0 (0)	0 (0)	2 (6)	**0.006**[h]

BP - Blood Pressure; BMI- Body Mass Index; CVA- cerebrovascular accident. MI- Myocardial Infarct; IHD- Ischaemic Heart Disease. * Benign controls: non-cancerous colonic polyps. ‡ Stable Coronary Artery Disease [SCAD]. Results were expressed as numbers and percentages, mean (SD) or median and interquartile range. ANOVA, Kruskal Wallis, Mann-Whitney and Chi Square were used as appropriate. † CRC vs HC p=0.208; CRC vs BD p=0.035 and SCAD vs all other groups p<0.001[d] CRC vs SCAD p=0.363. [h] CRC vs SCAD p=1.00. [c] CRC vs SCAD p=0.489.

Table 15: CECs, EPCs and their ratio in CRC versus controls

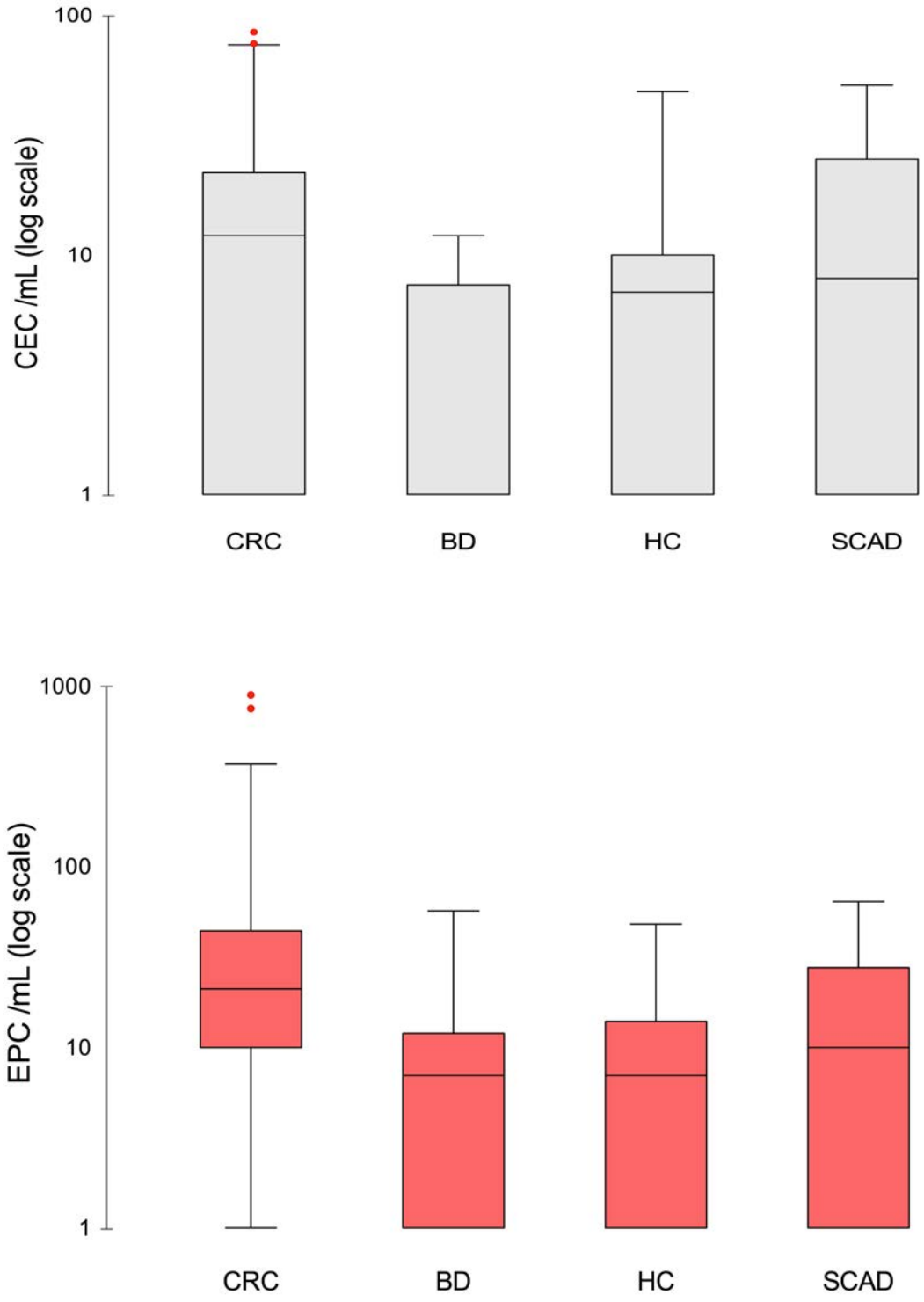
	Colorectal Cancer [CRC]	Healthy Controls [HC]	Benign Controls [BD]	Stable Coronary Artery Disease [SCAD]	p
Number (n)	154	29	26	33	
CECs (cells/ml)	12 (0-22)	7 (0-10)	0 (0-8)	8 (0-25)	**<0.001[a]**
EPCs (cells/ml)	21 (10-44)	7 (0-14)	7 (0-12)	10 (0-27)	**<0.001[b]**
EPC : CEC Ratio	1.4 (1.0-5.1)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-2.1)	**0.015[b]**
CD34+CD45- ($x10^3$ cells/ml)	0.80 (0.49-1.15)	0.95 (0.60-1.42)	1.05 (0.69-1.44)	1.56 (1.26-2.24)	**<0.001[C]**
WCC ($x10^6$ /ml)	7.17 (2)	5.73 (2)	5.76 (1)	6.38 (2)	**<0.001[a]**

Results were expressed as mean (SD) or median (interquartile range). Data was analysed by Kruskal-Wallis test, then log transformed for ANOVA and Tukey's post hoc test. [a] $p<0.05$ higher in CRC and SCAD than the other two groups, no difference between CRC and SCAD and no difference healthy controls to benign disease controls. [b] $p<0.05$ higher in CRC than all other groups, no difference between levels in the three other groups. [C] All CD34 positive monocytes were higher ($p<0.001$) in SCAD only.

Thus in testing hypothesis 1 there were:

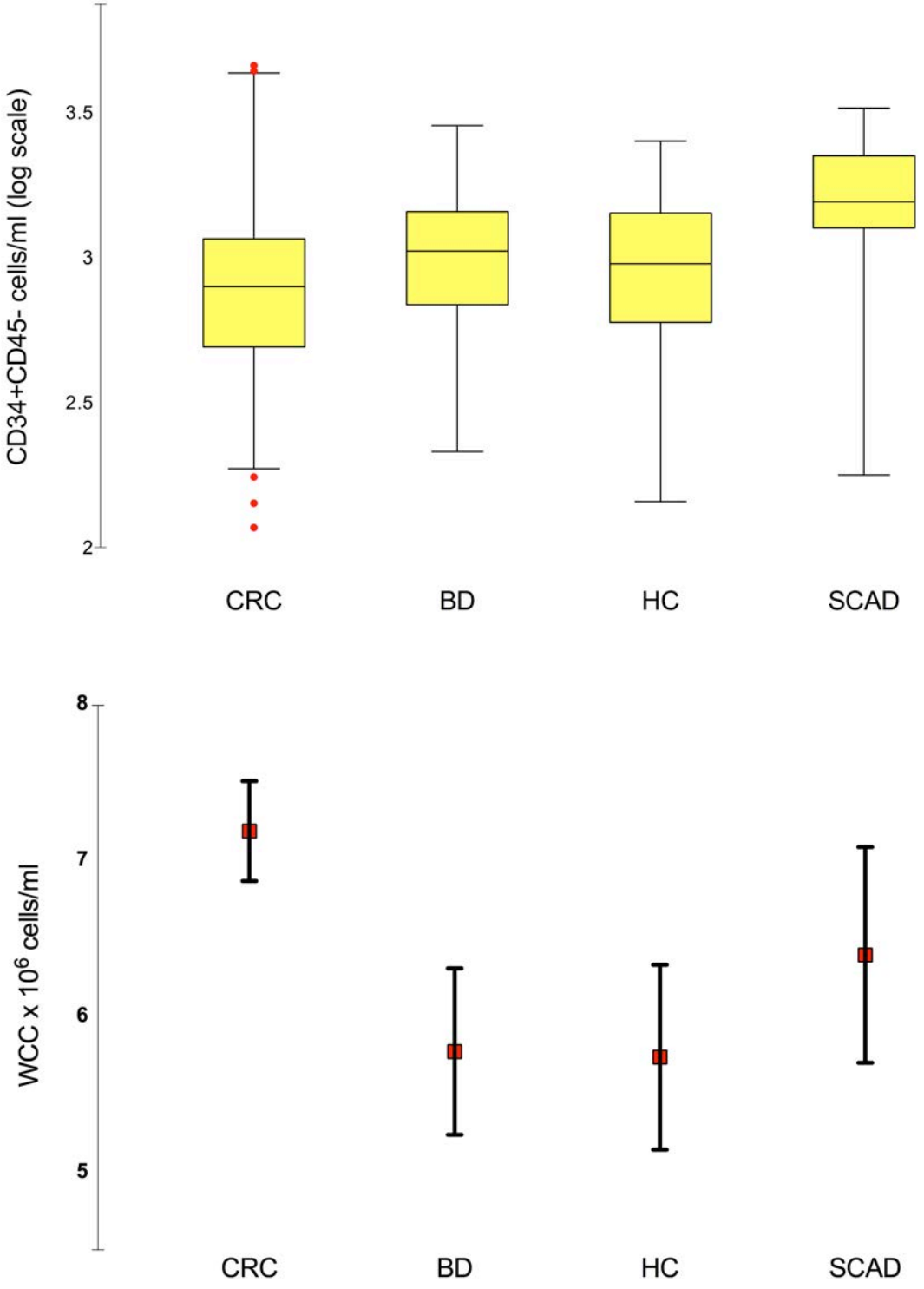
- raised CECs and WCC in CRC similar to levels in SCAD (figures 23 & 24)
- raised EPCs only in CRC (figure 23)
- EPC to CEC ratio was highest in CRC only (mathematically predictable)
- raised CD34+CD45- cells in SCAD only (figure 24).

Figure 23: Box (median with IQR) and whisker [2.5 to 97.5 percentile] plots of CECs (grey) and EPCs (red) in CRC versus controls



For both plots outliers are shown as red dots. CECs were higher in CRC and SCAD than the other two groups ($p<0.05$), with no differences of CRC with SCAD and BD with HC.

Figure 24: Box (median with IQR) and whisker [2.5 to 97.5 percentile] plot of CD34+CD45- cells (yellow) and mean WCC with 95% CI in CRC versus controls



Mean is shown as red box with 95% interval bar; box and whisker plots show outliers as red dots. For WCC $p<0.05$ in CRC and SCAD (but no difference between them) than the other two control groups. CPC was higher in SCAD only.

Correlations between cellular indices

While there was a correlation between CEC and EPC in SCAD (r=0.621, p<0.001), only the CRC group is reported as the low numbers in the other three groups gave considerable scope for type 1 and 2 statistical error, particularly with so many zero values. The sample size of n=154 gave the 1-beta power of 0.85 to detect at 2p<0.05 a correlation coefficient of 0.25 (387).

As EPC and CEC data were non-parametrically distributed, they were ranked by Spearman's method:

- EPC:CEC r=0.646, p<0.001
- CEC:WCC r=0.197, p=0.014
- EPC:WCC r=0.141, p=0.080

The EPC:CEC analysis was within power limits and therefore highly reliable (figure 25). However, CEC:WCC and EPC:WCC relationships had were correlated (r<0.25). The CD34+CD45- cells were strongly correlated to WCC (r=0.317, p<0.001, see figure 26), but not to EPCs (r=0.200, p=0.013) or CECs (0.152, p=0.062).

The study was powered to determine if clinical and demographic factors influenced the cell levels. A stepwise multivariate linear regression analysis was performed for CECs and EPCs versus the factors listed in Table 14 and are shown in Table 16.

Figure 25: Scatterplot showing the relationship between CECs and EPCs (log scales) for all CRC participants.

The mean is shown by the black line with 95% SE by grey dotted lines. Spearman's correlation was r=0.646, p<0.001.

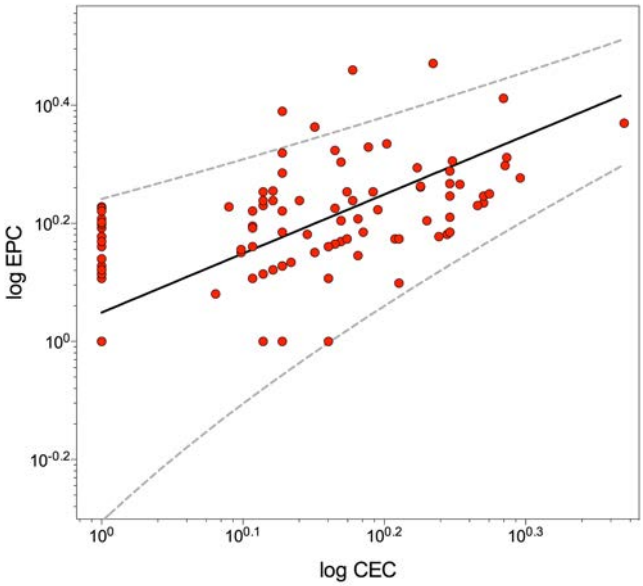


Figure 26: Scatterplot showing the relationship between WCC and CD34+CD45- cells for all CRC participants.

The mean is shown by the black line with 95% SE by grey dotted lines. Spearman's correlation was r=0.317, p<0.001.

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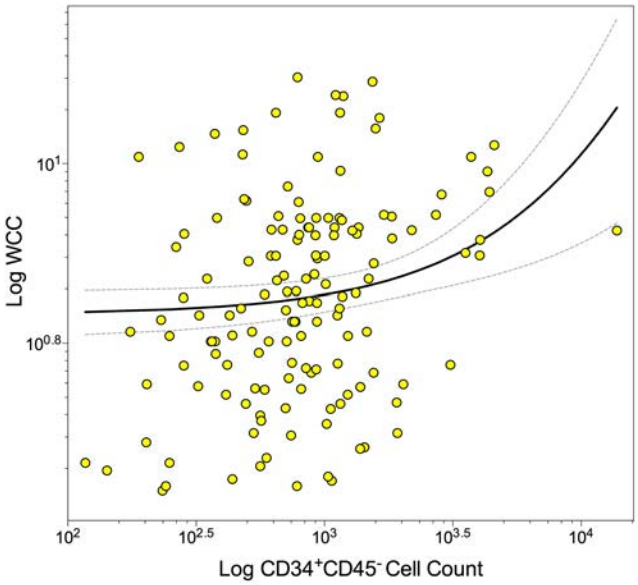


Table 16: Multiple regression analysis of the effect of clinical and demographic factors on CECs, EPCs, $CD34^+CD45^-$ cells and WCC.

	p values *			
Predictor	**CEC**	**EPC**	**$CD34^+45^-$**	**WCC**
Overall p value	0.293	0.482	0.790	0.241
Sex	0.482	0.405	0.510	0.334
Age	0.685	0.706	0.652	0.605
BMI	0.483	**0.030[a]**	0.580	0.770
Systolic BP	0.252	0.421	0.521	0.545
Diastolic BP	0.110	0.562	0.631	0.754
1st degree relative with CRC	0.127	0.213	0.541	0.324
Smoker	0.916	0.744	0.991	0.991
Hypertension	0.767	0.747	0.245	0.519
Ischaemic Heart Disease	0.326	0.365	0.278	**0.018[b]**
Previous TIA/stroke	0.287	0.307	0.340	0.104
Hyperlipidaemia	0.899	0.905	0.744	0.874
Diabetes Mellitus	0.767	0.676	0.335	0.422

* Analysis performed by stepwise multivariate linear regression. The Odds Ratio (95% CI) for BMI[a] was 1.8 (-0.4 to 2.5) and for IHD[b] 0.1 (-0.9 to 0.6).

Regression analysis showed no effects of age, sex, smoking, blood pressure or heart rate on either CEC or EPCs. Ischemic heart disease may have contributed to higher WCC (OR 0.1 with 95% CI -0.9 to 0.6, p=0.018), similar to the relatively higher levels seen in the SCAD group (table 19). No factors were identified as major influences on CECs (overall p= 0.293) or CD34+45- cells (overall p=0.790).

However, with the same analysis on EPCs in CRC, the participants' BMI (OR 1.8, 95% CI of -0.4 to 2.5, p=0.030) may have influenced the levels but overall not across the four groups (p=0.431). Once again, the relationship for the control groups could

not be examined, as they were not reliably powered (<35 per group). Next I examine the use of cellular markers as a test for CRC when compared to controls.

Cellular Markers as a test for CRC

All cellular markers reached significance on ROC analysis of above median levels in CRC when compared to that of the control groups (see table 17 & 18).

Table 17: ROC analysis of all cellular indices in CRC versus controls

	CRC vs ALL Controls		**CRC vs SCAD only [A]**	
	ROC area	p value	ROC area	p value
CEC	0.649 (0.579-0.719)	**0.0001**	0.551 (0.441-0.669)	0.362
EPC	0.708 (0.641-0.775)	**<0.0001**	0.646 (0.543-0.749)	**0.009**
CD34+45- cells	0.665 (0.594-0.737)	**<0.0001**	0.802* (0.720-0.884)	**<0.0001**
WCC	0.612 (0.504-0.719)	**0.044**	0.560 (0.441-0.679)	0.309

ROC area is shown as fraction with 95% Confidence Interval. [A] Comparisons were made with SCAD as a positive control group. * CD34+CD45- cells were more predictive of SCAD.

Table 18: Cellular indices in detecting CRC versus controls.

Marker	Level*	Sensitivity %	Specificity %	Likelihood Ratio
Cellular Markers in CRC versus ALL Controls				
CEC	>12 cells/ml	61.3	79.6	2.51
EPC	>21 cells/ml	53.2	72.7	2.05
CD34+CD45-	$<0.80x10^3$ cells/ml	26.4	50.0	1.50
WCC	$>7.17x10^6$ cells/ml	46.1	81.8	2.54
Cellular Markers in CRC versus SCAD only				
CEC	>12 cells/ml	44.1	63.6	1.41
EPC	>21 cells/ml	53.5	60.6	1.35
CD34+CD45-	$<0.80x10^3$ cells/ml	50.0	6.4	8.25
WCC	$>7.17x10^6$ cells/ml	46.1	69.7	1.46

* Above or below median (CEC, EPC, $CD34^+CD45^-$) and mean (WCC) levels.

Though the median EPC was higher in and therefore more predictive of CRC (ROC area 0.649, 95% CI of 0.641-0.775, p<0.001 in figure 27) than the controls it was not sensitive for the disease (for median value of >21 cells/ml, sensitivity and specificity were 53.3% and 72.4% respectively; see table 18). Remarkably CD34+CD45- cells were less in CRC (median 0.80, IQR 0.49-1.15 $x10^3$ cells) and a good predictor of SCAD (median 1.6, IQR 1.3-2.4 $x10^3$ cells) on ROC analysis (area 0.802, p<0.0001) with a sensitivity of 50% and specificity of 93.4% for $\geq 0.80x10^3$ cells/ml.

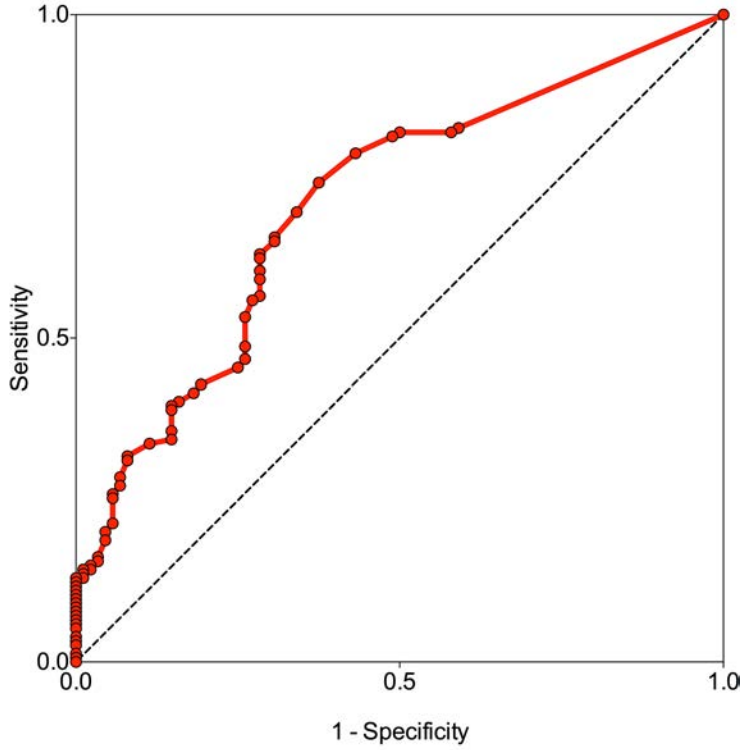
Figure 27: ROC Curve of EPCs in CRC versus all controls (in red) and CD34+CD45- cells in SCAD only (yellow).

EPCs in CRC versus ALL Controls

ROC Area=0.708
p<0.0001

<u>For EPCs > 21 cells/ml:</u>

Sensitivity= 53.2%
Specificity= 92.7%
LR= 2.05

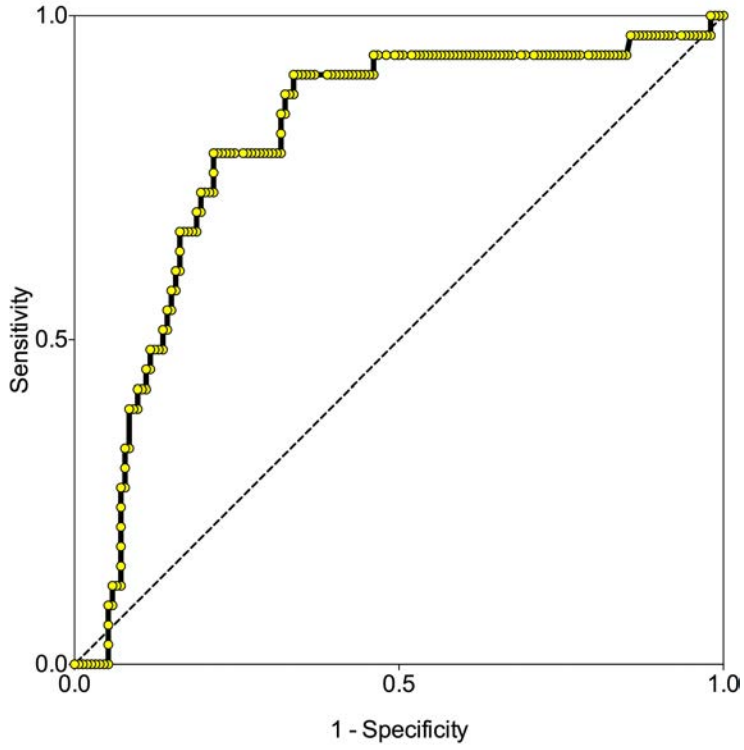


CD34+CD45- cells in SCAD versus CRC

ROC Area=0.802
p<0.0001

<u>For cells > 0.8 x 10^3/mL:</u>

Sensitivity= 50.0%
Specificity= 93.4%
LR= 8.25
[Good discriminator of SCAD]



Discussion

Validation of the FC Protocol

The preferred technique of 4-colour flow cytometry was used for multi-parametric detection of CECs and EPCs in the monocyte fraction of PB. While most agree to this approach, there was no consensus on definitions for CECs or EPCs. There were also agreements that, while CECs reported over the years may reflect a diverse population of cells given the variations in phenotypes, most express $CD45^-$, $CD34^+$, $CD31^+$, $CD146^+$, and $CD133^-$ (19, 388, 389). They were found in the $CD34^+$ gated fraction on FC. For this study CECs were defined by the expression $CD45^-$, $CD34^+$ and $CD146^+$. That is, CD45 negativity excluded leukocytes and progenitors, CD146 was heavily expressed on mature ECs (rarely on mature progenitor cells) and CD34 was found on most ECs in vivo (17, 139, 197, 390). There were no reports that detecting the presence or absence of more than 3 phenotypes improved the assays ability to detect these rare cells and differentiate them from other non-EC monocytes. Identification by FC with the chosen antigens with was validated by my colleague (21) against immunomagnetic separation (IMS), the previously preferred but laborious technique of quantification.

Having filled three channels ($CD45^-$, $CD34^+$ and $CD146^+$), the fourth was labelled for VEGFR-2 (CD309, also known as KDR) events and hence simultaneously detected EPCs ($CD45^-/CD34^+/CD309^+$, see below). This approach excluded non-EC $CD34^+$ cells such as small population of progenitor and stem cells (both of which also heavily express CD133). CD 31 was excluded as its immuno-fluorescent antibody

bound to the majority of leukocytes, platelets and haematopoietic progenitors (391). Also, a small number of in vitro cancer EC lines do not express CD31 or CD105 (339, 392). The protocol detected similarly accepted levels of CECs in healthy participants (median 7, IQR 0-10) and those with SCAD (median 8, IQR 0-25) (24, 91). This contrasted with the results of Mariucci et al (393) of more than 100 CECs per ml in healthy subjects by a similar protocol and higher than levels detected by IMS. They did not report on the assay variability or attempts to avoid false positives from large platelets proposed by Strijbos et al (394).

EPCs probably represent a heterogeneous group of cells. Popular markers, as outlined in section 1.2, included CD34, CD105, CD133, CD309, CD144, CD 146, and CD45 (dim). While the ideal phenotype may appear questionable, the choice was reasonably straightforward. Firstly a minority express CD146, counter to the argument of its exclusivity to mature ECs (20, 197). Secondly, CD133 expression was lost with maturation and most importantly its function was largely unknown. It was also found on haematopoietic and other progenitors, thus raising the possibility that non-EC stem cells were detected in error by other authors (20, 395, 396). Therefore EPC was defined as $CD45^-$/ $CD34^+$ /KDR^+ since KDR, though initially low in $CD45^-/CD133^+/CD34^+$ cells, is up-regulated and CD133 down-regulated as the cell matures. I would agree with other authors that CD133 positive cells were best described as forerunners to the EPC or circulating progenitor cell, [CPC] (24, 336, 343). The CD105 marker was not chosen as it was up-regulated by angiogenic ECs and may therefore be found on CECs released from the rapid vascular turnover of tumours (397). That is, with 3 channels already occupied for CEC detection ($CD45^-$, $CD34^+$ and $CD146^+$) by FC the fourth identified KDR expression for EPC

quantification. My colleagues used a similar strategy to detect CECs and EPCs in prostate cancer and from this I developed my assay (140). Though there were no significant differences with healthy controls, curiously they reported much higher than expected levels of both cells (median [IQR] of 28 [11-43] and 32 [18-82] respectively) compared to other published studies (17, 24). Having examined this protocol in detail I suspected the gating strategy did not differentiate entirely between the two cell populations or 'double-counted' i.e. some EPCs were counted as CECs, hence the higher than expected levels reported.

To improve the assay's ability to differentiate between CECs and EPCs pro-angiogenic HUVECs (expressing CD34 and KDR but not CD146 and CD45) was used with an antibody minus 1 protocol to reduce the false positive events of the gating strategy. The preparation protocol was maintained and tested on blood from 7 participants with Dukes' D stage not undergoing chemotherapy, assuming that both CECs and EPCs would be high. That is, processing a 0.2 ml sample of EDTA blood within 2 hours of collection by the following 5 sequential steps:

1. Incubation with flourochromes,
2. Incubation with lysing/fixative solution,
3. Centrifugation and decanting of supernatant,
4. Re-suspension with PBS, centrifugation and decanting of supernatant,
5. Re-suspension in PBS ready for FC.

This 'reduced' the median [IQR] levels of CECs and EPCs of healthy participants from 28 [11-43] and 32 [18-82] respectively to 0 [0-8] and 7 [0-12] respectively in my

study. The CEC levels were in keeping with quantification results by IMS. It was difficult to establish a ‘true’ level of EPCs in healthy controls as numerous cellular definitions existed and hence varying numbers were reported.

I was not able to reproduce the inter- and intra-assay variability of below 17 % of Widemann et al and my colleagues (24, 140, 398). I believe the protocol to be robust and the high coefficients of variability of 17 to 39% to reflect the difficulties in detecting extremely rare events. Comparisons with other studies were generally inconclusive as results were expressed as means with standard deviation (though the data was typically non-parametric) or events only, rather than cells per ml, the units chosen for my study. Other studies, including Mancuso et al, expressed their results in cells or events per µl, which translated to less than 1 cell per millilitre even when expected to be well above this in cancer (399).

There may be other potential causes for the differences in numbers reported:

1. Quantity of blood analysed: that is the more blood analysed the more likely rare events were detected. Mancuso et al and Furstenberger et al analysed more than 2 mls of blood and reported observer variability under 16%. My colleagues, who also analysed 0.2 ml of blood, also had lower variability, which probably resulted from the higher than expected levels detected (140), as already discussed above. Simply, the more cells to be found the smaller the inter- and intra- observer variability. I therefore determined the effect of volume and found no differences after analysing 0.2, 0.4, 0.6 or 1.0 ml of blood from healthy participants. It certainly took longer to analyse a larger sample volume (approximately 5.6 minutes for every 0.1ml) by FC. Furthermore, I found no

significant difference between the volume of the sample analysed and a detection limit of 1 million total events.

2. Sampling error, as proven by Goon et al, specifically traumatic blood letting, may displace ECs from veins and into the sample collected (21). To avoid this one observer drew all samples and all traumatic ones (which occurred on 5 occasions only) were discarded and repeated 24 hours after initial sampling.
3. Mancuso et al suggested that the blood-lysis step with or without fixative might destroy other circulating ECs, though they reported much lower than expected levels in healthy participants after omitting this step (399). I was unaware of any other study on CECs and EPCs to support this argument. Blood lysis is an important step to reduce the scattering of the laser light by haemoglobin molecules during FC. Recently, questions were raised on the reagent FACS Lyse, as used in my study (400). The report concluded that, when compared to other agents, it significantly reduced the number of CD45+ CD34+ blast cells detected by standard FC protocol. The reasons for this were unclear.
4. Wash procedures may dispense with rare cells, but once again to my knowledge had never been demonstrated in CEC and EPC enumeration. To reduce variability all samples were analysed within 4 hours, blood was drawn from no more than 5 participants at any given time (maximum number that could be processed without breaching the 4 hour target) and strict adherence to the protocol was maintained to minimise the potential errors above.

In summary the multi-parameter FC protocol, through careful assay reproduction, had acceptable intra-assay variability (all specimens processed by one user only) and was therefore robust at identifying and discriminating between EPCs and CECs.

CECs and EPCs in CRC versus control groups

I found higher levels of both EPCs and CECs in CRC compared to control groups except CEC, which alongside the WCC, was comparable to the SCAD group. As discussed in section 1.2 disturbances in CEC and EPC levels in PB were well known in SCAD (91, 401) but less so in CRC, isolated typically to metastatic disease only (197, 402). The data of Boos et al on acute coronary syndrome and endothelial dysfunction showed similar CEC levels by the immune-magnetic separation (CD146 beads) technique in both healthy and SCAD participants, the latter used specifically as a disease control (91). Having applied similar criteria for recruitment I found slightly more CECs in my SCAD group ($p<0.05$) than that of Boos et al. The result probably reflected the higher accuracy of enumeration by FC against the IMS method of counting beads under microscopy and extrapolating to a wider field. Notably the participants in my study were on average older by 10 years but there was no published data to support a theory that CEC levels, unlike EPCs, increased with age. Alternatively, patients with on-going EC damage were unintentionally included.

Comparing levels of EPC in SCAD was less straightforward. Typically EPCs were elevated in the presence of acute vascular injury e.g. acute coronary syndrome or unstable angina (403, 404). In SCAD levels were usually at baseline similar to that of healthy patients (386). This was certainly the case in my study. Pellicia et al recently challenged the limited role of EPCs postulated by Werner and proved that higher tertiles of EPCs were predictive of further cardiovascular events (401). Their study, using my chosen definition for EPC by FC detection, expressed levels as percentages of the mononuclear fraction and therefore comparisons with my study

(units of cells/ml) were limited. Arguably there were more exclusion criteria to my study, but a number of factors may affect EPC levels in both studies including aging, smoking, dyslipidemia, hypertension and end stage renal failure (405-409).

Rowand et al, in validating an automated IMS CD146 technique, and Beerepoot et al respectively found 5 and 3 times as many CECs in progressive tumours (mCRC included) compared with healthy participants (143, 146). Much attention has been given to the value of the changes in CEC and EPC levels in monitoring the response to, progression with and survival from chemotherapy for mCRC (410). However, at the time of submitting this thesis there were no studies on their role in predicting the stage, progression and survival of CRC before treatment. Furthermore, after a thorough literature search, this was the first to compare their levels in CRC (regardless of stage) with that of SCAD. They both share some risk factors (e.g. smoking, diabetes mellitus), which may contribute to the disturbances of both cellular markers though, as discussed later, were not significant after stepwise linear regression. While both conditions and their risk factors may occur concurrently in some patients, there were no risk associations between them and when compared to the general population (411). Disturbances of the markers were known in both diseases of similar age groups. However, I found fifty percent more CECs and twice as many EPCs ($p<0.001$ for both) in CRC, and hence both, though not diagnostic, were potentially valuable markers for CRC prognostication.

Another first was the analysis of these markers in benign colonic adenomas (BD), or the ‘pre-cancerous’ group as per the adenoma-carcinoma sequence hypothesis (412). The question asked by its inclusion and comparison with CRC was whether

circulating factors of EC activity were altered before malignant transformation. As predicted and on the basis that adenomas (identified histologically by incomplete cellular differentiation) were slow-growing benign neoplasias with low angiogenesis activity (413), levels were similar to that of the HC group (median [IQR] CECs of 7 [0-10] and EPCs of 7 [0-14] cells/ml). This difference supported the theory by Goon et al that tumour invasion alongside angiogenesis increased CECs (92). The adenoma to carcinoma transformation is a slow process found in polyps more than 1cm in size (only 10% versus 1% of those less than 1 cm) with substantial villous changes (>25% of the architecture) and highly dysplastic changes; typically this was less than 10% of all polyps (414). Ideally adenomas with high-grade dysplasia without invasion (the feature that distinguished them from cancer) should be considered truly 'pre-cancerous' i.e. the step before malignant transformation. However such polyps were rare. In fact there were only three potential recruits with highly dysplastic polyps during the 18-month study period; as is often the case, the invasion status was uncertain, probably detected in 'malignant transformation' but not truly malignant by histological characteristics and were therefore excluded. To collect a well powered group would require at least a 5 to 10-year study, and as there was no published data of circulating markers of EC activity in this group (probably reflecting the difficulties with recruitment), levels were likely to be similar to the BD or HC group.

The WCC in CRC and SCAD, though within normal limits and of similar levels, was higher than that of HC and BD participants ($p<0.001$). Elevated inflammatory markers such as WCC, monocytes and neutrophil-lymphocyte ratios have long been described in both conditions, but their predictive value in disease progression and mortality remained inconclusive (415, 416). There were no other reports of WCC in

SCAD exceeding that of healthy controls. In a Japanese cohort by Takeda et al of SCAD versus acute coronary syndrome, WCC were comparable to the levels found in this study but not dissimilar to their healthy age-matched controls (417). This may reflect population differences of WCC, variations to the definition and/or inclusion criteria for this group and/or the inclusion in my study of participants with unrecognised unstable coronary artery disease with EC dysfunction.

Differences in the levels of all $CD34^{+}$ cells were also found but their role in both health and disease was uncertain. Asahara first reported that progenitors expressing CD34 and KDR (CD309) differentiated into ECs and from which the theory of postnatal vasculogenesis was hypothesised (147). However there may be others roles of progenitors in angiogenesis than simply ‘adding’ to the endothelial layer. Yoder et al examined by clonal analysis the proliferation and differentiation outcomes of monocytes from peripheral blood and found not all subsets of EC-antigen expressing cells formed vessels (418). These were dubbed endothelial colony forming cells (ECFC), which did not mature into ECs and appeared to promote maturation of other similar monocytes (called colony-forming units of EC type or CFU-EC) into ECs. A recent well-conducted study by Bellows et al suggested that such cells expressing CD34 but not CD45 and CD31 were elevated in CRC; the authors called these mesenchymal stromal cells [MSC] (419). In brief MSCs are cells derived from the embryonic mesoderm with high capacity for cloning and, when cultured in the laboratory, can differentiate into muscle, cartilage, adipose and fibroblast cells (420). This versatility is currently being exploited extensively for tissue regeneration towards autologous organ replacement (421). The flaw with the assumption of Bellows et al was that MSCs, for which there is no single marker of

identification, arguably do not express CD34. Asahara et al referred to Yoder's ECFC as non-haematopoietic EPCs, and although not examined for CD34 expression, were not derived from HSCs and were therefore from an alternative source (147). Nevertheless, I support the theory of Bellows et al of a substantive role of CD34 expressing cells in tumour angiogenesis, but suggest a similar function to ECFCs (if not the same subtype) of promoting differentiation of EPCs into ECs.

In a comprehensive review by Sydney et al (120) a compelling argument for the expression of CD34 expression on all progenitors, including those of MSC origin, was given. They cited Ferraro's work on highly proliferative colonies of MSCs expressing CD34 that eventually matured into fibroblasts at which stage CD34 was no longer expressed (422). Apart from ECs, CD34 was commonly used for haematopoietic stem cell identification but was also co-expressed by other normal cells (e.g. dermal dendrocytes, fibroblasts, interstitial cells of Cajal) and numerous tumours e.g. acute lymphoblastic leukemia, blood vessel tumour, thymoma (120, 423-425). The marker was linked to a number of normal cellular activities mainly modulating adhesion, proliferation and differentiation (426-428). On this basis I propose that progenitor mononuclear cells in PB that express CD34, have other non-EC mature endpoints in tumours, but further clarification on the diversity of this population by co-expression of other mesenchymal markers required a more in-depth study.

Cohen et al reported the clinical and genetic features of CD34+ cells (which they called progenitor cells) in the large community-based healthy population of the Framingham Heart Study (429). While they may have included all positive events, e.g. CD34+ microparticles and therefore did not specifically identify CD34+CD45-

mononuclear cells (as CD45 flourochromes were not used), an inverse relationship to older age, smoking and female sex was reported. A positive association was seen with weight, cholesterol levels and the use of statins. Risk factors alone may account the significantly higher levels of CD34+CD45- cells in SCAD than in all other groups in my study as all patients had hypercholesterolemia, though historically were well controlled at the time of sampling their blood.

While some $CD34^{+}CD45^{-}$ circulating progenitors eventually become EPCs (i.e. some were 'immature' EPCs) there numbers were quite considerable in all groups of my study (1-2 x 10^{-3} per ml) and their eventual identity and function at maturity cannot be clearly ascertained by the published literature. Yoder et al suggested only 2 possible outcomes (CFU-EC and ECFC) but to date there was no assay to separate CD45- cells from the CD34 fraction of monocytes. Nevertheless, in my study there were higher numbers of $CD34^{+}CD45^{-}$ cells ($p<0.005$) in SCAD but less so in CRC ($p>0.05$) compared to other groups, including healthy controls. Bellows et al found much higher levels in CRC than healthy patients, though they compared a smaller cohort of 45 participants of an average age of 54 years (versus 154 with average age of 72 years in my study) to 26 younger participants and were therefore not matched for age. Cangiano et al reported much higher levels of these cells over several days after acute myocardial ischemia, necrosis and revascularisation with angioplasty (430). Once again, as with many other studies, a comparison with my study was difficult as they reported proportions of MCs and not numbers relative to blood volume measured (cells/ml). However, there were no differences of these cells in their control group of SCAD (by definition similar to my group) to that of their healthy participants. This raised the question once again as to whether patients in my SCAD

group where truly ‘stable’ i.e. some may have on-going ischemia. The smaller size of the HC and BD groups may account for the slightly higher levels but the IQRs were quite similar to the CRC group and therefore adequately powered. Assuming I have not unknowingly committed a type 1 error, I theorised differing roles of these cells, mainly regeneration of the ‘dysfunctional’ vasculature in SCAD versus modulation (mainly promotion) of neo-angiogenesis and/or cellular proliferation in cancer.

My study was the first to report a correlation of $CD34^{+}CD45^{-}$ cells with the WCC ($r=0.314$, $p<0.001$), and therefore a potential role in tumour growth as well as angiogenesis, rather than purely a mathematical relationship. Neoplastic proliferation is dependent on inflammatory cytokines by immune cells e.g. monocyte derived macrophages or dendritic cells, NK cells from lymphocytes and mast cells from eosinophils (431). Though not within the remit of this thesis, the correlation suggested immune cells in CRC may stimulate (by cytokines) the increase in $CD34^{+}CD45^{-}$ cells which, as proposed by Bellows et al, may well be MSCs that eventually become mesenchymal cells e.g. fibroblasts, within the tumour. However further in vitro clarification is required.

Correlations between levels of CEC and EPC, as found in my study ($r=0.646$, $p<0.001$), have also been reported in breast and prostate cancer (24, 140). The authors inferred that the rise of CECs from tumour invading into blood vessel created a void subsequently replaced by EPCs. In its simplest form it would account for the correlations but a more complex mechanism driven by the need for angiogenesis within a hypoxic tumour environment may offer a better explanation. This is

discussed in more detail in sections 2.5.2 and 2.5.3 on the relationships with disease stage and other plasma markers respectively.

A number of patient co-morbidities, mainly linked to vascular insult/injury were shown to alter levels of both EPCs and CECs. They include unstable angina, MI, rheumatoid arthritis, uncontrolled hypertension, and smoking (104, 161, 162, 432, 433). However in my study, after multivariate stepwise and univariate linear regression analysis, only BMI was linked to levels of EPCs in CRC. This was the first result of its kind and no plausible reason was found, as reports generally on obesity and EPCs were conflicting. Müller-Ehmse et al suggested reduced numbers and function of these cells predisposed to cardiovascular disease (434). Graziani et al however found that morbidly obese patients with higher EPCs (as defined in my study) and without insulin resistance were protected against atherosclerosis (435). It was possible that within my study there was more diversity to the $CD34^{+}CD45^{-}KDR^{+}$ cell fraction in CRC patients with a BMI greater than 29. Bellows et al found that patients with a BMI greater than thirty had significantly higher levels of circulating cells expressing $CD34^{+}CD45^{-}CD31^{-}$ (MSCs) but not cells of $CD34^{+}CD45^{-}CD31^{+}$ (CECs) and $CD34^{+}CD45^{dim}CD31^{dim}$ (CPCs, which they called ‘EPCs’) phenotypes (419).

As a study extracting endothelioid phenotypes from CD34+ circulating cells, there were two significant findings, which to my knowledge were not previously reported. Firstly, of all the cellular markers measured in all groups, EPCs were higher in and therefore more predictive ($p<0.001$ on ROC analysis) of CRC compared to the controls, including SCAD, the positive control. This suggested that EPCs might have roles in repair and angiogenesis other than remodelling the architecture of the inner

layer of blood vessels. While there may be limited clinical application, measuring their levels may offer a laboratory method of discriminating EC activity between the two conditions in experimental models of vascular biology. More importantly there may be a clinical role for CD34+CD45- cells to detect the presence of un-recognised coronary disease in CRC patients. That is, levels of these cells may help to risk stratify CRC patients with concurrent but unrecognised cardiovascular disease whose extensive treatment may include potentially harmful general anaesthesia (i.e. bowel resection) and /or cardiotoxic chemotherapy (e.g. oxiloplatin and Bevacuximab). However, further characterisation of this risk was beyond this thesis.

In summary, significantly higher levels of CECs and EPCs were found in CRC compared with controls except CECs, which, alongside WCC were comparable to the SCAD group. Higher EPCs, in discriminating between CRC and SCAD, may have practical applications in experimental models. Higher CD34+CD45- cells may be useful in identifying CRC patients with unrecognised coronary artery disease.

The relationship of the cellular markers and the CRC stage is explored next.

3.1.2 Cellular markers in staging CRC

Abstract

Background: CECs and EPCs were suggested as biomarkers for monitoring treatment of metastatic CRC but little was known of their changes with each stage. Levels were hypothesized to be associated with the CRC stage.

Methods: CECs (CD45-CD146+CD34+) and EPCs (CD45-CD34+KDR+) were prospectively enumerated by 4-colour flow cytometry in 154 CRC participants. The stage was determined from the reports of the hospital's histopathology and radiology departments, and confirmed at multidisciplinary team meetings. Reports were generated as per the guidelines of the Royal College of Pathologists (second edition) and Royal College of Radiologists (first edition).

Findings: Both CECs and EPCs independently increased in a linear trend with Dukes' stage ($P<0.05$) and a modified AJCC, but were not significant when adjusted for each other. There was also a linear trend ($p<0.05$) of EPCs with tumour differentiation.

Conclusion: EPCs and CECs were important to the vascular biology of CRC and its stage. This may have implications for predicting disease outcomes.

Background

CECs and EPCs were proposed as biomarkers to monitor response to treatment of metastatic CRC (28, 146, 201, 351) but little is known of their clinical value in staging. I hypothesized that levels of these cellular markers were associated with, and therefore, informative of the cancer's stage before surgery.

Methods

The subjects and methodology were similar to that described for testing hypothesis 1 in section 3.1.1. Colorectal cancer (n=154) participants were recruited (as detailed in section 2.1, page 94) and pre-treatment cellular markers were measured in peripheral blood by FC (section 2.2.2, page 98). Histology was reported in accordance with the minimum dataset guidelines of the Royal College of Pathologists on reporting CRC [see appendix 2, page 252] (285, 373). Radiology was guided by the standards of the Royal College of Radiologists, first edition (374). Stage was obtained from the reports of the hospital's relevant departments, and confirmed at the multi-disciplinary meetings.

Statistics

This was effectively the same sample size estimation as for hypothesis 1 with four distinct groups i.e. Dukes stages A, B, C and D. Therefore 25 participants for each

stage were recruited for total of 100. However, tumour stage could not be pre-determined without resection. Accordingly, I aimed to recruit to excess, i.e. to circa 150, and ultimately towards correct staging ratios. Hence, far more CRC patients in hypothesis 1 were recruited, giving more power and confidence. It followed that with n=150, the study was powered to detect a correlation coefficient of 0.25, at $2p<0.05$ and 1-beta = 0.8. By definition, the disease stage progressed in a stepwise manner i.e. from good to bad. Analysis of variance (ANOVA) was inappropriate as the groups were not independent and Altman's test of linear trend of ordered groups was applicable (380). That is, the Dukes' system is of an ordered linear trend: stage B is worse than stage A, C is worse than B and D is worse than C.

Results

There was a statistically significant upward trend for CECs and EPCs with advancing Dukes' stage (see table 19, Figures 28). Despite the clear rise in WCC with Dukes' stage, this was not significant. The better marker of advanced stage was determined by ordinal regression analysis (see Table 20). Although both CECs and EPCs independently increased with CRC stage, neither was significant when adjusted for each other. However given the close mathematical relationship between CECs and EPCs i.e. $r=0.646$, $p<0.001$, one may not perform better than other (see figure 25).

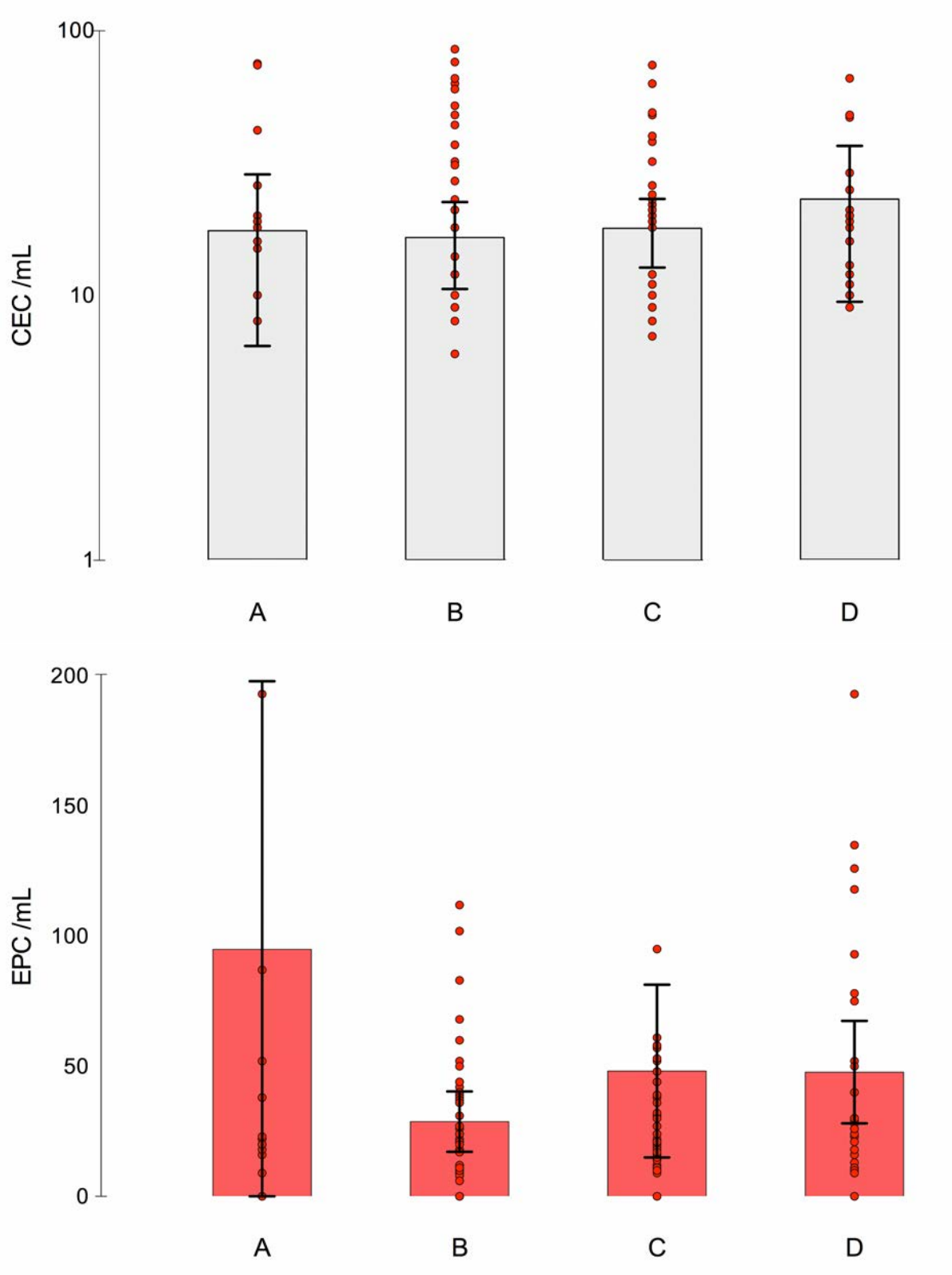
Table 19: CEC and EPC counts and Dukes' Stage

	Dukes' A	Dukes' B	Dukes' C	Dukes' D	P (trend)
Number (n)	19	54	49	32	
Age (years)	73 (11)	74(10)	72 (9)	71 (9)	n.a.
Male (n,%)	11 (58)	26 (50)	23 (49)	22 (71)	0.170*
Female (n,%)	8 (42)	26 (50)	24 (51)	9 (29)	
CECs (cells/ml)	10 (0-18)	12 (2-23)	11 (0-22)	20 (10-29)	**<0.05**
EPCs (cells/ml)	20 (0-26)	20 (11-37)	21 (10-44)	30 (20-93)	**<0.01**
WCCs (10^6/ml)	6.79 (1.59)	6.93 (2.06)	7.41 (1.85)	7.44 (2.33)	<0.1
CEC: EPC Ratio	0.9 (0.8-1.1)	1.0 (0.8-1.0)	1.0 (0-2.8)	0.9 (0.9-1.0)	n.a.
CD 34^+CD45^- cells*	0.9 (0.7-1.2)	0.7 (0.4-1.0)	0.8 (0.5-0.8)	0.8 (0.4-1.4)	n.a.

Results are expressed as numbers and percentages, mean (SD) or median (interquartile range). P (trend) values from Altman's linear trend of ordered groups. n.a. = not appropriate as no clear linear trend, so analysis not attempted. *Chi Squared test. * $CD34^+CD45^-$ cells x 10^3/ml.

The linear trends of CECs and EPCs are illustrated in figure 28 below.

Figures 28: Linear trend of CECs, EPCs and Dukes' stage (p<0.05)



For both figures data points are shown as red dots, mean as block bars and 95% CI interval bars.

Table 20: Ordinal regression – Which marker(s) related better to Dukes' stage?

Factor	Odds Ratio	P value
CEC	1.01	0.275
EPC	1.00	0.368
CD34+CD45- cells	1.00	0.233
WCC	1.34	0.093

Model fit: Chi-Square=5.286, p=0.259; Pearson's Goodness-of-fit p=0.420

Next I determined if other classifications, with modification, showed any correlations.

Modified Dukes' classification

I examined other models that may offer better discriminators of disease severity and/or prognosis. Using the same principles above, I tested the hypothesis that CECs and EPCs performed better when the Dukes' stages were modified into 3 groups: bowel wall involvement (Dukes' A+B), spread to lymph nodes (Dukes' C) and spread to distant organs (Dukes' D). Unlike Dukes' stage, this reclassification did not reveal any significant ordinal or linear trend (table 21).

Table 21: CEC and EPC counts according to lymphatic and metastatic spread.

	Localised disease & no spread to lymph nodes Dukes' A+B	Localised disease with spread to lymph nodes Dukes' C	Metastasis to other distant organs Dukes' D	P of linear trend
Number (n)	73	49	32	-
Age (years)	73 (10)	72 (9)	71 (10)	n.a
Male (%)	38 (52)	29 (58)	15 (68)	0.410*
Female (%)	35 (48)	23 (42)	7 (32)	
CEC /mL	11.5 (0-21)	14 (0-22.5)	11 (0-23)	n.a.
EPC /mL	20 (9-43)	21 (11-40)	28.5 (12-47)	>0.2
WCC $x10^6$ cells/mL	7.0 (2.1)	7.3 (1.8)	7.3 (2.0)	n.a.
EPC:CEC ratio	1.1 (0.5 – 5.9)	1.14 (0.7 – 4.0)	1.75 (0.6 – 5.5)	>0.1
CD 34^+CD46^- cells[A]	0.8 (0.5-1.1)	0.9 (0.7-1.2)	0.8 (0.4-1.4)	

Results are expressed as numbers, percentages, mean (SD) or median (interquartile range).
P values from Altman's linear trend of ordered groups. n.a. = not appropriate as no clear linear trend.
*Chi Squared test. [A] $CD34^+$ cells x 10^3/ml.. No differences on ordinal regression analysis (not shown).

Localised disease with of lymph node spread (Dukes' A,B&C) was examined against metastatic disease (Dukes' D). Once again there were no differences by either comparative analysis or ordinal regression. That is, the cellular indices were not good discriminators of distant spread (see table 22 below).

Table 22: Cellular markers in localised versus distant organ spread in CRC.

	Localised disease Dukes' A+B+C	Metastasis Dukes' D	p-value	Ordinal Regression
Number (n)	122	32	-	-
Age (years)	72 (10)	71 (10)	0.920	0.480
Male (%)	67 (52)	15 (68)	0.410	0.130
Female (%)	58(48)	7 (32)		
CEC /mL	12 (0-21)	11 (0-23)	0.392	0.148
EPC /mL	20 (9-43)	29 (12-47)	0.966	0.440
EPC:CEC ratio	1.13 (0.5 – 5.7)	1.75 (0.6 – 5.5)	0.774	0.236
CD 34^+ CD45- cells/ml	0.50 (0.79-1.11)	0.80 (0.39-1.38)	0.529	0.925
WCC $x10^6$ cells/mL	7.11 (1.95)	7.43 (2.23)	0.405	0.450

Results are expressed as numbers, percentages, mean (SD) or median (interquartile range).
P values from Chi Squared or t-test; ordinal regression model fit was acceptable at p=0.608 with Pearson's Goodness-of-Fit of p=0.243.

The AJCC system was also modified; otherwise ten sub-stages would under power the analyses. Therefore tumours with poorer prognoses i.e. less than 50% 5-year survival were pooled together (see table 13). That is, sub-stage T4 N2 M0 of stage IIIC (27-47% 5-year survival) was pooled with stage IV (8% 5-year survival). This gave the following groups n= 19 (AJCC I), 54 (AJCC IIA, IIB), 45 (AJCC IIIC) and 36 (AJCC IIIC & IV). The results of the markers are outlined in table 23 below.

Table 23: CEC and EPC counts according to modified AJCC Classification

	Modified AJCC Stages & expected 5-year survival				
	I 97%	**IIA+IIB 72-88%**	**IIIA+IIIB+IIIC‡ 69-88%**	**IIIC* + IV 8-47%**	**p (trend)**
Number (n)	19	54	45	36	
Age (years)	72 (11)	73 (10)	72 (9)	73 (9)	n.a.
Male n (%) Female n (%)	12 (63) 7 (37)	26 (53) 23 (47)	21 (45) 26 (55)	27 (69) 12 (31)	0.123^
CECs (cells/ml)	10 (0-19)	10 (0-22)	11 (0-22)	19 (9-23)	**<0.05**
EPCs (cells/ml)	18 (0-38)	21 (9-38)	21 (11-44)	28 (13-75)	**<0.05**
WCC (10^6/ml)	6.7 (2.2)	6.9 (1.9)	7.3 (1.8)	7.6 (2.2)	**<0.05**
EPC: CEC Ratio	1.5 (1-1.8)	1.1 (1-6.9)	1.5 (1-4.8)	1.5 (1-6.2)	n.a.
CD 34+CD45- cells	0.9 (0.7-1.2)	0.7 (0.4-1.0)	0.8 (0.5-0.8)	0.8 (0.4-1.4)	n.a.

Results are expressed as numbers, percentages, mean (SD) or median (interquartile range). P values are of Altman's linear trend of ordered groups. n.a. = not appropriate as no clear linear trend and hence analysis was not attempted. ^Chi Squared test. No differences on were found on ordinal regression (not shown). ‡ IIIC - T3 N1 M0 + T3 N2 M0 + T4 N1 M0. * IIIC- T4 N2 M0.

As with Dukes' Stage, a significant upward trend of CECs and ECPs was found. Next other histological determinants of prognosis were examined.

The table 24 below shows additional tumour characteristics. Differentiation, tumour type and vascular invasion, are associated with poorer prognoses (285). Only 4 patients of the 127 who underwent surgery had incomplete or indeterminate resection margins (another poor prognostic marker) and were therefore excluded.

Table 24: Details of the tumour characteristics other than Disease Stage

	All Cancer Patients	
Features	**n**	**%**
Number	154	100
Diagnosis		
Screening	12	8
Symptomatic	142	92
Family history of colorectal cancer	40	35
Anatomical position		
Right (caecum +ascending)	53	34
Transverse & descending	19	12
Sigmoid	25	16
Rectum	55	36
Unknown	2	1
Histological Grade*	122	100
Well differentiated	19	4
Well/Moderate	39	44
Moderate	56	45
Poor	8	7
Tumour Type*		
Adenocarcinoma	122	82
Mucinous adenocarcinoma	5	16
Unknown	27	2
Vascular Invasion*		
Yes	25	19
No	85	62
Unknown	12	19
Metastatic*	32	20

*Histology was available for the 148 patients that underwent surgical excision; biopsy results were not included. Five patients of the 32 with metastatic disease underwent palliative resections.

The histological features that did influence the levels of the cellular markers (not shown) were: size, site, depth of invasion relative to the serosa, relationship with peritoneal reflection (for rectal cancers), number of nodes excised and number of nodes involved. However more CECs and EPCs were found with poorly differentiated tumours though not significantly (table 25). An Altman's linear trend ($p<0.05$) was seen with EPCs and progression of tumour differentiation. The smaller power in the poorly differentiated group may increase the likelihood of type 1 and 2 statistical errors. Remarkably both markers were non-significantly higher in the unreported vascular invasion group, and probably resulted from observer error.

Table 25: Cellular markers, tumour differentiation and vascular invasion.

	Differentiation				Vascular Invasion		
	Well	**Well to Moderate**	**Moderate**	**Poor**	**No**	**Yes**	**?**
n	19	39	56	8	85	25	13
CECs	12 (0-22)	10 (0-19)	12 (0-19)	19 (0-30)	**10 (0-21)**	**11 (0-22)**	**22 (8-30)**
EPCs	**15 (11-24)**	**20 (9-36)**	**22 (9-44)**	**54* (17-97)**	**21 (10-40)**	**12 (7-40)**	**22 (13-44)**
WCC	7.8 (2.5)	7.3 (2.3)	6.9 (1.8)	7.3 (1.1)	7.2 (1.9)	7.2 (2.3)	7.2 (2.3)
$CD34^+$ $CD45^-$	1.1 (0.4-1.6)	0.8 (0.4-1.2)	0.8 (0.4-1.2)	0.9 (0.7-1.2)	0.8 (0.4-1.2)	0.7 (0.5-1.1)	0.8 (0.4-1.2)

Levels displayed as medians with IQR cells/ml, WCC as mean with sd x 10^6 cells/ml and $CD34^+CD45^-$ cells as median with IQR x 10^3 cells/ml. P values were by Kruskall Wallis and * Positive linear trend of ordered groups, $p<0.05$. The histologists in 13 patients did not report on vascular invasion.

The significant results of multivariate and univariate analysis of CECs and EPC s for both patient and tumour variables are shown in table 26 below.

Table 26: Regression analysis of CECs and EPCs showing only the significant correlations when all variables were analysed.

	CEC		EPC	
	r^2 (p)	**OR (CI) p**	**r^2 (p)**	**OR (CI) p**
BMI	-	-	**0.156 (0.041)**	126 (0-10^8) 0.112
No. of nodes*	-0.09 (0.092)	**0.1 (0-1.2) 0.048**	-	-
CEC	-	-	**0.417 (0.001)**	**4.1 (2.1-8.0) <0.001**
EPC	**0.394 (0.001)**	**1.1 (1.1-1.2) 0.001**	-	-
WCC	**0.205 (0.011)**	8.7 (0.8-98) 0.505	-	-

r^2=Pearson correlation by multiple regression; Univariate regression analysis for odds ratio [OR] with confidence interval; significance was p<0.05. Factors without correlations were indicated by (-). The model fit for both CECs and EPCs was: r^2 = 0.276, p=0.004. Other non-significant factors (not shown) were: age, sex, smoking, blood pressure, temperature, SCAD, hypertension, DM, screening, family history of CRC, tumour site, vascular invasion, tumour differentiation, number of involved lymph nodes, CD34+CD45-cells, Dukes' and mAJCC stage. * Number of lymph nodes with CRC spread

Both EPCs and CECs correlated with each other after multivariate and univariate analyses. As already shown in section 3.2, BMI correlated with EPCs while the proposed model fit for univariate analysis showed higher CECs with more lymph node involvement. The later must be interpreted with caution, as nodal yield was inconsistent from variations in patient anatomy, 'incomplete' excision at surgery or under-sampling by the pathologist. Similarly regression analysis for Dukes' stage showed only a correlation with CEC (OR 1.0, CI 1.0-1.0, p=0.032), which did not remain with regression against the modified AJCC (not shown).

Discussion

To date Dukes' and AJCC-TMN staging of CRC are the best determinants of prognosis and are therefore used to inform the need for further oncological treatment where suitable. Both depend on surgical resection (stage I-III or Dukes' A, B & C disease) with CT staging or invasive biopsies (radiology-guided or endoscopic) for confirmation of metastatic CRC detected by CT, as surgical resection may be inappropriate (285). Diagnostic and prognostic tests of CRC that were minimally invasive with high specificity and sensitivity were certainly elusive. I therefore theorised that CECs and EPCs, though altered in various other diseases, may a clinical role in prognostication. Some authors suggested changes during chemotherapy for metastatic CRC were predictive of treatment outcomes, though results of 'success' (e.g. improved PFT versus OS) varied. What had not been studied previously, and the basis of this hypothesis, was that pre-treatment levels may change with advancing disease and was therefore predictive of CRC stage.

On first glance there appeared to be increasing levels of CECs and EPCs with advancing stage (the highest observed in Dukes' D), but there were no significant differences by ordinal regression. However the suspicion was verified by Altman's linear trend of ordered groups ($p<0.05$), and confirmed the hypothesis that markers increased with tumour progression from one stage to the next. This was further explored in section 5.2.4 on plasma and cellular markers with CRC stage.

Modifying stages did not reproduce this trend. Comparisons included analysis by oridinal regression of CRC as three groups: disease confined to the bowel wall

(Dukes' A plus B), lymph node spread (Duke's C) and metastases. Similarly there were no differences between localised disease (Dukes' A+B+C) and metastatic disease. Pooling T4N2M0 from stage III with stage IV disease of the AJCC-TMN classification (which I called the mAJCC) produced a more distinct upward trend of CECs and EPCs ($p<0.05$). Stage III disease, as with Dukes' C, was characterised by nodal involvement and the sub-category above was associated with a poorer 5-year survival rate similar to metastatic disease (AJCC stage IV). Another first was the finding that CECs and EPCs increased in a linear trend ($p<0.05$ by Altman's method for ordered groups) with tumour differentiation regardless of tumour stage. Poorly differentiated status was a predictor of disease recurrence after resection (285).

Only CECs correlated to Dukes' stage on multivariate and univariate regression, which did not remain after analysing the mAJCC stages (i.e. pooling T4N2M0 from stage 3 (or Dukes' C) to stage 4 (Dukes' D) disease. However a nearly significant ($p=0.049$) link between BMI and mAJCC (OR 0.9, CI 0.9-1.1) was found i.e. patients with more advance disease had lower BMIs. Although data on weight changes were collected, for most it was not reliably quantifiable and therefore excluded from the analysis. Otherwise, there were no significant findings when all measured cellular indices were analysed against tumour characteristics such as method of detection (screening versus symptomatic), site of origin, size of tumour, vascular invasion or tumour grade. I postulated higher EC activity was reflected histologically by the presence of tumour cells within blood vessels, or vascular invasion. That is, displaced ECs by invasion or translocation of tumour into blood vessels increased CEC levels and 'mobilised' more EPCs to repair the damage. However, there were less EPCs with vascular invasion, though non-significantly (median 12 [IQR 7-40]

cells/ml versus 21 [10-40] with no invasion; p=0.113). Although the status was unknown in 19% of the specimens examined, the finding, which was not previously reported, suggested that the presence of tumour within the blood vessels might suppress EPC mobilisation from the bone marrow and/or transformation into the EPC phenotype. Neither theory could be supported by the data, even less so for the later, as numbers of CD34+CD45- cells (assuming they were circulating precursors to the EPC phenotype) were not elevated by vascular invasion. Therefore further in vitro studies were required and other possible explanations are suggested in section 5.2.4 when plasma and cellular markers are discussed together.

Goon et al reported the first successful attempt at utilising pre-treatment levels of circulating endothelioid cells as predictors of disease stage in breast cancer (Goon 2009). CECs and CPCs (circulating progenitor cells with phenotype $CD45^{-}$ $CD34^{+}CD133^{+}$, which the authors described as the forerunner to EPCs) increased and decreased respectively with advancing stage as assessed by both the Nottingham Prognostic Index (3 stages) and the AJCC-TNM (4 stages) criteria. Unlike my study, they also reported correlations of the CEC with NPI, tumour size, histological grade, vascular invasion and screen-detected cancers. They mainly inferred that CEC was related to tumour bulk. This was not as evident in my study given that tumour size did not correlate with the cellular markers, but rather indirectly by the positive Altmans' linear trend with Dukes' stage. That is the findings supported my suspicion that both EPCs and CECs were influenced more by angiogenic activity or the angiome, than increasing tumour load by advancing stage.

There were certainly more CECs and EPCs with metastasis to distant organs, while spread to lymph nodes had little effect on levels when compared to disease localised to the bowel wall. This suggested that a critical volume of endothelial and angiogenic activity must be reached before the circulating median levels doubled at progression to Dukes' D stage. It was possible that this volume was achieved at Dukes' C stage when levels were similar to Dukes' B stage. That is increased angiogenesis 'consumed' more EPCs and hence did not produce the further rise anticipated in moving to Dukes' C disease. Alternatively, there was shift to metastasis rather than angiogenesis, as blood vessel growth was less of a priority for the tumour after it achieved its critical mass (436). Similarly CECs from vascular invasion did not rise significantly as metastasis favoured the lymphatic rather than haematogenous route.

As all patients in my study with Dukes' D stage had at least liver metastases, the organ involved rather than disease load and neovessel demand were linked to the increase in CECs and EPCs found. However, I support the view that the priority once again shifted to angiogenesis, having achieved spread, to aid the growth of new seedlings (436, 437). With 'chaotic' neoangiogenesis in tumours sited at other organs, immature vessels added more CECs to the circulation while more EPCs were mobilised to repair the damage (339). Other tumour changes with liver metastasis may further contribute to the rise in CEC and EPC levels. Hanrahan et al established variable angiogenic activity when levels of VEGF and VEGFR subtypes were measured in the adenoma-carcinoma sequence and site of metastasis (438). Localised and lymph node disease expressed higher levels of VEGF-A mRNA, VEGFR-1 and VEGF-R2. With metastatic disease, mainly to the liver, there was a 'shift' in expression to VEGF-C mRNA and the VEGFR-2 receptor. Generally VEGF-

C promoted angiogenesis via VEGFR-2 and was comparatively higher in lymphatic spread than liver metastases (439, 440).

In summary although there were linear positive trends of CECs and EPCs with advancing Dukes' stage and tumour differentiation, they did not inform on the disease stage itself. Nevertheless, the trends had implications for the vascular biology of CRC as EC activity varied with each stage and may therefore be important determinants of its prognosis (as examined in section 5.2.6).

All pre-treatment plasma and cellular indices in CRC are analysed next.

3.1.3 Plasma & cellular markers in CRC

Abstract

Background: Markers of endothelial activity were altered in cancer and other diseases. In CRC the relationship between factors of the endotheliome (vWf, sE-selectin) and angiome (VEGF, angiogenin) with EPCs and CECs were unknown. All indices were hypothesized to be higher than controls and correlations of plasma with cellular markers to be associated with the CRC stage.

Methods: Prospective study of 154 CRC participants compared to 26 participants with benign colonic polyps (BD), 33 with stable coronary artery disease (SCAD) and 29 healthy (HC) age-matched controls. CECs (CD45-CD146+CD34+) and EPCs (CD45-CD34+KDR+) were enumerated by 4-colour flow cytometry. All plasma markers were measured by ELISA assays. The CRC stage was determined by the hospital's histopathology and radiology departments, and confirmed at multidisciplinary team meetings.

Findings: All indices were higher in CRC versus the controls except vWf and sE-selectin, which were comparable to the SCAD group. VEGF correlated with CECs ($r=0.407$, $p<0.001$) and EPCs ($r=0.530$, $p<0.001$). Angiogenin, VEGF and EPCs (all $p<0.001$), were more predictive of CRC. Except for Dukes' A (stage 1), only angiogenin and VEGF were higher in all Dukes' & mAJCC stages ($p<0.001$) when compared to SCAD. Like EPCs and CECs, angiogenin rose in a linear order with advancing stage. This relationship remained on ordinal regression analysis after

adjusting for all other indices (p=0.001). EPCs and WCC were higher only in stages C and D (p<0.05). CD34+CD45- cells and sE-sel remained unchanged at all stages. *Conclusion:* There were disturbances of all measured indices of the endotheliome and angiome in CRC. However, only angiogenin may be a potential biomarker of diagnosis. Along with CECs and EPCs, it progressed with disease stage and may therefore be important for the risk stratification of CRC.

Background

CECs and EPCs were proposed to monitor response to treatment of metastatic CRC (28, 146, 201, 351). Angiogenin (267), VEGF (284, 366), vWf (11, 23) and sE-selectin (78, 369) were also suggested to be of diagnostic and prognostic value. As with most biomarkers of EC activity, they were also altered in many diseases including cardiovascular disorders and other cancers (22, 92, 140, 386). For CRC, their value across all stages, performance against cardiovascular disease and differences with pre-cancerous disease, i.e. colonic polyps, were unknown. I hypothesised that levels were higher in CRC than healthy (HC) and benign polyp (BD) controls, but comparable to those with stable coronary artery disease (SCAD). The later was also used as a positive disease control. The markers and their correlations with each other were hypothesised to be associated with, and therefore, informative of the cancer's stage before surgery.

Method

Participants were recruited as detailed in section 2.1. CECs and EPCs were measured in peripheral blood by 4-colour flow cytometry (section 2.3.1) and all plasma markers (vWf, sE-sel, VEGF & angiogenin) by ELISA assays as summarized in sections 2.3.3 to 2.3.6. Pre-treatment levels were quantified in CRC (n=154) and compared to the 26 BD, 29 HC and 33 SCAD control groups. As detailed in the methods of section 3.1.2, the stage was obtained from the reports of the hospital's relevant departments, and confirmed at the multi-disciplinary meetings.

Statistics

The power calculation was the same as section 2.3.1 (page 117) for testing hypothesis 1. That is, I determined the correlations of plasma markers of the endotheliome (vWf and sE-sel), and the angiome (VEGF, angiogenin) with CECs and EPCs from the participants recruited to hypothesis 1. Results were expressed as numbers and percentages, mean and standard deviation (SD) or median and interquartile range (IQR). Analyses between groups were performed using ANOVA, Kruskal Wallis, Mann-Whitney, t-test and Chi Square as appropriate. Correlations were obtained by Spearman's rank method, and for the clinical variables, by stepwise multiple regression. Analysis of variance (ANOVA) was inappropriate for changes with CRC stages as each were dependent on the previous in moving from one stage to the next. Altman's test of linear trend of ordered groups was applicable (380). A two-tailed p value < 0.05 was considered statistically significant.

Results

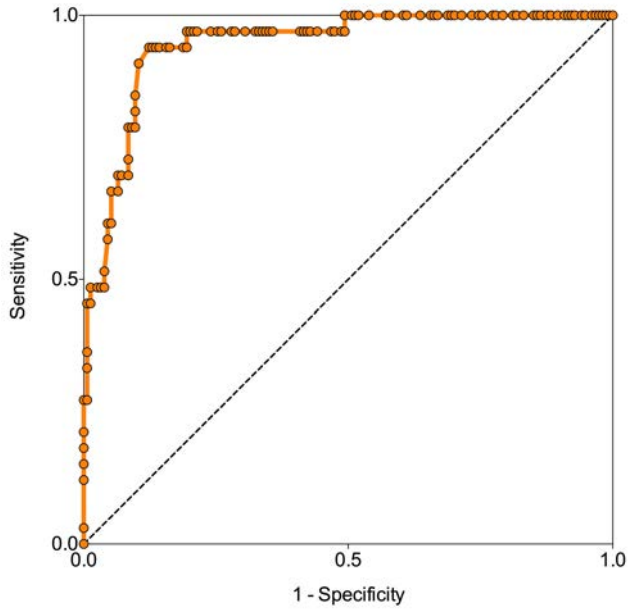
All indices were higher in CRC versus the controls except for vWf and sE-sel in SCAD (Table 27 below). The variables were then tested by ROC analysis for their diagnostic accuracy of CRC The ROC curves showed that, like EPCs, there were less false positives for angiogenin and VEGF (figure 29).

Table 27: Plasma markers in CRC versus Control Groups

	Colorectal Cancer [CRC]	**Healthy Controls [HC]**	**Benign Controls [BD]**	**Coronary Disease [SCAD]**	**p**
Number	154	29	26	33	
vWf [IU/dL]	**119 (26)[a]**	97 (17)	110 (31)	**128 (48)[b]**	**<0.001**
sEsel [ng/mL]	**27 (18-34)[a]**	20 (16-28)	18 (16-26)	**31 (28-41)[b]**	**<0.001**
VEGF [pg/mL]	**195 (30-703)[a]**	30 (0-65)	32 (0-82)	90 (23-110)	**<0.001**
Angiogenin [ng/mL]	**312 (248-367)[c]**	140 (55-176)	143 (107-175)	137 (78-160)	**<0.001**

Data presented as mean (standard deviation) or median (interquartile range), overall p value by ANOVA or Kruskall-Wallis. Post-hoc Tukey's test found [a] higher levels in CRC than controls except [b] SCAD; [c] higher in CRC compared to the other three groups, $p<0.05$.

Figure 29: ROC curves for Angiogenin & VEGF in CRC versus controls



Angiogenin

Area under curve: 0.938

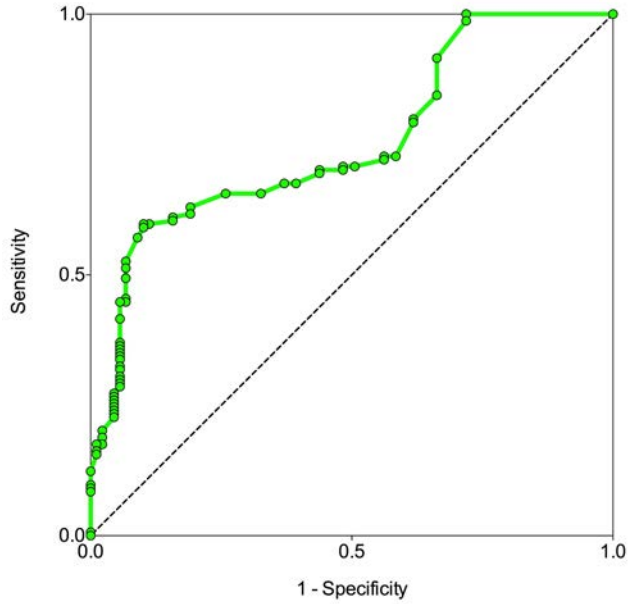
P value: <0.001

For Angiogenin > 312 ng/ml:

Sensitivity: 98 %

Specificity: 50 %

Likelihood Ratio: 1.9 (0.9-2.2)



VEGF

Area under curve: 0.732

P value: 0.001

For VEGF > 195 pg/mL

Sensitivity: 93 %

Specificity: 49 %

Likelihood Ratio: 1.8 (1.0-2.1)

That is, higher VEGF and angiogenin were predictive of CRC. The next analysis determined the relationships with CECs, EPCs, WCC and CD34+CD45- cells by correlation (table 28). As in hypothesis 1, only the CRC group was assessed, as the low numbers in the controls were likely to result in types 1 and 2 statistical errors.

Though results were similar when all groups were included, the correlations with VEGF were within power limits and therefore highly reliable in CRC.

Table 28: Correlation matrix of plasma and cellular markers in CRC.

	CECs	EPCs	WCC	CD34+CD45-
vWf	r = 0.050 p = 0.542	r = 0.153 p = 0.059	r = 0.062 p = 0.445	r = 0.085 p = 0.293
eSelectin	r = -0.010 p = 0.945	r = 0.000 p = 0.995	r = 0.059 p = 0.464	r = 0.013 p = 0.871
VEGF	**r = 0.407** **p < 0.001**	**r = 0.530** **p < 0.001**	r = 0.141 p = 0.081	**r = 0.169** **p = 0.035**
Angiogenin	r = 0.049 p = 0.546	r = 0.144 p = 0.075	r = 0.134 p = 0.097	r = -0.067 p = 0.409

r= Spearman's correlation coefficient.

VEGF therefore was an important determinant of CECs, EPCs and CD34+CD45- cells. Scatterplots of the relationships between CECs to VEGF, and EPCs to VEGF, are shown in Figures 30 and 31. Logarithm (base 10) of the raw data was performed as the data had a strong non-parametric distribution. The EPC/VEGF plot (r=0.530) and hence correlation was more convincing than the CEC/VEGF results (r = 0.407).

Figure 30: Relationship between CECs and VEGF in CRC. By Spearman's test r=0.407, p<0.001, with mean (solid) and 95 % SE (dashed) lines shown.

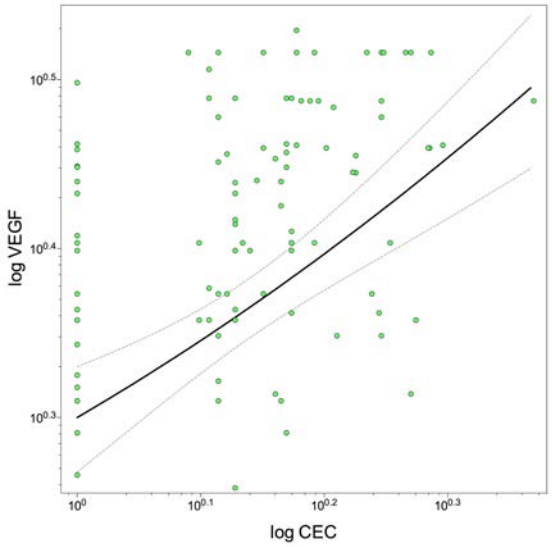
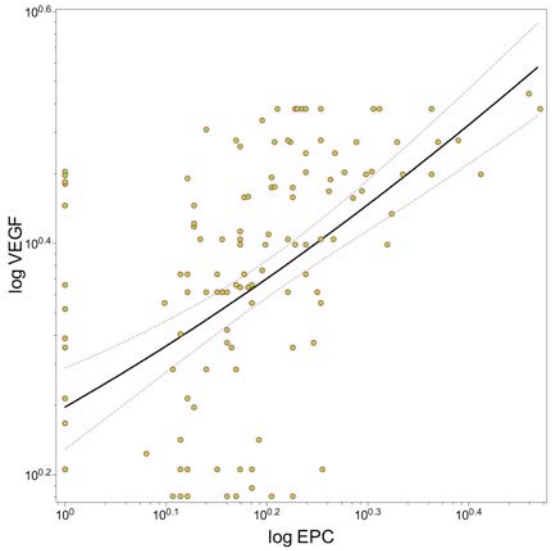


Figure 31: Relationship between EPCs and VEGF in CRC by Spearman's test r=0.53, p<0.001, with mean (solid) and 95 % SE (dashed) lines shown.



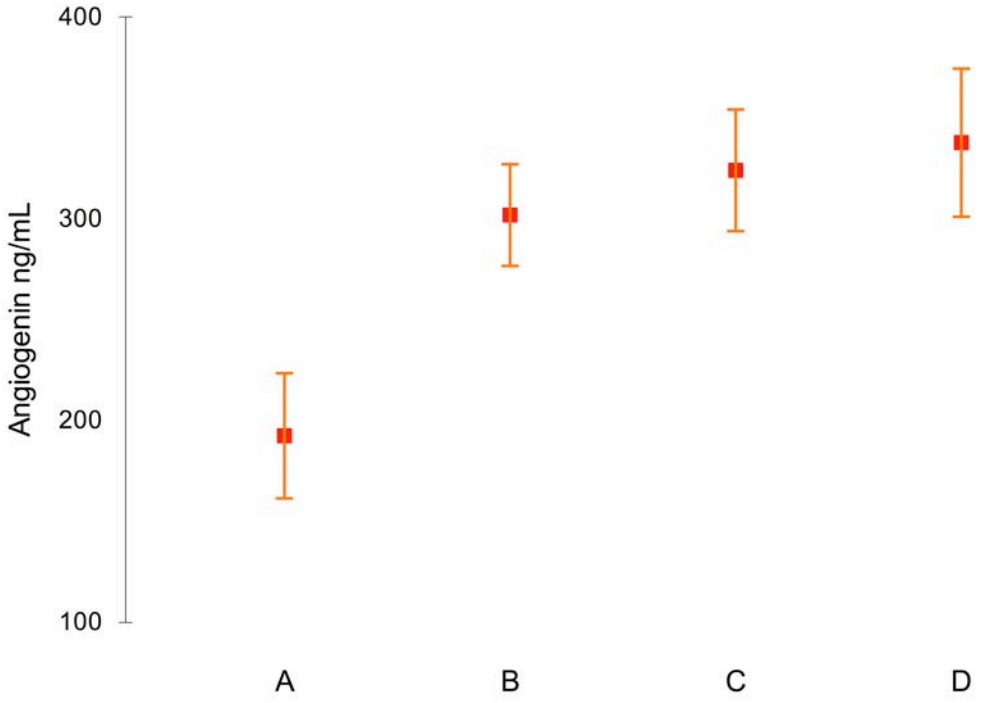
The relationships between the plasma markers and Dukes' stage are shown in Table 29. As for EPC and CEC, ANOVA was inappropriate as groups were not independent and hence Altman's linear trend of ordered groups was used instead. Only angiogenin rose in a linear order with Dukes' stage (figure 32 below).

Table 29: Plasma markers and Dukes' Stage

	A	B	C	D	P (trend)
Number (n)	19	54	49	32	
vWf [IU/dL]	115 (23)	117 (22)	115 (26)	129 (34)	< 0.1
sE-sel [ng/mL]	28 (22-32)	29 (20-37)	24 (11-32)	25 (7-34)	> 0.2
VEGF [pg/mL]	120 (10-560)	188 (5-610)	220 (10-885)	265 (5-742)	> 0.2
Ang [ng/mL]	254 (190-290)	319 (242-353)	322 (264-374)	356 (293-396)	**< 0.01**

Results expressed mean and standard deviation or median and interquartile range.
Approximate p value of Altman's linear trend of ordered groups'. Table B4 in Altman (1991).

Figure 32: Relationship between Angiogenin and Dukes' stage p (trend) <0.01, mean (red dot) with 95% CI (error bars).



As in hypothesis 2, I compared the indices in those with localised disease (confined to the bowel wall), involvement of lymph nodes and spread to other organs (table 30).

Table 30: Plasma markers in local, node and metastatic disease

	Localised disease	**Lymph nodes involved**	**Metastasis to other organ**	
	Dukes' A+B	**Dukes' C**	**Dukes' D**	**P**
Number (n)	77	53	24	-
vWf [IU/dL]	117 (22)	115 (26)	129 (34)	>0.1
sE-sel [ng/mL]	29 (21-36)	24 (11-32)	25 (7-34)	n.a.
VEGF [pg/mL]	180 (30-559)	220 (10-885)	265 (5-742)	>0.2
Ang [ng/mL]	291 (91)	322 (264-374)	356 (293-396)	**<0.01**

Results are mean (SD) or median (interquartile range). P values are from Altman's linear trend of ordered groups. n.a. = not appropriate as no clear linear trend, so analysis was not attempted.

Once again only angiogenin showed a significant trend with disease progression.

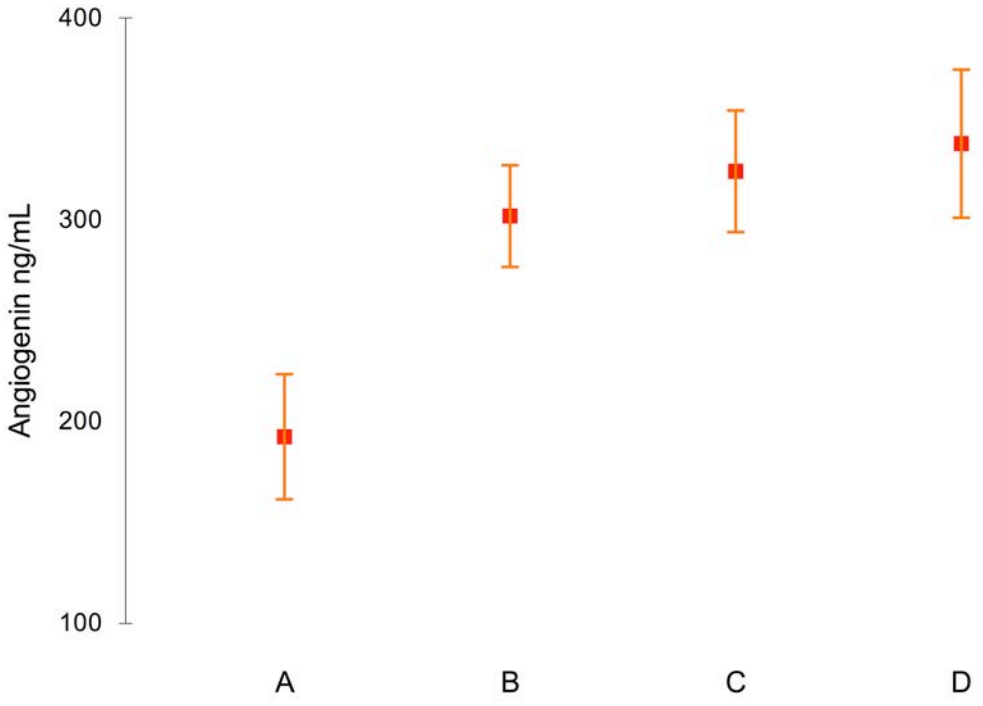
For the AJCC-TMN classification, as with hypothesis 2, I pooled the poorer prognostic tumours from stage IIIC (T4N2M0) with stage IV (5-year survival of less than 50%). Again only angiogenin showed a linear trend (table 31, figure 33).

Table 31: Plasma markers versus modified AJCC stage

	I	IIA+IIB	IIIA+IIIB+IIIC‡	IIIC*+ IV	p (trend)
Number (n)	19	54	42	39	
vWf [IU/dL]	115 (23)	117 (22)	114 (24)	128 (23)	n.a.
sEsel [ng/mL]	28 (22-32)	29 (20-37)	24 (18-34)	25 (16-30)	n.a.
VEGF [pg/mL]	120 (10-560)	188 (5-610)	363 (80-1200)	190 (23-750)	n.a.
Ang [ng/mL]	254 (190-290)	319 (242-353)	325 (102)	332 (100)	**<0.01**

Results are mean (SD) or median (interquartile range). P values are from Altman's linear trend of ordered groups. n.a. = not appropriate as no clear linear trend, so analysis not attempted. Abbreviations are as per table 27. ‡ IIIC are stages T3 N1 M0 + T3 N2 M0 + T4 N1 M0. * IIIC is T4 N2 M0.

Figure 33: Relationship between modified AJCC score and angiogenin (mean in red dot with 95% CI error bars).



Only vWf showed ordinal regression when localized disease, inclusive of lymph node spread, was compared with metastatic disease (see table 32 below).

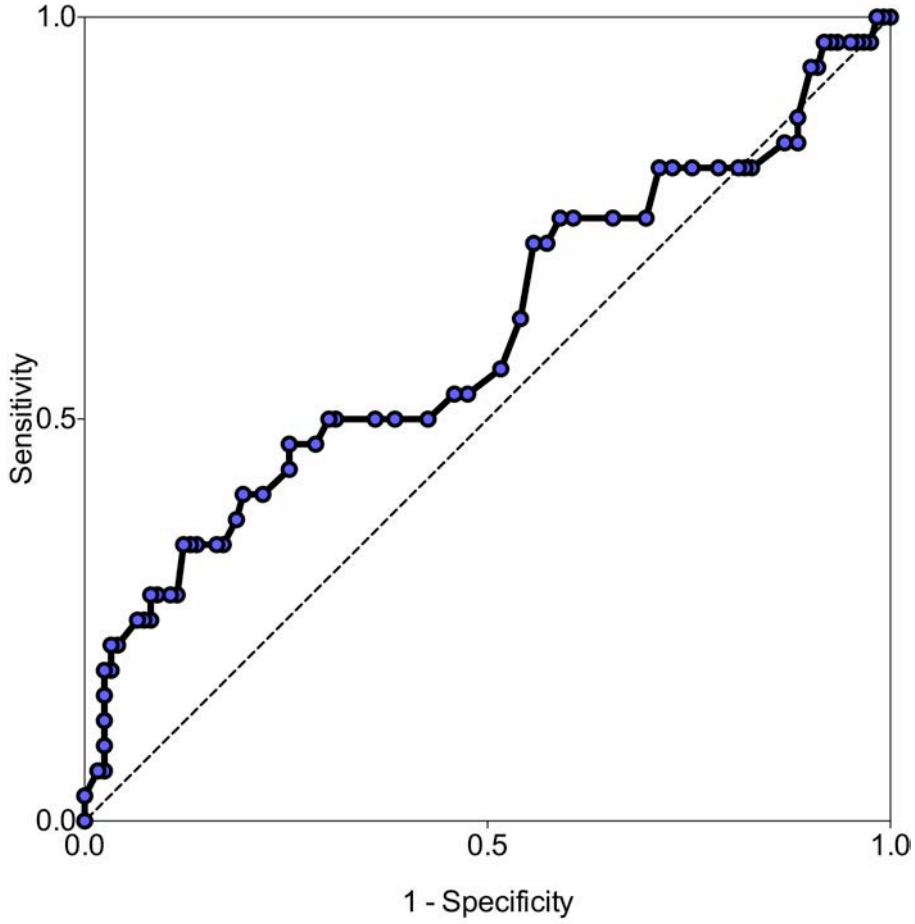
Table 32: All indices in localized versus metastatic CRC

	Localised disease Dukes' A+B+C	Metastasis Dukes' D	p-value	Ordinal Regression
Number (n)	122	32	-	-
Age (years)	72 (10)	71 (10)	0.920	0.323
Male (%)	67 (52)	15 (68)	0.410	0.130
Female (%)	58(48)	7 (32)		
CEC /mL	11.5 (0-21)	11 (0-23)	0.392	0.159
EPC /mL	20 (9-43)	29 (12-47)	0.966	0.509
EPC:CEC ratio	1.13 (0.5 – 5.7)	1.75 (0.6 – 5.5)	0.774	0.285
CD 34^+ CD45- cells/ml	0.50 (0.79-1.11)	0.80 (0.39-1.38)	0.529	0.787
WCC $x10^6$ cells/mL	7.11 (1.95)	7.43 (2.23)	0.405	0.537
vWf [IU/dL]	118 (22)	127 (29)	**0.009**	**0.045**
sEsel [ng/mL]	27 (21-36)	24.5 (16-32)	0.153	.0199
VEGF [pg/mL]	225 (30-833)	177 (21-699)	0.911	0.674
Ang [ng/mL]	314 (98)	310 (101)	**0.014**	0.186

P-values were by Mann-Whitney test or t-test. For ordinal regression model fit p=0.161; Pearson's Goodness-of-fit p=0.080.

Although vWf was significantly higher in Dukes' D compared to all other stages, it did not perform as a good discriminator of metastatic CRC (for vWf > 118 IU/dl sensitivity was 41%, specificity 78%, ROC area=0.602 [p=0.075, LR=1.6]; see figure 34).

Figure 34: ROC curve for vWf in Dukes' D versus ALL other stages.



I then compared the stages with the positive control (SCAD) to determine if there was a difference that was more significant in CRC for all the measured indices (table 33).

Table 33: Differences between measured indices of Dukes' Stage and SCAD.

	Dukes' Stage versus SCAD (p values) [1]			
	A	**B**	**C**	**D**
n	19	54	49	32
Age (years)	73 (11)	74 (10)	72 (9)	71 (9)
CEC cells/ml	0.752	0.796	0.249	0.217
EPC cells/ml	0.156	0.129	**0.007**	**0.004**
WCC 10^6/ml	0.444	0.213	**0.021**	**0.046**
CEC:EPC	0.617	0.763	0.436	0.248
$CD34^+CD45^-$*	**0.002**	**<0.001**	**<0.001**	**<0.001**
vWf [IU/dL]	0.252	0.151	0.096	0.914
sEsel [ng/mL]	**0.009**	0.062	**<0.001**	**<0.001**
VEGF [pg/mL]	0.074	**0.018**	**<0.001**	**<0.001**
Ang [ng/mL]	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Results were p values [significance <0.05]. SCAD: Stable coronary artery disease. * There were less CD34+CD45- cells in CRC than SCAD.

Only angiogenin was higher in all CRC stages while EPCs, WCC, and VEGF were higher only in stages C and D when compared to the positive SCAD control. Conversely CD34 cells and sEsel were lower regardless of stage. The results were similar with the modified AJCC classification (not shown).

Summary of the cross-sectional analyses

There were raised CECs in CRC (that are comparable with levels in SCAD), raised EPCs only in CRC and the EPC/CEC ratio was high only in CRC. EPCs strongly correlated to CEC levels and body mass index. While CECs, EPCs and the WCC all increased across Dukes' stage, none of the measured markers correlated to the disease on multivariate or univariate regression analysis.

As expected, all four plasma markers (vWf. sE-sel, VEGF, angiogenin) were raised in CRC compared to HCs, and some were comparable to those in SCAD. However, only VEGF correlated to both CECs and EPCs, while angiogenin progressively increased with progression of Dukes' stages and the modified AJCC. Results of ordinal regression analysis of angiogenin, VEGF, CECs, EPCs, and Dukes' stage is shown table 34. This unequivocally found that the relationship between angiogenin and Dukes' stage remained after adjustment for the other indices (p=0.001).

Table 34: Ordinal Logistic Regression of Dukes' Stage and measured factors.

Predictor	Z	P
CECs	-0.81	0.419
EPCs	-0.44	0.66
VEGF	-0.04	0.969
Angiogenin	**-3.18**	**0.001**

Discussion

Angiogenesis is essential to CRC growth and metastasis, hence its measurement and that of EC activity may inform on disease stage and prognosis (267, 441, 442). This thesis was the first practical approach on the correlation of plasma with cellular markers of the endotheliome and angiome. It compared levels in participants with CRC, SCAD, benign polyps and those who were otherwise healthy (recruited to hypothesis 1). In CRC and SCAD (positive control) I hypothesized that the mobilisation of EPCs was linked to angiogenesis as determined by plasma VEGF and angiogenin levels (i.e. the angiome). Also expected were abnormal levels of sectin and vWf, measures of EC activity or the endotheliome. Perturbation of CEC levels was also expected to correlate with all measured plasma indices. That is, tumour growth by vascular invasion (aided by sE-selectin) displaced ECs from vessels (from which effete ECs released vWf) and/or with high angiogenic activity (high VEGF and angiogenin levels) produced immature blood vessels that easily shed CECs (92, 235) With the exception of VEGF on EPCs, no correlations between my chosen cellular and plasma markers were previously studied in CRC (20, 147).

All plasma marker, as with CECs and EPCs, were significantly higher in CRC than the controls, except vWf and sE-selectin which were similar to the SCAD group. For the later, levels were also more or less the same as those from studies on acute coronary syndrome in which SCAD was the disease control (22, 443). Both factors were described by the authors as indicators of EC dysfunction or damage and were markedly increased with acute coronary occlusion. Nevertheless, vWf on its own, as described by Schellerer et al and now confirmed in this study, was not a useful

biomarker of CRC (23). It also disproved the diagnostic value of sE-selectin suggested by Uner et al (78). Only VEGF and, more so, angiogenin were good discriminators of CRC from all the other groups studied (that is, for a VEGF > 195 pg/mL sensitivity = 49%, specificity = 93%, ROC area of 0.732, p=0.001; for angiogenin >312 ng/mL sensitivity = 75%, specificity = 98%, ROC area of 0.938, p<0.001). This raised the possibility of angiogenin as a good diagnostic tool for CRC, but, like VEGF, was also elevated in other cancers e.g. breast cancer (24).

Correlations were calculated in the CRC group only, since the numbers were within power limits. The findings in the smaller sized controls were more likely to result in types 1 and 2 statistical errors. Significant results were found with VEGF only; that is, there were correlations to CECs (r=0.407, p<0.001), CD34+CD45- cells (r=0.169, p=0.035) and more so, EPCs (r=0.530,p<0.001). Asahara et al first reported that EPCs were partially mobilized from the bone marrow by VEGF while, for cancer, correlations were reported in lung and hepatocellular carcinomas only (147, 190, 216). As for metastatic CRC, neither CECs nor EPCs were linked to VEGF levels but these findings of Beereport et al included other solid organ tumours (146).

Based on the study of Asahara et al (147), I hypothesized a relationship of VEGF with EPCs but not CECs; however, I found correlations with both. This probably resulted from the inclusion of all disease stages or was ‘mathematical’ from the correlation that existed between CECs and EPCs described in section 5.2.2. Since this was not reported in other cancers [breast and prostate (24, 140)], alterations in CECs were more likely the result of angiogenesis in CRC given VEGF was the ‘gold standard’ test of the angiome. It would also support the hypothesis that CEC levels

increased as they were displaced from invasion into vessels by the growing tumour. As tumour growth demanded blood supply (probably by hypoxic factors) more angiogenic factors, like VEGF, were released, which gave rise to immature vessels with ‘loose’ basement membranes that shed ECs, further increasing CEC levels.

The positive correlation of VEGF with CD34+CD45- cells, not examined previously by other authors, probably resulted from the presence of EPCs within this fraction. Alternatively VEGF may mobilize (from the bone marrow or otherwise) CD34+ ‘precursors’ of other non-endothelial cells (CD34+CD45-KDR-) important to CRC growth e.g. fibroblasts (419). However, the fate and phenotype(s) of this fraction of cells could not be determined from my study or the published data.

vWf

The lack of a correlation with CEC to the ‘gold standard’ of EC damage, vWf, was unexpected and did not fit with the model of the endotheliome I proposed (89, 122). That is, I expected tumour invasion into blood vessels to release and/or damage ECs. CECs were mature cells shed from the vasculature, which on apoptosis should increase levels of vWf. This non-correlation was not unique to CRC, as it was also not found in prostate and breast cancer (24, 140). A criticism of my study was that ABO blood group, a major determinant of vWf levels (444), were not collected. However, I anticipated that vWf, as recently suggested by Schellerer et al (though their findings were not yet published when I formulated this hypothesis), should be high from increased EC activity in cancer regardless of blood type (23). To detect

influences of ABO blood type in CRC would require a much larger cohort than my study. Also, it was possible that vWf was cleared significantly within the portal circulation to discern any detectable differences in the peripheral circulation (445). Although vWf was significantly higher in Dukes' D stage, ROC analysis proved it was only a fair discriminator of metastatic disease.

sE-selectin

The results of Ito et al of a trend of sE-selectin with disease stage, the highest levels in Dukes' D (n=11), may have overestimated the effect from under-powering with only 54 participants recruited as this result was not reproduced in my study (369). Soluble E-selectin (also known as CD-62E, ELAM-1 or LECAM2) is a transmembrane adhesion protein activated on ECs by cytokines and bind to glycolipids and glycoproteins of leukocytes during inflammation (446). Attachment to sialyl Lewis carbohydrate-ligands (specifically x) on tumour cells was shown in CRC lines to facilitate haematogenous metastasis (447). The expression on ECs and the subsequent increase in plasma levels were highly inducible by cancer-derived cytokines e.g. TNF- and interleukins, and VEGF (448, 449). On these findings alone, the hypothesised correlations with VEGF, and CEC, the latter I proposed as a marker of vascular invasion and metastasis, were rejected. As the highest levels were found in SCAD not CRC, vascular dysfunction in atherosclerosis with a greater inflammatory effect, may be a more potent stimulator of sE-selectin expression (22, 377). Alternatively, apoptosis of CECs was less frequent than in SCAD (22). As no marker of apoptosis (e.g. sFas or sFasL) was measured in my study, I was unable to

conclude that CEC death resulted in either increased vWf or sE-Sel but the absence of correlations suggested more viable cells remained in the circulation than SCAD. This may have been less so in metastatic disease as vWf, but not sE-Sel, levels were similar to that of SCAD. There may be a role for vWf in identifying Dukes' D stage where conventional methods (CT, PET-CT or MRI) failed and therefore better inform therapeutic strategies but further conclusions were beyond the remit of this thesis.

Angiogenin

The trend of increasing levels of CECs and EPCs with Dukes' and mAJCC-TMN stage described in section 5.2.3 was also hypothesised to occur with the measured plasma markers. However, only angiogenin displayed this quality and remarkably more so ($p<0.01$) than with either circulating cell ($p<0.05$). Shimoyama in a smaller study (34 CRC patients) similarly described changes in serum and tissue levels that varied with disease stage, with the highest concentrations found in Dukes' B participants (267). My study was adequately powered to support this trend but was measured in the participant's plasma. There were no reports in the literature, unlike VEGF, of differences between serum or plasma levels, or the contribution, if any, of intra-cellular angiogenin e.g. from leukocytes or platelets. The mechanisms of angiogenin in angiogenesis were unclear. This 14kDa tumour-derived ribonuclease protein was first isolated from the supernatant of a CRC cell line (352, 450). It interacted with the endothelium and smooth muscles of blood vessels to induce a range of EC responses including migration, invasion, proliferation and formation of vascular 'tubes'. It primarily stimulated tPA release when bound to surface actin on

ECs. Plasmin, from the enzymatic breakdown of plasminogen by tPA, then degraded basement membrane and extracellular matrix to allow ECs to penetrate perivascular tissues and facilitate neovascularisation. Angiogenin adhered more rapidly to HT-29 human CRC cells compared to other ECM proteins, hence a role in metastasis by aiding EC attachment prior to cancer migration across the vessel wall was postulated by Hu et al (358, 359, 362). Tumour cell proliferation was also shown in this cell line to involve tyrosine kinase pathway and increased RNA activity with angiogenin translocation (endocytosis) into the nucleus. Its physiological substrates were largely unknown but interactions with the intracellular cytoskeleton and the ECM protein fibulin-1 supported the theory of aiding cell adhesion towards metastasis (451, 452).

VEGF

VEGF expression was highlighted by a Ferrara et al (229) as the ‘best’ biomarker to guide treatment as, was often assumed but only recently proven by Jin et al (specifically VEGF-C), levels reflected tumour size in CRC (453). There was sufficient evidence that VEGF on its own had limited clinical application but its inclusion, as a marker of the angiome, was to determine its clinical relationship with markers of EC activity (the endotheliome) and specifically with CECs and EPCs. The relationship had not been examined with the cellular definitions proposed, which I considered the ‘best’ fit based on the evidence to date. On initial inspection, median VEGF (-A) levels in my study appeared to increase with disease stage, though not significantly, and were similar to other reports of the last three decades (454-456). However, unlike angiogenin, levels tended to be higher in serum than in paired plasma, and

presumed to arise from stores within platelets released during clotting (270, 284, 457). Hence, anti-coagulated plasma may more accurately reflect ‘true’ circulating levels, rather than serum contaminated by ‘accumulated’ stores from platelets, and therefore VEGF was indeed independent of Dukes’ stage. Platelets, which can be higher in advanced disease, were recognised depots for and hence transporters of angiogenic and growth factors via the peripheral circulation to cancer cells (458, 459). Like plasma, there were also inconsistencies of the serum levels reported between studies and therefore the differences were not necessarily from platelet contamination. Rudge et al proposed that the accuracy of assays may improve by chemical traps to stop its rapid breakdown during blood storage as VEGF otherwise had a half-life of only minutes (460). I proposed that a ‘trap’ already existed within platelets themselves, and this quantity, if measurable, was the best reflection of VEGF secreted by tumours. A few studies on the quasi-differential content measured plasma (free VEGF) versus serum (containing free and stored VEGF) levels (461, 462). Otherwise, I could not find published assays to test this hypothesis.

In summary levels of CECs, EPCs, angiogenin and VEGF were good measures of angiogenic activity (the angiome) and therefore were important to the understanding of the vascular biology of CRC. However, angiogenin alone was the best discriminator of the disease and its stage. Hence, angiogenin may be a useful biomarker of CRC detection and risk stratification. Of the other measures of the endotheliome, only vWf was significantly higher in Dukes’ D stage which may also be useful in identifying metastatic disease and hence inform treatment strategies.

The longitudinal studies are next, starting with the serial changes of markers in CRC.

3.2 Longitudinal Studies

3.2.1 Serial changes of cellular and plasma markers in CRC

Abstract

Background: Serial changes to CECs, EPCs and VEGF were proposed to predict outcomes after CRC treatment. However the overall value of these changes, along with that of other markers of the endotheliome (vWf, sE-selectin) and the angiome (angiogenin) were unknown. I hypothesized that all markers fell after surgery and the changes with treatment strategies were important determinants of outcomes.

Methods: Sixty-eight participants treated for CRC were recruited and three subgroups were compared: surgery only (n=16), surgery followed by standard chemotherapy (n=32), and surgery followed by standard chemotherapy plus anti-VEGF therapy (Bevacizumab, n=20). Peripheral blood was taken before surgery and again at 3 and 6 months. CECs (CD34+CD45-CD146+) and EPCs (CD34+CD45-KDR+) were measured by 4-colour flow cytometry and the plasma makers by ELISA.

Findings: All markers fell after surgery at 3 months ($p<0.05$). CECs and EPCs fell by 3 months but returned to pre-surgery levels at 6 months ($p<0.05$). VEGF remained lower throughout except after anti-VEGF, increasing to pre-surgery levels. After standard chemotherapy, sE-sel was higher than baseline.

Conclusions: The changes with CECs and EPCs regardless of treatment strategy after surgery, VEGF with anti-angiogenic therapy, and sE-selectin and angiogenin with standard chemotherapy may have clinical and pathophysiological implications.

Background

Studies on the serial changes of CECs and EPCs were isolated to metastatic CRC and reported as good predictors of outcomes (28, 143, 201, 463). A fall in EPCs during treatment was associated with improved progression-free survival (28, 147, 201) while Ronzoni et al proposed the pre-treatment levels, not the fold changes, as better surrogates (201). Low VEGF was reported by Hyodo et al as predictive of non-responders to chemotherapy (274), but later refuted by Berglund et al (464). The value of the changes in vWf, sE-sel and angiogenin after surgery were unknown.

Having described the changes of markers of the angiome and endotheliome in CRC stage (sections 3.1.2 & 3.1.3), their changes over time after surgery, with or without adjuvant therapy were analyzed. All markers were predicted to fall after surgery. Participants receiving adjuvant treatment (that is, they had histological predictors of recurrence as stated section 1.4.2, page 72) were hypothesised to have fewer changes than those treated with surgery only (as most had favourable histology), though not planned in my original hypothesis.

Methods

Participants treated for CRC and monitored thereafter, were recruited. Peripheral blood was taken before surgery and again at 3 and 6 months. CECs (CD34+CD45-CD146+) and EPCs (CD34+CD45-KDR+) were measured by 4-colour flow cytometry and the plasma makers by ELISA assays as for the cross-sectional studies

(described in section 2.2, SOPs detailed in appendices 3 to 7). The exclusion criteria of section 2.1.3 (page 95) were also applied to the repeat measurements.

The options for those needing adjuvant chemotherapy were diverse but nearly all received the modified de Gromant regime (folinic acid and 5-fluorouracil [5-FU] or its oral equivalent, capecitibine), while only 4 with Dukes' C had FOLFOX (5-FU, folinic acid and oxaloplatin). These two regimes were evidence-based, and protocolled by the oncology departments; they were therefore referred to as 'Standard Chemotherapy' (see section 1.4.3 and appendix 8, page 291). Another group received anti-VEGF therapy, predominantly as part of the QUASAR 2 trial, and for metastatic disease (Dukes' D). The trial period overlapped with my study and fortunately the hospital from which most of my participants were drawn, was one of the top 3 recruitment centers in the UK. It allowed the effects on the markers to be measured with the addition of the anti-VEGF agent Bevacuximab to standard chemotherapy. Therefore three categories were analysed (see figure 29):

1. Surgery alone i.e. no adjuvant treatment was needed
2. Surgery plus adjuvant therapy with standard chemotherapy (intravenous 5-fluorouracil [5-FU]) or Capecitabine (oral 5-FU equivalent)
3. Surgery plus adjuvant therapy with anti-VEGF (Bevacuximab, Avastin®).

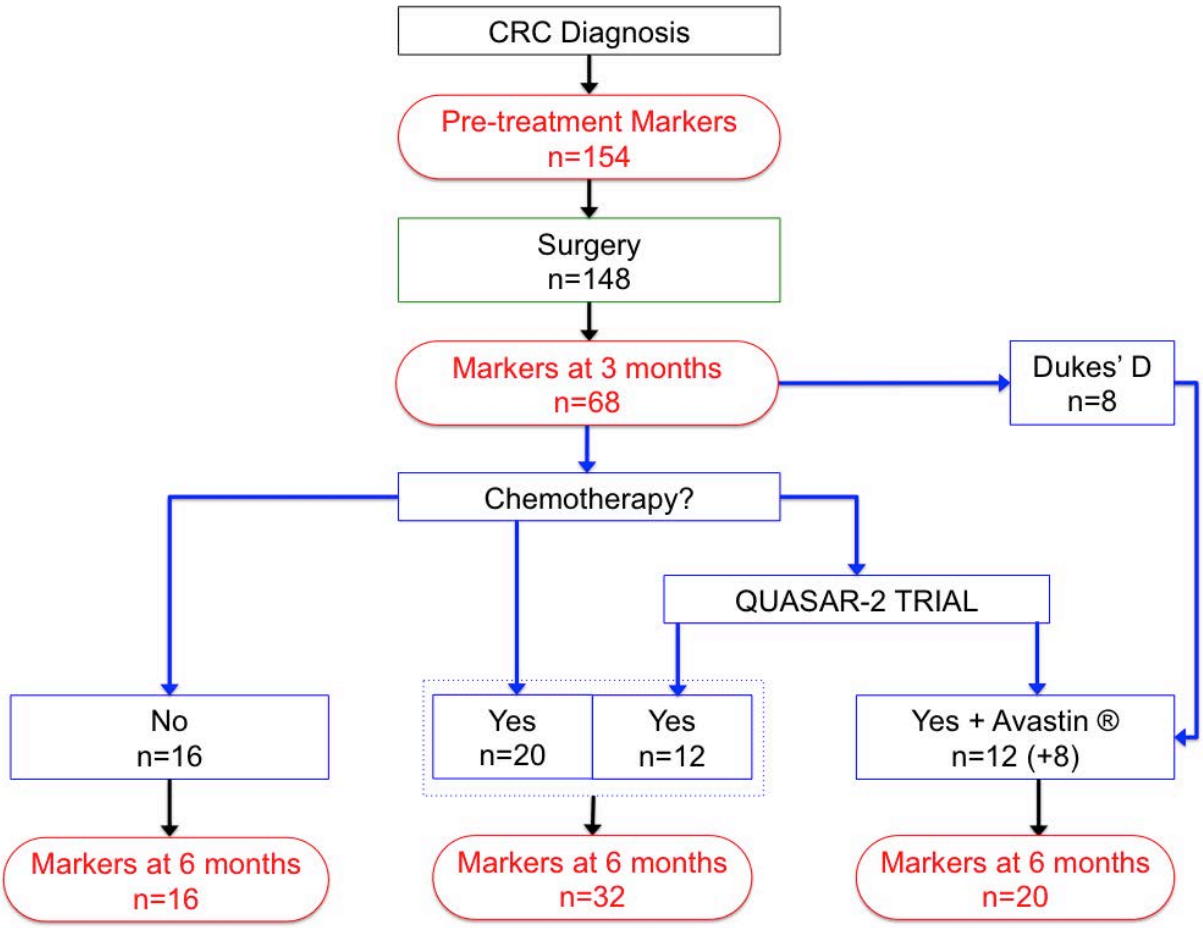
After adjuvant treatment, markers were typically measured 2 to 4 weeks after completion but excluded those with significant biochemical or haematological abnormalities (see 'exclusion criteria, section 2.1.3, page 95). For Dukes' D disease treatment continued beyond 6 months where applicable, and therefore, levels were

measured while on a break at six months and 2 to 4 weeks after the last cycle. Of the 154 patients recruited, 114 were approached to participate. Those on adjuvant treatment unable to complete their course were excluded. After applying the exclusion criteria (section 2.1.3 page 95), and receiving consent, 68 were recruited. Subgroups were as follows (see figure 29): 1). n=16 for surgery only, 2). n=32 for surgery followed by standard chemotherapy, and 3). n=20 for surgery followed by standard chemotherapy plus anti-VEGF therapy (Bevacizumab, Avastin®). Changes of the indices from pre-surgery to 3 months (range 2.6 – 3.8 months, before starting adjuvant treatment, if given) and then to 6 months (range 5.5 – 8.7 months, and where applicable, 2-3 weeks after completing chemotherapy) were analysed.

Statistics

For the power calculation, surgery was predicted to reduce the CEC and EPC counts by a third, as found in by Goon et al in breast cancer (24). That is from a median (IQR) of 3 (1.5 – 5.0) cells/mL CEC levels were expected to fall to 2 (0.5 – 3.5) cells/ml after treatment. If numbers fell in 21 patients, but rose in 4 patients (25 participants in total), then the calculated paired t-test would be significant at p=0.003. Results were expressed as numbers and percentages, mean and standard deviation or median and interquartile range. Analyses between groups were performed using ANOVA, Kruskal-Wallis, Mann-Whitney, t-test and Chi-squared as appropriate. Data over time was analysed by repeated measures (two-way) analysis of variance (for parametric data) or by Friedman’s method (for non-parametric data).

Figure 35: Study outline of numbers recruited to the serial groups.



Results

Clinical and demographic data are shown in table 34. The 'serial' group was effectively matched to the 'non-serial' group, except in sex, CECs and sE-sel levels (see table 34 and 35). The unmatched factors were not influenced by Dukes' stages (p=0.630). The differences were probably from multiple testing and therefore not knowingly a selected subgroup unrepresentative of CRC.

Table 35: Tumour characteristics of the serial versus the non-serial groups

	All Cancers	Non-serial Group	Serial Group	P value
Number	154	86 (56%)	68 (44%)	
Age (SD) years	73 (9)	73 (10)	70 (8)	0.064
M:F ratio	86:78	37:31	49:37	**<0.001**
Diagnosis				
Screening	12	7	5	1.00
Symptomatic	142	79	63	
Family history of CRC*	40	28	12	0.313
Histological Grade				
Well differentiated	7	3	4	0.857
Well/Moderate	67	39	28	
Moderate	70	38	32	
Poor	10	6	4	
Tumour Type				
Adenocarcinoma	126	79	57	0.219
Mucinous	24	13	11	
Unknown	4	4	0	
Vascular Invasion				
Yes	29	18	11	0.149
No	96	48	48	
Unknown	29	20	9	
Dukes'				
A	19	8	12	0.630
B	54	31	23	
C	49	29	20	
D	32	18	13	

Values were expressed as number of cases and p values by Chi-Squared test.
* First degree relatives with CRC. SD-standard deviation

Table 36: Differences of measured indices of the endotheliome and angiome between the serial and non-serial groups before treatment.

	Non-serial Group n=86	Serial Group n=68	P value
CEC cells/ml	12 (0-30)	10 (0-20)	0.295
EPC cells/ml	24 (12-45)	17 (2-40)	0.090
CD34+CD45- x 10^3 cells/ml	0.80 (0.44-1.18)	0.79 (0.55-1.15)	0.602
WCC (10^6/ml)	7.09 (2.06)	7.28 (1.93)	0.476
EPC:CEC ratio	1.5 (1.0-4.8)	1.1 (1.0-9.7)	0.809
vWf [IU/dL]	117 (24)	121 (30)	0.810
sE-sel [ng/mL]	23 (16-32)	30 (24-37)	**0.003**
VEGF [pg/mL]	180 (30-610)	310 (30-771)	0.333
Ang [ng/mL]	321 (122-376)	305 (262-352)	0.404

Data presented as mean (standard deviation) or median (interquartile range), p value by t-test or Mann-Whitney's with significance <0.05.

The results after surgery in the 68 patients are shown in table 36. As hypothesised, all measured markers were significantly reduced except sE-selectin (p=0.067 at 3 months, p=0.973 at 6 months), vWf (p=0.113 at 6 months) and WCC (p=0.176 at 6 months). This reduction was consistent with the hypothesised effect from 'debulking' of the cancer. The unchanged indices of EC activation (vWf) were still higher (p<0.01) than those of the HC group (table 27).

Table 37: Changes in the measured indices of the endotheliome and angiome in all 68 patients before and (3 and 6 months) after treatment.

	Pre-treatment	3 months	6 months	Overall P value
CEC cells/ml	10 (0-20)	0 [A*] (0-10)	9 [B*, C] (0-11)	**<0.001**
EPC cells/ml	17 (2 - 40)	0 (0-9)	10 [B*, C] (2-19)	**0.003**
WCC x 10^6 cells/ml	7.29 (1.93)	5.39 [A*] (1.47)	5.76 [C*] (1.67)	**<0.001**
EPC:CEC ratio	1.1 (1.0-9.7)	1.0 [A] (1.0-1.0)	1.0 [B] (1.0-7.7)	**0.016**
CD 34+ CD45- x 10^3 cells/ml	0.81 (0.62-1.24)	0.83 (0.62-1.31)	0.64 (0.32-0.94)	0.081
vWf [IU/dL]	121 (30)	110 [A] (25)	120 [B] (25)	**0.033**
sEsel [ng/mL]	30 (24-36)	28 (16-32)	25 [C] (19-32)	0.567
VEGF [pg/mL]	310 (30-771)	23 [A*] (12-51)	40 [B*, C] (20-534)	**<0.001**
Ang [ng/mL]	305 (261-352)	242 [A*] (179-294)	323 [B*] (249-396)	**<0.001**

Data is presented as mean (standard deviation) or median (interquartile range) and p values between time points by ANOVA of repeated measures. P values of < 0.05 were [A], [B] (from 3 to 6 months) and [C] (from pre-treatment to 6 months). P values of <0.001 were [A*], [B*] (from 3 to 6 months) and [C*] (from pre-treatment to 6 months).

Participants who required adjuvant therapy had tumours with local organ invasion, perforation, lymph node spread, vascular invasion or distant metastases (285). Hence as a group with poor prognostic factors and as hypothesized, significantly more perturbed indices were found except of CD34+CD45- cells (table 37).

Table 38: Preoperative characteristics of participants after surgery receiving no further treatment versus those receiving adjuvant therapy.

	Surgery only n=16	**Surgery + Adjuvant treatment n=52**	**P value**
Vascular Invasion			
Yes	0	34	**<0.001**
No	16	11	
Unknown	0	7	
Dukes'			
A	10	2	**0.003**
B	6	17	
C	-	20	
D	-	13	
CEC cells/ml	4 (0-19)	12 (0-20)	**0.002**
EPC cells/ml	14 (0-39)	19 (10-42)	**<0.001**
CD34+CD45- cells/ml	0.92 (0.64-1.22)	0.83 (0.53-1.22)	0.395
WCC (10^6/ml)	6.68 (1.57)	7.47 (2.04)	**<0.001**
EPC : CEC ratio	1.08 (0.82-18.65)	1.13 (1.00-5.63)	**<0.001**
vWf [IU/dL]	115 (22)	123 (31)	**<0.001**
sEsel [ng/mL]	30 (24-35)	30 (23-37)	**<0.001**
VEGF [pg/mL]	120 (10-494)	374 (33-1128)	**<0.001**
Ang [ng/mL]	295 (267-350)	306 (253-357)	**<0.001**

Data presented as mean (standard deviation) or median (interquartile range), overall p value by t-test, Mann-Whitney, or Chi-Squared test; significance of $p<0.05$.

For patients undergoing surgical follow-up only (table 38), all indices fell after 3 months except WCC, sE-sel and vWf. CECs rose after 6 months (p=0.045).

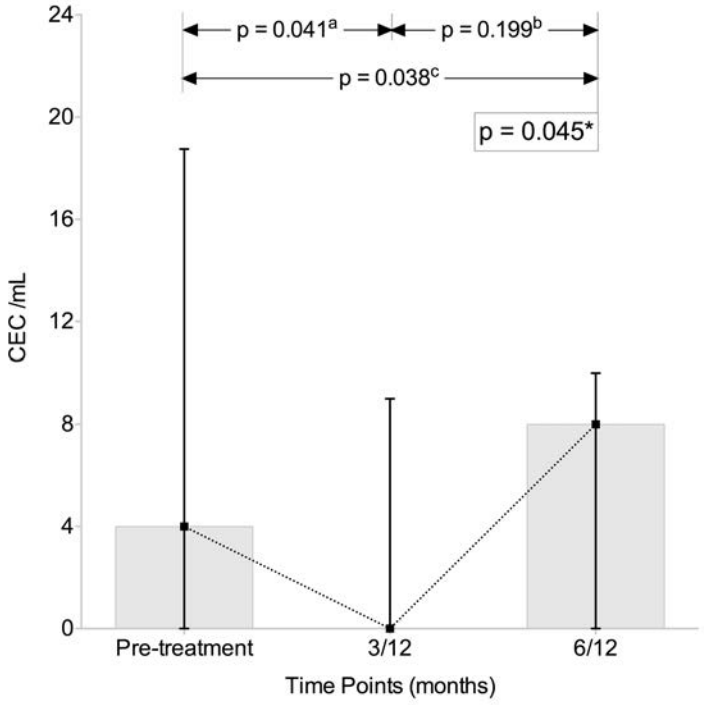
Table 39: Research indices in 16 patients in Group 1 (Surgery only)

	Baseline	3 months	6 months	P value
CECs (cells/mL)	4 (0-19)	0 (0-9)	8 (0-10)	**0.045**
EPCs (cells/mL)	13 (0-39)	0 (0-9)	8 (0-10)	**0.036**[a]
vWf (IU/dL)	115 (22)	106 (19)	118 (30)	0.441
sE-selectin (ng/mL)	31 (9)	26 (9)	29 (11)	0.302
VEGF (pg/mL)	120 (10-494)	23 (6-23)	16 (5-82)	**0.024**[b]
Angiogenin (ng/mL)	308 (75)	235 (97)	300 (96)	0.061
CD34+CD45- cells (cells/mL)	0.9 (0.6-1.2)	1.1 (0.7-1.6)	0.9 (0.5-1.3)	0.395
WCC (x10^6/mL)	6.7 (1.6)	5.7 (1.7)	6.2 (1.3)	0.175

Data are mean (standard deviation) or median (interquartile range). P value by ANOVA, general linear model or Tukey's post-hoc test. [a] $p<0.05$ baseline to 3 month. [b] $p<0.05$ baseline to 3 month and 6 month.

There were no significant changes in vWf, sE-sel, angiogenin, CD34+CD45- cells or WCC. CECs fell significantly overall, except between 3 and 6 months (figure 36).

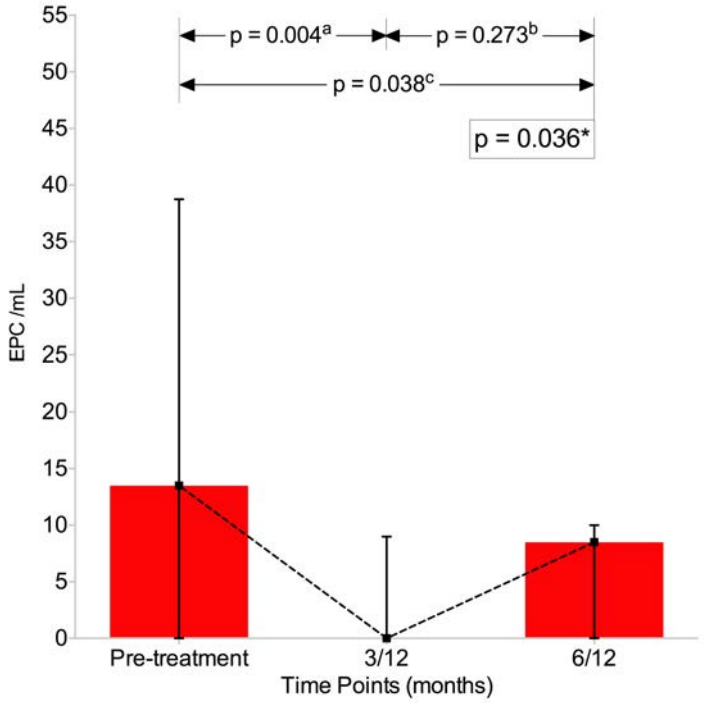
Figure 36. Serial Changes in CECs: Surgery only.

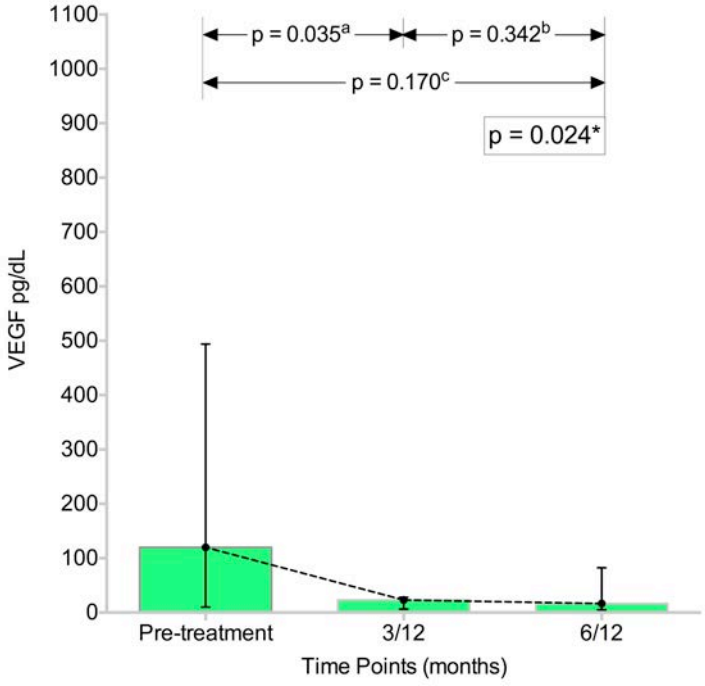


* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively. Boxes= median, Interval bars= Interquartile ranges.

EPCs and VEGF fell from baseline to 3 months (both $p<0.05$). At 6 months, VEGF remained low ($p<0.05$), but the EPC was similar to baseline (see figure 37).

Figure 37: Serial changes in EPCs & VEGF for Surgical Treatment only.





* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively.
Boxes= median, Interval bars= Interquartile ranges.

The picture was quite different when the indices were examined for those undergoing standard chemotherapy after surgery (table 39).

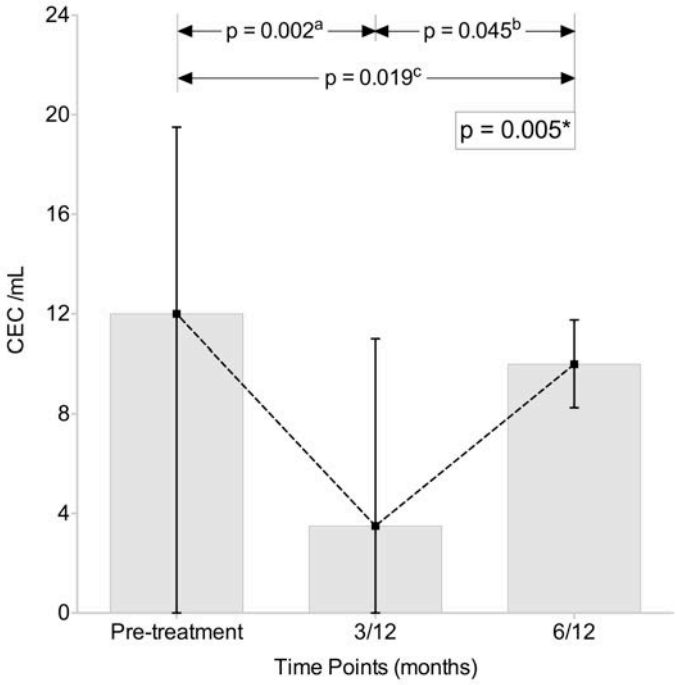
Table 40: Research indices Group 2 (Surgery + chemotherapy), n=32.

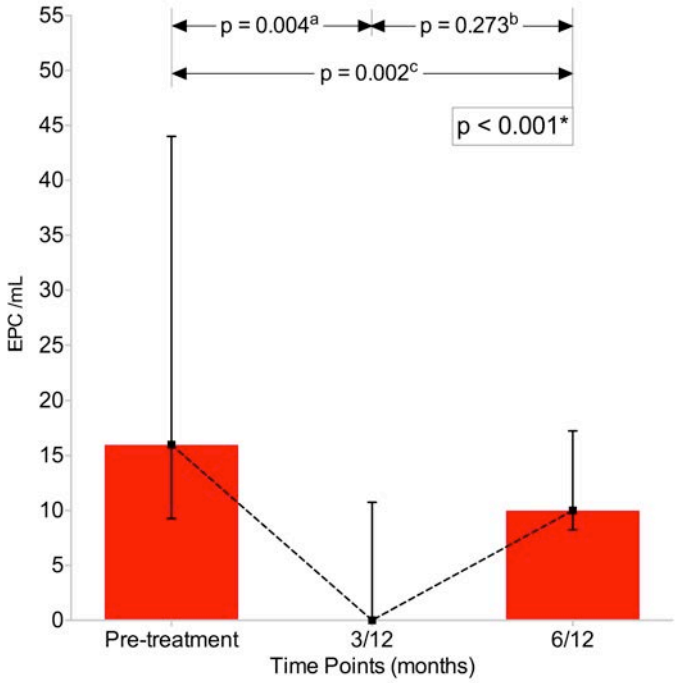
	Baseline	3 months	6 months	P value
CECs (cells/ml)	12 (0-19)	3 (0-11)	10 (0-12)	**0.019**[a]
EPCs (cells/ml)	16 (9-44)	0 (0-11)	10 (8-15)	**<0.001**[b]
vWf (IU/dl)	123 (33)	106 (25)	119 (28)	0.08
sE-sel (ng/ml)	34 (12)	38 (9)	24 (10)	**0.007**[c]
VEGF (pg/ml)	320 (30-1200)	30 (20-287)	30 (21-310)	**0.001**[d]
Angiogenin (ng/ml)	282 (97)	231 (93)	346 (109)	**<0.001**[e]
CD34+CD45- cells (x 10^3 cells/ml)	0.81 (0.40-1.31)	0.83 (0.62-1.21)	0.74 (0.44-1.12)	0.304
WCC (x10^6/ml)	7.93 (2.22)	5.44 (1.31)	5.82 (2.00)	**<0.001**[d]

Data are mean (standard deviation) or median (interquartile range). P value by ANOVA/general linear model/Tukey's post-hoc test. [a]p<0.05 baseline to 3 month point. [b]p<0.01 baseline to 3 months, p<0.05 3 months to 6 months. [c]p<0.05 3 moths to 6 months. [d]p<0.01 Baseline to 3 months and to 6 months. [e]p<0.05 Baseline to 6 months, p<0.01 3 months to 6 months.

CECs were lower at 3 months (p<0.05), but no different to baseline at 6 months. EPCs fell at 3 months (p<0.01) but returned to near baseline at 6 months (p<0.05). VEGF were lower at 3 and 6 months (both p<0.01, see figure 39) and sE-sel was lower at 6 months versus baseline and 3 months (p<0.05). Angiogenin and WCC fell from baseline to 3 months and increased at 6 months (figure 40).

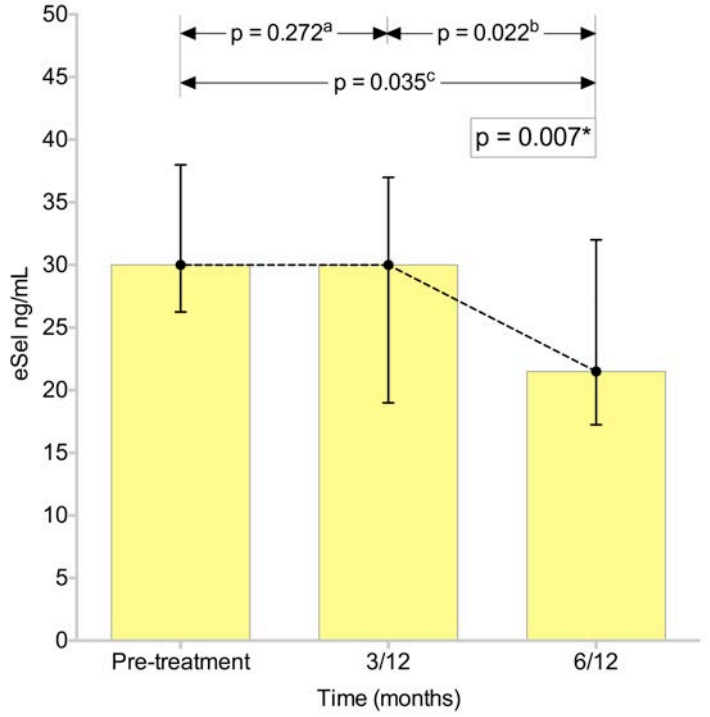
Figures 38: Serial changes in CECs and EPCs for Surgery + Standard Chemotherapy (n=32).

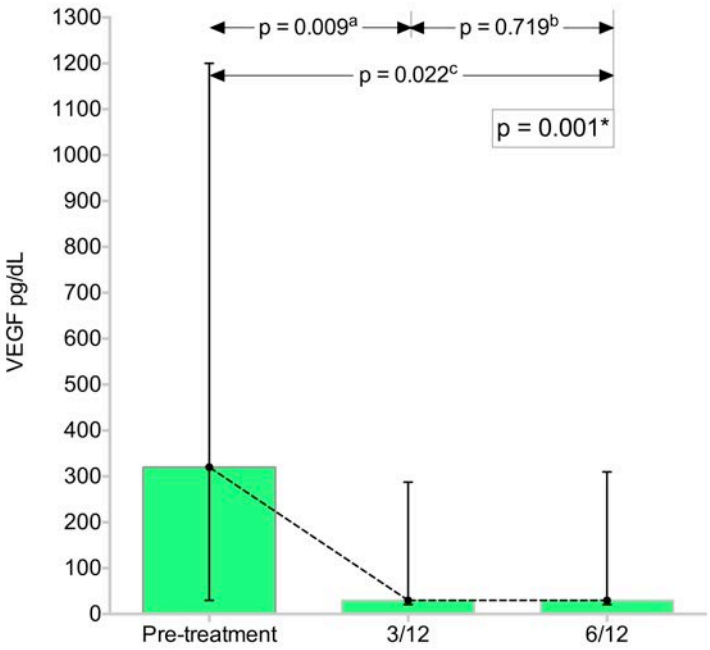




* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively.
Boxes= median, Interval bars= Interquartile ranges.

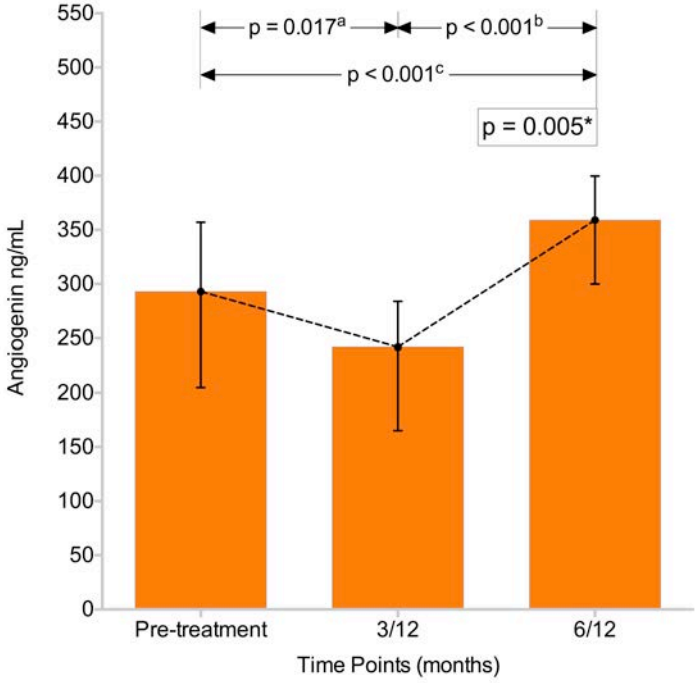
Figures 39: Serial changes in eSel and VEGF for Surgery + Standard Chemotherapy (n=32).

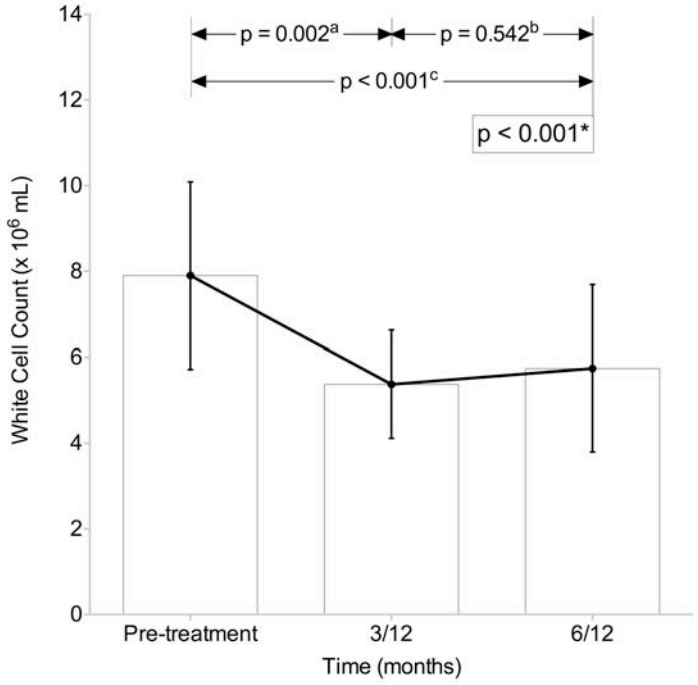




* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively.
Boxes= median, Interval bars= Interquartile ranges.

Figure 40: Serial changes in Angiogenin and WCC for Surgery + Standard Chemotherapy (n=32).





* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively.
Boxes= median or mean, Interval bars= Interquartile ranges or standard deviation.

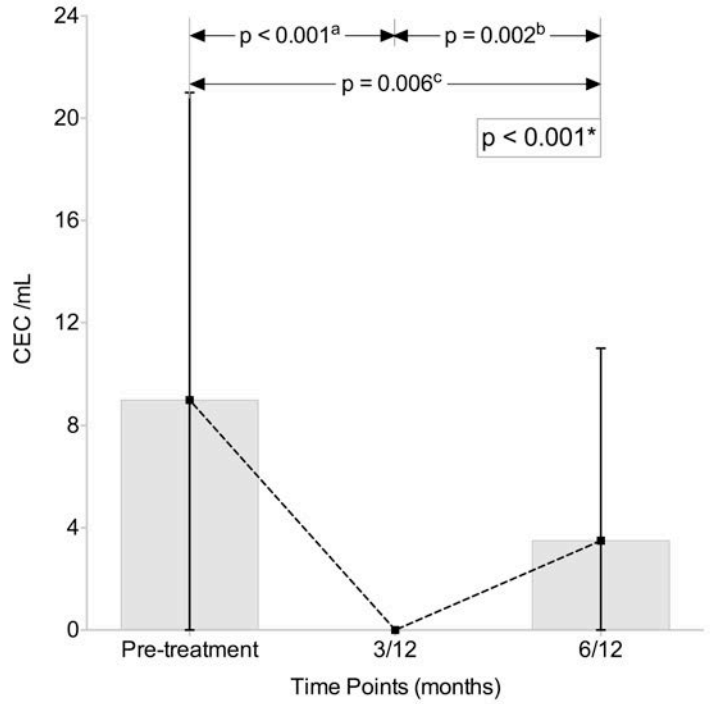
Bevacuximab added to the chemotherapy regime is examined in table 40. CECs numbers fell to 3 months (p<0.01), then increased to 6 months (p<0.05). EPC and VEGF levels fell at 3 months and then increased at 6 months (all p<0.01). There were no changes in vWf or angiogenin. The CD34+CD45- cells were lower at 6 months than at baseline, and the WCC fell at 3 months (figures 41 – 43).

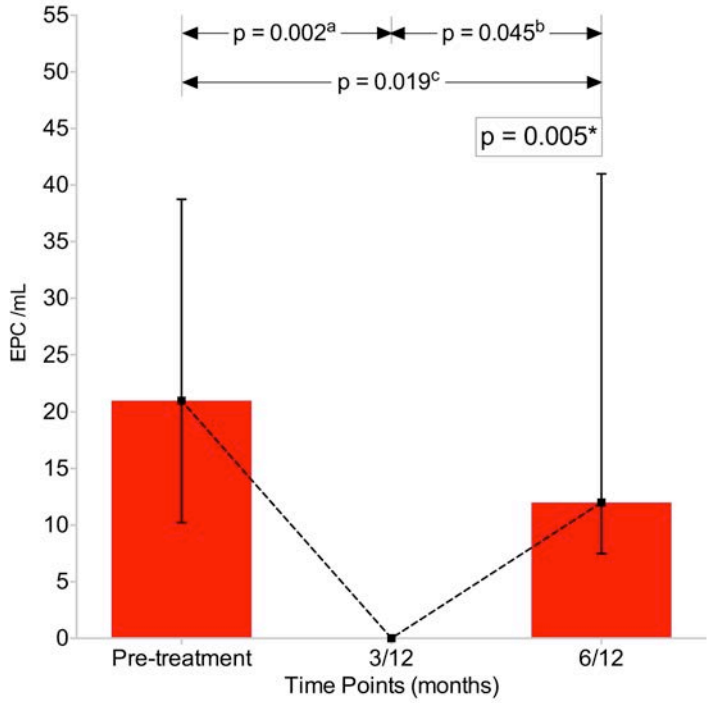
Table 41: Research indices in 20 patients: surgery followed by standard chemotherapy and anti-angiogenic therapy

	Baseline	3 months	6 months	P value
CECs (cells/mL)	21 (7-60)	0 (0-13)	11 (7-48)	**0.005**[a]
EPCs (cells/mL)	21 (10-39)	0 (0-0)	12 (7-32)	**<0.001**[b]
vWf (IU/dL)	121 (30)	122 (26)	122 (15)	0.943
sE-selectin (ng/mL)	26 (8)	22 (15)	29 (12)	0.195
VEGF (pg/mL)	500 (40-1000)	30 (23-55)	770 (630-1900)	**<0.001**[b]
Angiogenin (ng/mL)	319 (97)	255 (117)	317 (87)	0.074
CD34+CD45- cells (cells/mL)	0.8 (0.6-1.1)	0.6 (0.4-1.1)	0.3 (0.2-0.5)	**0.003**[c]
WCC (x10^6/mL)	6.7 (1.5)	5.1 (1.7)	5.3 (1.4)	**0.003**

Data are mean (SD) or median (IQR). P value by ANOVA/general linear model/Tukey's post-hoc test [a] p<0.01 Baseline to 3 months, p<0.05 3 to 6 months. [b] p<0.01 Baseline to 3 months and baseline to 6 months. [c] p<0.01 Baseline to 6 months.

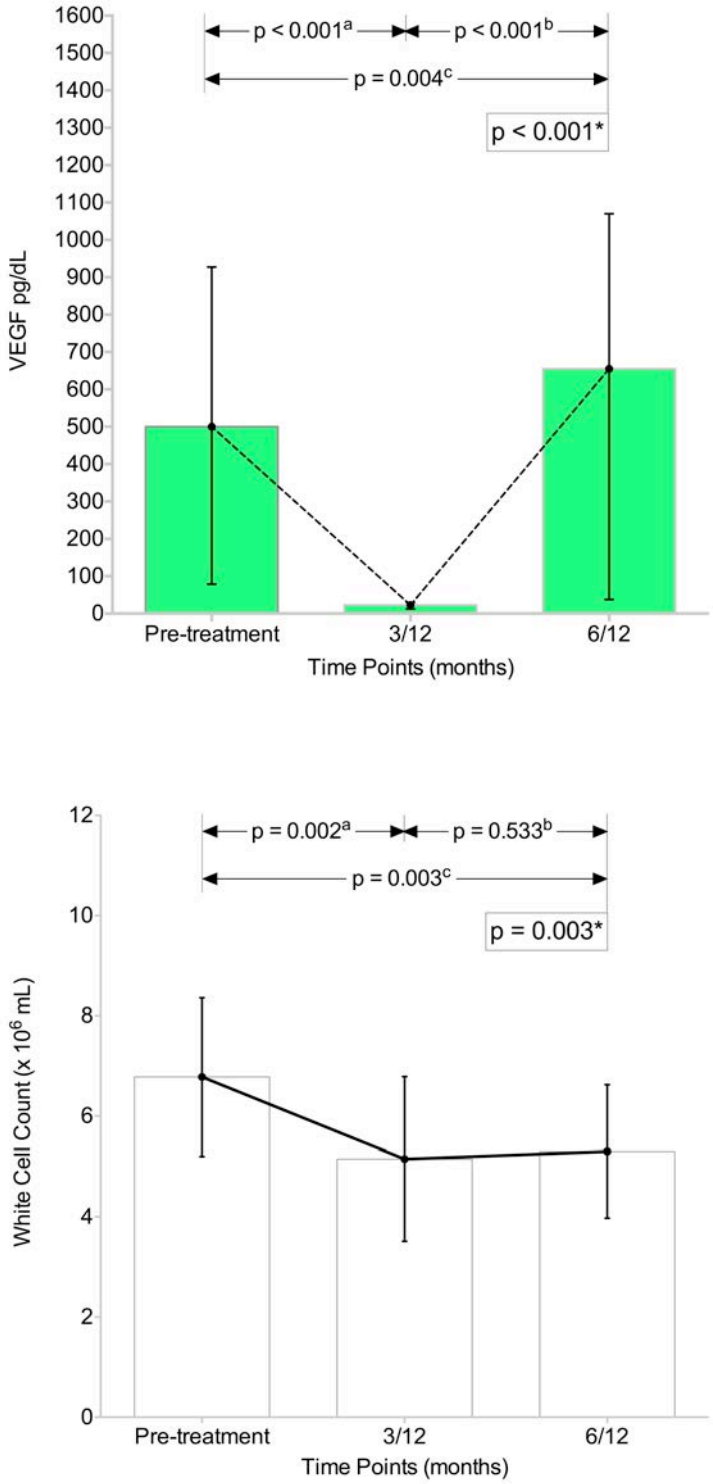
Figure 41: Serial changes in CECs and EPCs for Surgery + Standard Chemotherapy + Bevacizumab (n=20).





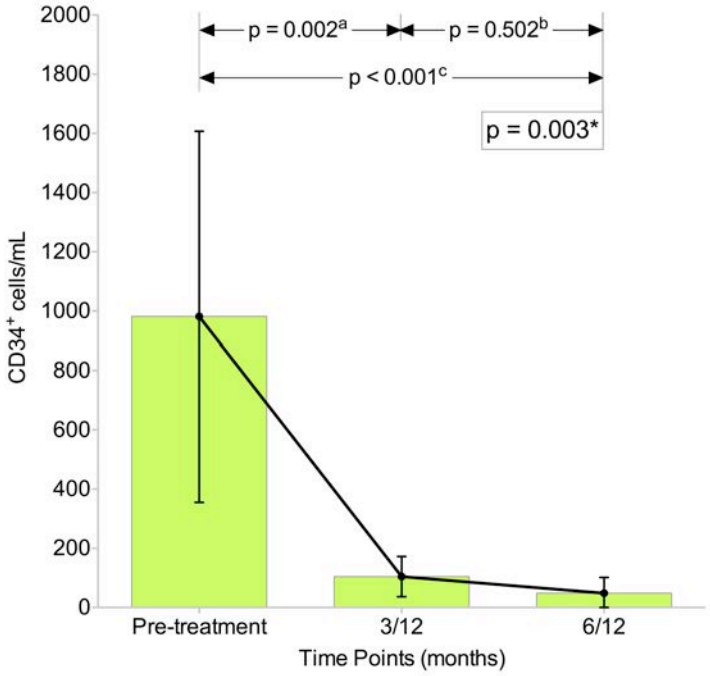
* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively. Boxes= median, interval bars= IQR.

Figure 42: Serial changes in VEGF and WCC for Surgery + Standard Chemotherapy + Bevacizumab (n=20).



* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively. Boxes= median or mean, Interval bars= IQR or S.D.

Figure 43: Serial changes in CD34+CD45- cells for Surgery + Standard chemotherapy + Bevacizumab (n=20).



* Overall p value by value by ANOVA/general linear model/Tukey's post-hoc test.
[a b c] p values by Wilcoxon test between 0 to 3 months, 3 to 6 months and 0 to 6 months respectively. Boxes= mean, Interval bars= S.D.

Relationships were examined between indices regardless of treatment modality. At baseline, significant Spearman correlations were of EPCs with CECs (r=0.70, p<0.001), VEGF with CECs (r=0.55, p<0.001) and VEGF with EPCs (r=0.69, p<0.001). These relationships were still present at 3 months, though less so, at r= 0.52 (p=0.001), r=0.28 (p=0.019) and r=0.25 (p=0.037) respectively. The latter two may be false negatives as they were only powered for r>0.4. At 6 months only CEC with EPC (r=0.45, p=0.001) and VEGF with EPCs (r=0.25, p=0.037) were significant.

Discussion

At submission, this thesis was the first comprehensive study of a panel of cellular and plasma markers of EC activity with CRC treatment. Having found elevated EPCs, CECs, angiogenin, VEGF and to a lesser extent vWf in mCRC, the effect of surgery, with or without adjuvant treatment, was analysed. This tested the hypothesis that levels fell to that of healthy controls at 3 months after surgery and remained low at 6 months. Debulking of CRC certainly reduced all measured indices at 3 months, similarly reported by Goon et al, in breast (24, 140). From the literature, the changes of these markers regardless of CRC stage and with adjuvant therapy, was reported only for VEGF (456) and vWf (14), though their relevance was inconclusive.

CEC and EPC measurements were isolated to studies on metastatic disease only but changes with treatment informed outcome, probably as they reflected Dukes' Stage (28, 143, 201, 463). Cross-comparisons between studies were hindered by the lack of consensus on the definitions with the 'best fit' of CD markers (217, 268, 399, 465). Nevertheless, EPCs, given their partial mobilisation by VEGF, may be good surrogates of anti-VEGF treatment, with Matsusaka et al reporting better outcomes when levels (CD45-CD31+CD34+CD133+ cells) fell below 0.04% at day 4 of treatment (28, 147, 201). Ronzoni measured changes at baseline before chemotherapy and then again at the third and sixth cycles, with baseline levels of CEC < 40 /ml associated with better outcomes (201). Variable and random time points were chosen by other studies on anti-VEGF treatment, probably based on the convenience of sampling with each cycle. Nevertheless, most reported pre-treatment or baseline levels were better indicators of response (441, 466). The choice of time

points in my study was deliberate to avoid the possible effects of chemotherapy on EC activity during treatment. Therefore, they were measured before and after treatment, except in Dukes' D disease when treatment continued, hence levels were measured while on a break at six months and 2 weeks after the last cycle.

Strikingly, after surgery with or without adjuvant standard chemotherapy and regardless of the addition of Bevacizumab, both CECs and EPCs increased nearly to that of baseline median levels. This was unexpected given the correlation of both markers to VEGF, which, as shown in this study, fell with tumour debulking. The correlation with VEGF also continued up to 6 months and more so with EPCs (albeit measured in smaller groups). The effect in participants who underwent surgery without adjuvant therapy may reflect angiogenesis for physiological repair rather than residual tumour (only 1 participant with recurrence at 2 years). This may also occurred with the addition of adjuvant therapy or the result of drug-related injury to and subsequent repair of the endothelium. Nevertheless, chemotherapy, once completed, did not appear to hinder angiogenesis in physiological repair required after surgery, as reflected by a rise in EPC levels.

While the WCC generally fell after surgery and remained low regardless of treatment, CD34+CD45- cells fell only when anti-VEGF therapy was included with standard chemotherapy. The reasons for this were unrelated to EPCs or VEGF (both of which were higher at 6 months) and therefore uncertain. As postulated in 5.2.4, the CD34+CD45- fraction may include other progenitors e.g. of fibroblasts, and their demand in repair, probably from the blockage of angiogenesis, was also reduced. Once again this was the first study on this fraction of CD34+ cells and their reduction

may be more clinically relevant to the effect of anti-VEGF than measuring VEGF alone. That is, tumour excision reduced levels of VEGF in all groups but not in those receiving anti-VEGF. The higher levels at 6 months may be a rebound phenomenon, as bloods may have been taken after completing Bevacizumab and when the therapeutic effect (half-life of 20 days, range 11-50 days) at blocking VEGF was significantly diminished (467). Other authors with similar findings could not suggest this, presumably as they, like me, did not measure drug and VEGF levels together after treatment. Yang et al also reported a paradoxical increase in VEGF and plausibly suggested that the ELISAs measured both free and antibody-bound VEGF (468). I was unaware of any assay separately measure the two factors.

With angiogenin found to be a good ‘identifier’ of CRC and its stage in this study, unsurprisingly it fell at 3 months but inexplicably rose to pre-treatment levels at 6 months in all groups except in standard chemotherapy for whom levels exceeded that of the baseline. This result was not previously reported and, while more likely from residual tumour at 6 months, may also have arisen from the effects of treatment on other somatic cells (469). Levels of sE-sel and vWf also fell after surgery similar to that reported by Gil-Bazo et al and Sato et al respectively (14, 15). Unlike the other markers already discussed, the differences were not as apparent and showed no clear pattern. This may reflect the poorer capacity of these markers to measure EC activity, the damaging effects on ECs by standard chemotherapy with or without Bevacizumab, or the unpredictable disturbances to the EC from surgery.

In summary, within the limitations of the small numbers of each group, changes with some of the measured indices were highly significant though not entirely explicable. A fall in CD34+CD45- cells may have clinical importance as a better discriminator of the effectiveness of anti-VEGF treatment than EPCs or CECs, both of which fell at 3 months and rose again at 6 months. Their measurements at two time points after treatment were not as informative as levels before surgery.

Disease progression and survival are examined next.

3.2.2 The Endotheliome and Angiome in CRC Prognosis

Abstract

Background: Pre-treatment levels of the markers of the endotheliome and angiome individually may predict outcomes after CRC treatment. However their collective value was unknown. I hypothesized that the markers together and measured before surgery, were important determinants of outcomes than any single test.

Methods: Peripheral blood from 154 participants with CRC was analyzed before surgery. CECs (CD34+CD45-CD146+) and EPCs (CD34+CD45-KDR+) were measured by 4-colour flow cytometry and the plasma makers by ELISA.

Findings: Pre-surgery CECs and EPCs levels were higher in participants with recurrence in 2 years. However, Dukes' and more so mAJCC stages were still the best independent predictors of progression free survival (PFS) and time to progression (TTP). Mathematical models that combined mAJCC stage with CECs and EPCs (when greater than median levels) may predict TTP within 2 years. With adjuvant therapy, there were no significant fold changes of CECs, EPCs or angiogenin in participants with poorer TTP or PFS outcomes.

Conclusions: Models incorporating markers of the endotheliome before surgery with the CRC stage may better predict progression within 2 years than Dukes' or mAJCC staging alone. As a clinical tool, the models may aid risk stratification and, alongside traditional determinants, decisions for adjuvant treatment.

Background

Having described the importance of the markers of the angiome and endotheliome in CRC staging (sections 3.1.2 & 3.1.3), their value in determining outcomes was analyzed. Fewer CECs and EPCs in metastatic CRC were previously reported as good predictors of progression (28, 143, 201, 463). A fall in EPCs during treatment was associated with improved PFS (28, 147, 201) while Ronzoni et al proposed the pre-treatment levels, not the fold changes, as better surrogates (201). Disease progression was also associated with levels of VEGF (366), vWf (16, 470), angiogenin (364) and less so, sE-selectin ((369). The collective value of these factors in predicting outcomes after surgery was unknown. Therefore I hypothesized that, when measured before surgery, and regardless of adjuvant strategies, the markers together were important determinants of outcomes than any single test.

Methods

The markers measured in participants with CRC recruited to test hypotheses 1 to 3 for the cross-sectional studies, were analyzed. That is, peripheral blood taken before surgery was tested by 4-colour flow cytometry for CECs (CD34+CD45-CD146+) and EPCs (CD34+CD45-KDR+); the plasma makers were quantified by ELISA assays (detailed in section 2.2, the exclusion criteria in section 2.1.3 and the methods of sections 3.1.2 and 3.1.4). Data on outcomes were prospectively collected over the 2-year period after surgery (see details in section 2.3.2, page 102-106).

Statistics

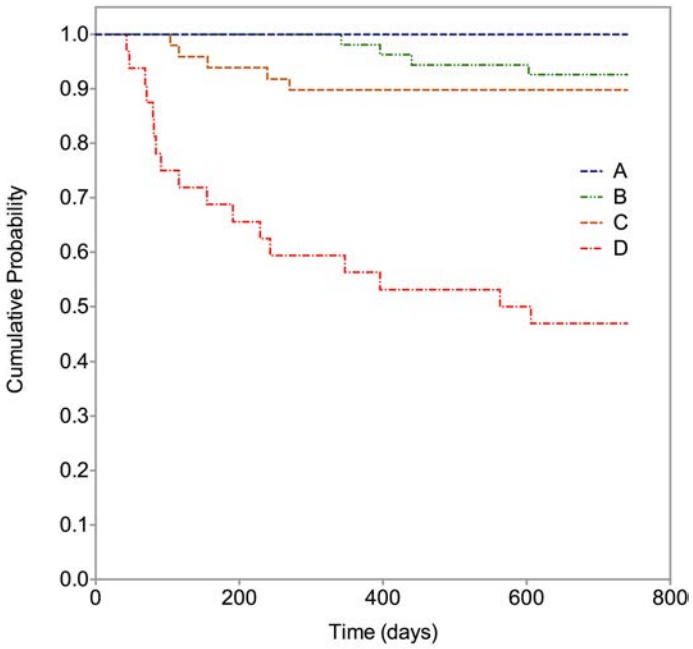
Data was presented as mean and standard deviation (when normally distributed) or median and inter-quartile range (non-parametric distribution). The Chi-squared test was used for categorical data. Data was analysed using binary regression and Cox proportional hazards were estimated, and outcomes over time presented as Kaplan-Meier plots. All analyses were performed on SPSS and figures produced by Prism 6 package. A $p<0.05$ was taken to assume significance.

Results

Survival

The cumulative 2-year mortality (OS) is shown on the Kaplan Meier chart, figure 44, and was consistent with previous reports (471). OS after 740 days for stages A, B, C and D were 100%, 92.6%, 89.8% and 46.9% respectively ($p<0.001$).

Figure 44: Kaplan Meier Plots of 2-year cumulative survival and Dukes' Stage



Progression

Progression for this study, also referred to as TTP, was defined according to the widely accepted criteria of Eisenhauer et al (381, 382). It used 'measurable' evidence (X-rays, USS, CT, MRI, PET-CT or biopsy) of one of the following:

- Local recurrence or metastases after surgery with curative intent and/or
- For Dukes' D, increasing tumour size of involved organs and/or new metastases, specifically of at least a 20% increase in the sum of diameters of lesions.

Those with disease progression within 2 years were found to have significantly higher levels of CECs and EPCs only (see table 41). However both were poor on ROC analysis. For CEC > 10 per ml. sensitivity was 59%, specificity 66%, and ROC area of 0.621 with p=0.351 LR=1.08; for EPCs > 12 per ml, sensitivity was 67%, specificity 50%, and ROC area of 0.639 with p=0.110 LR=1.33).

Further analysis of the indices by binary logistic regression did not reveal any correlations with disease progression or histological features of poor prognosis (not shown). Only Dukes' and mAJCC stages remained the best independent predictors of recurrence within 2 years (table 45). Both retained significance after adjustment for other indices i.e. Dukes' stage OR was 2.28 (95% confidence interval 1.01-5.14), p=0.047; for mAJCC stage OR was 4.78 (1.98-11.55) and p<0.001.

Table 42: Pre-treatment markers and CRC progression within 2 years.

	Preoperative Values		
	No Progression	**Progression**	**P value**
Number	103	51	
Sex (male/female)	54/49	32/19	0.225
Dukes' stage n: Stage A/B/C/D	17/48/37/1	2/6/12/31	**<0.001**
Modified AJCC stage n: 1,2,3,4 [1]	19/35/44/5	0/6/11/34	**<0.001**
Treatment group n: A/C/S [2]	18/39/45	11/35/6	**<0.001**
CEC cells/ml	10 (0 - 21)	19 (9-29)	**0.019**
EPC cells/ml	20 (9-38)	30 (12-61)	**0.004**
WCC (10^6/ml)	6.97 (2.01)	7.60 (1.94)	0.066
EPC:CEC ratio	0.5 (0-1)	0.5 (0.1-1.0)	0.964
vWf [IU/dL]	116 (25)	125 (29)	0.060
sE-sel [ng/mL]	28 (19-34)	25 (18-34)	0.566
VEGF [pg/mL]	190 (30-560)	330 (30-778)	0.253
Ang [ng/mL]	300 (101)	330 (93)	0.069

Data presented as mean with standard deviation, median with interquartile range, or as number of patients, analysed by t test, Mann-Whitney or the chi-squared test respectively.

[1] I/IIa+ IIb/ IIIA+IIIB+IIIC‡ /IIIC* + IV.

Table 43: Measured Indices and CRC stage in predicting recurrence in 2 years.

	Odd's ratio	95% confidence interval	P value
Treatment group	0.86	0.42 – 1.78	0.688
Dukes' stage	**2.30**	**1.00 – 5.30**	**0.049**
Modified AJCC stage	**4.62**	**1.88 – 11.33**	**0.001**
Angiogenin	1.21	1.01 – 2.17	0.061
EPCs	1.33	0.87 – 2.03	0.194
CECs	1.08	0.73 – 1.61	0.700

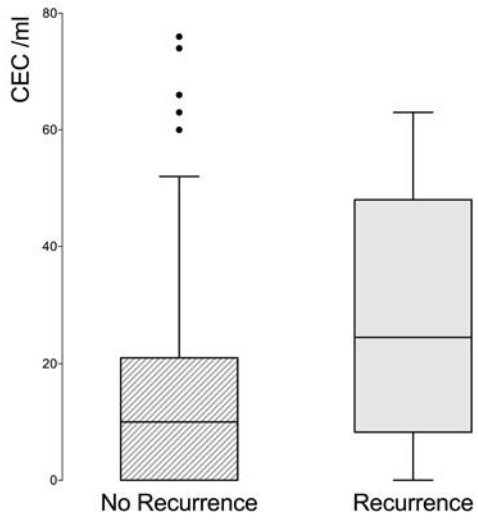
CECs and EPCs before surgery followed by adjuvant chemotherapy were higher in the progression group (p=0.015 and 0.012 respectively, table 43, figure 45). Once again, however, only Dukes' and mAJCC were more significant predictors by binary logistic regression (not shown), and with ORs similar to that of table 39.

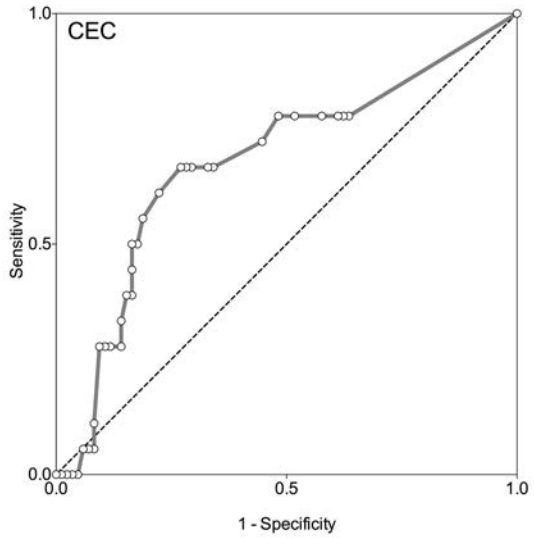
Table 44: Markers before surgery and 2 year recurrence with adjuvant therapy.

	No Progression n=85	Progression n=24	P value
CEC cells/ml	10 (0 - 21)	25 (8-48)	**0.015**
EPC cells/ml	19 (0-36)	43 (12-55)	**0.012**
WCC (10^6/ml)	6.99 (2.10)	7.72 (1.27)	0.157
CD34+CD45- cells	0.8 (0.5-1.1)	0.7 (0.6-1.1)	0.559
vWf [IU/dL]	116 (26)	115 (11)	0.060
sEsel [ng/mL]	27 (19-35)	28 (20-34)	0.594
VEGF [pg/mL]	195 (30-618)	552 (53-1375)	0.196
Ang [ng/mL]	312 (106)	313 (75)	0.974

Data presented as mean with standard deviation, median with interquartile range Significance of p<0.05 by t-test or Mann-Whitney.

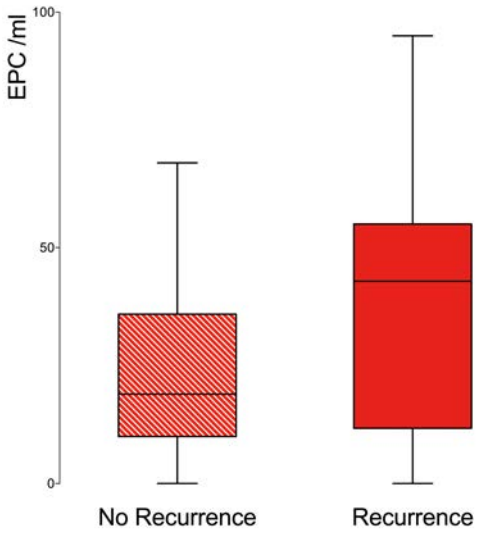
Figure 45: Box and Whisker Plots and ROC curves- pre-treatment CECs (grey) and EPCs (red) to predict 2 year recurrence after adjuvant chemotherapy.

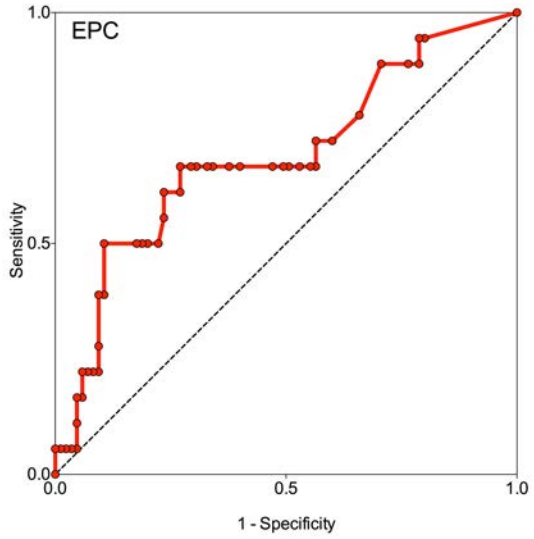




CEC: p=0.015 (Mann-Whitney). Tukey Box & Whisker plots

ROC area 0.638 (p=0.017). For > 12 cells/ml Sensitivity=78%, specificity=49%, LR=1.8



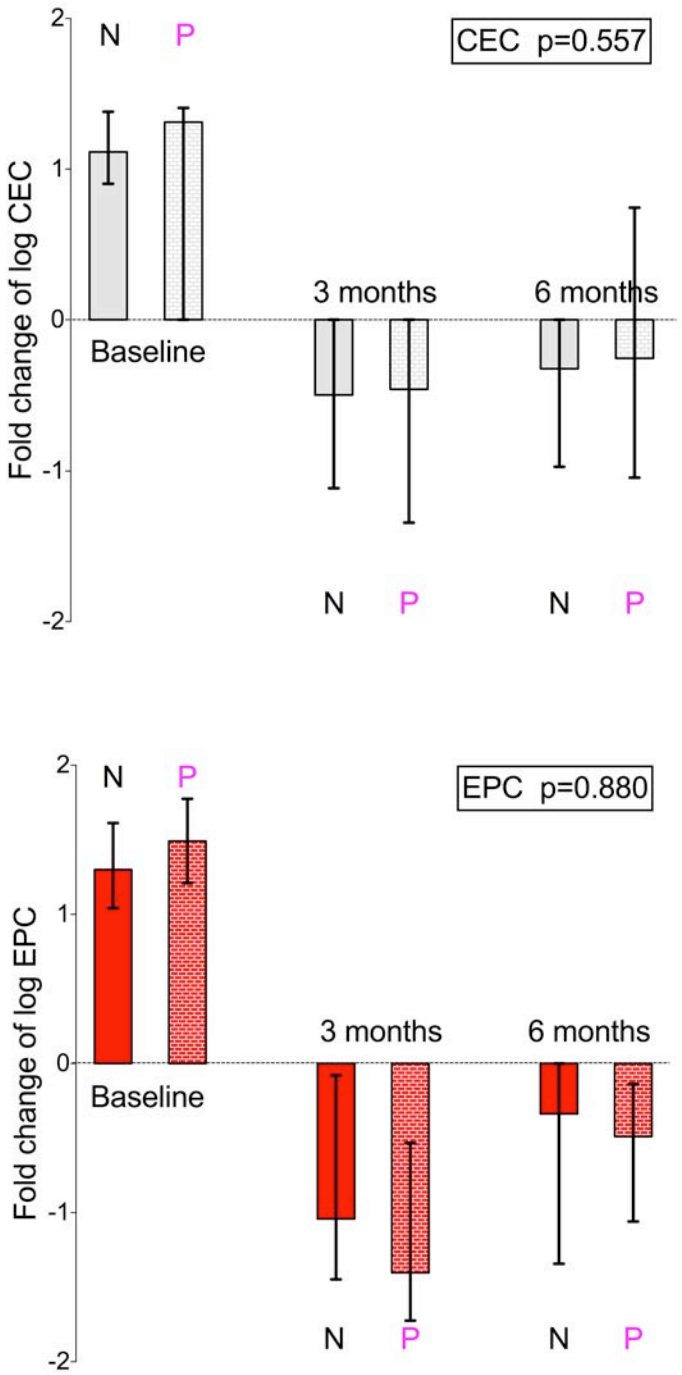


EPC: p=0.012 (Mann-Whitney) Tukey's Box & Whisker plots

ROC area 0.687 (p=0.012). For > 22 cells/ml: Sensitivity=78%, specificity=34%, LR=1.3

For adjuvant therapy, fold changes for CECs or EPCs were not significant (figure 46).

Figure 46: Fold changes of CECs and EPCs after adjuvant chemotherapy.



P= progression, N= no progression. Median shown as box, and IQR as interval bar. No significant differences between progressive and non-progressive disease groups (Mann Whitney or t-test).

The relationship of the indices with other definitions of outcomes was analysed. Having examined progression (TTP) and survival (OS), PFS was also tested for the relationship with the indices relative to their median and tertile levels.

The progression free survival (PFS) rates after 740 days for Dukes' stages A, B, C and D was 100%, 92.6%, 89.8% and 46.9% respectively (median 740, 418, 156 and 116 days respectively, $p<0.001$). The PFS after 740 days for mAJCC stages 1 to 4 were similar to Dukes' stages: 100%, 92.6%, 91.1% and 50.0% respectively (mean times were 740, 418, 198, 110 days respectively, $p<0.001$).

First, outcomes over the 740 days relative to the median levels of the indices were analysed. Significant outcomes for TTP and PFS were found with comparisons above and below the median values of CEC, EPC and angiogenin (see table 44). Kaplan Meier (cumulative survival) charts are in figure 46. Only CECs>12 /mL and EPCs >22 mL were significantly associated with poorer TTP and PFS over 2 years. Angiogenin > 308 ng/mL was also associated with poorer PFS outcomes only.

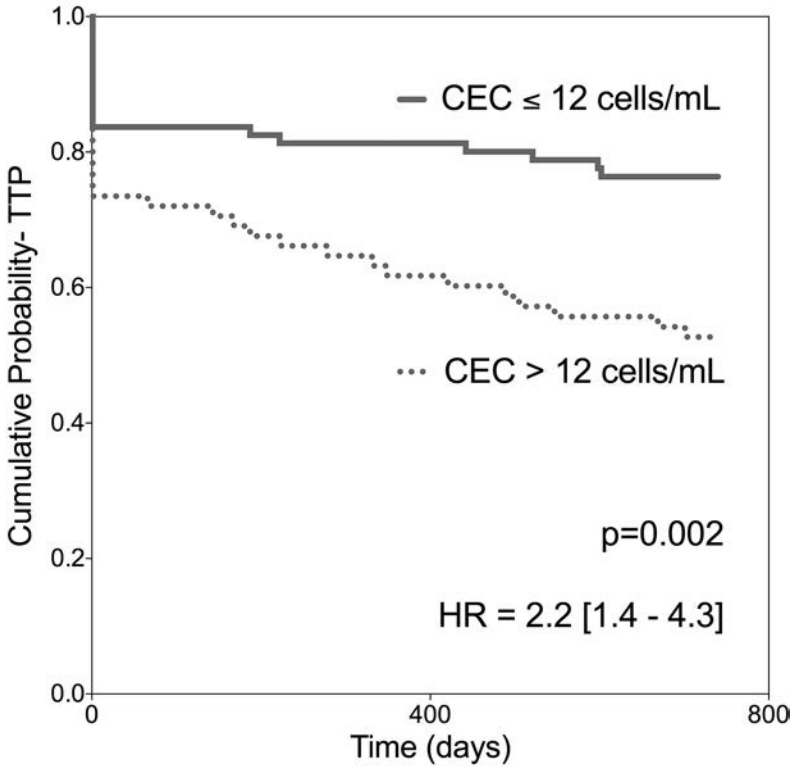
Table 45: Survival/progression analyses above/below median of indices.

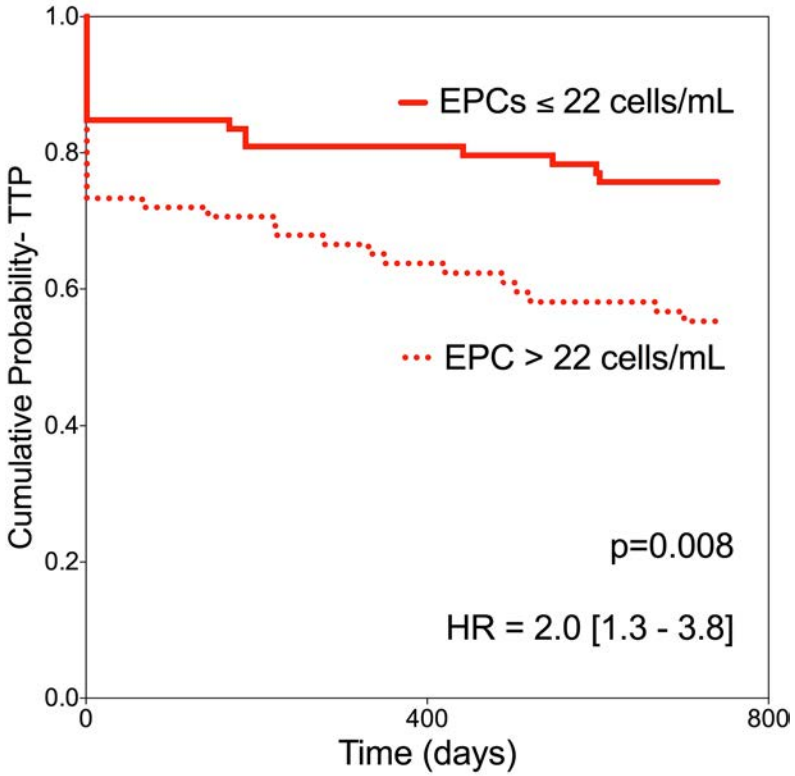
	Number	< > Median/ mean	p values		
			Time to Progression (TTP)	Overall Survival (OS)	Progression Free Survival (PFS)
CEC (cells/mL)	86 68	≤ 12 > 12	**0.002**	0.884	**0.015**
EPC (cells/mL)	79 75	≤ 21 > 21	**0.008**	0.554	**0.017**
vWf (IU/dL)	79 75	≤ 114 > 114	0.853	0.844	0.786
sE-sel (ng/mL)	81 73	≤ 27 > 27	0.172	0.304	0.098
VEGF (pg/mL)	78 76	≤ 195 > 195	0.455	0.947	0.541
Angiogenin (ng/mL)	77 77	≤ 308 > 308	0.088	0.095	**0.020**

P values obtained from cumulative survival over time by Kaplan Meier method (log rank). WCC and CD34CD45- cells were excluded - no significant findings and not part of the hypothesis.

The cumulative survival charts are shown for CECs and EPCs in figure 46.

Figure 47: Time to progression (TTP) over 2 years relative to median CEC (grey) and EPC (red) levels.





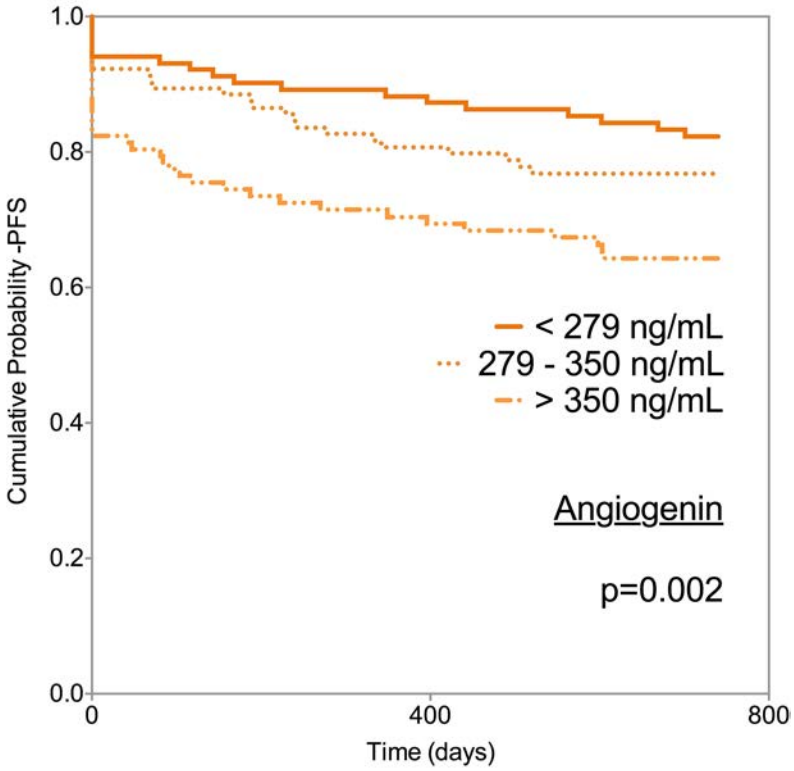
Next, the survival/progression over 2 years was analysed against tertiles of the measured indices (table 48). CECs (p=0.008) and EPCs (p=0.049) levels were not as significant by log rank analysis against their performance above and below median levels (p of 0.002 and 0.008 respectively, figure 46). However PFS was worse when angiogenin was >350ng/ml (see figure 48).

Table 46: Survival and progression analyses by tertiles of measured indices

			p value		
	Number	**Tertiles**	**TTP**	**OS**	**PFS**
CEC (cells/mL)	51 49 54	< 7 7 – 19 > 19	**0.008**	0.738	**0.026**
EPC (cells/mL)	47 55 52	< 12 12 – 35 > 35	**0.049**	0.273	0.396
vWf (IU/dL)	50 51 53	< 108 108 – 122 > 122	0.208	0.937	0.347
sE-sel (ng/mL)	48 52 54	< 20 21 – 30 > 30	0.350	0.382	0.214
VEGF (pg/mL)	50 52 52	< 80 80 – 499 > 499	0.190	0.362	0.123
Angiogenin (ng/mL)	51 52 51	< 279 279 – 350 > 350	**0.021**	**0.032**	**0.002**

Tertiles were genrerated from SPSS and p values of cummulative survival by Kaplan Meier method. WCC and CD34+CD45- cells were excluded as there were no significant findings and were not part of the original hyoptheses.

Figure 48: Angiogenin tertiles and progression free survival (PFS).



In summary, CECs and EPCs were significantly higher pre-surgery for participants with disease progression within 2 years but Dukes' or modified AJCC stage remained the best independent predictors. CECs>12 /ml and EPCs >22 ml may be useful to predict progression (TTP), and angiogenin >350ng/ml poorer PFS outcomes.

From the results above a mathematical model was developed to test whether the indices, when combined with Dukes and mAJCC stage improved their prognostic value by identifying those with potentially poorer TTPs and PFS. For TTP, levels with CECs>12/ml were assigned '1' which was added to Dukes' and mAJCC. Similarly this was also done for EPC (>22 cells/ml) and for PFS angiogenin levels >350ng/ml.

The composite stage was then tested against disease recurrence, death and all events (see table 48). For example EPCs of 30/ml, scored 1 and added to the stage, such as Dukes' 3 (C) or mAJCC 4, giving a score of 4 and 5 respectively.

Table 47: Performance of mathematical models against Dukes' and AJCC in predicting time to progression and progression free survival.

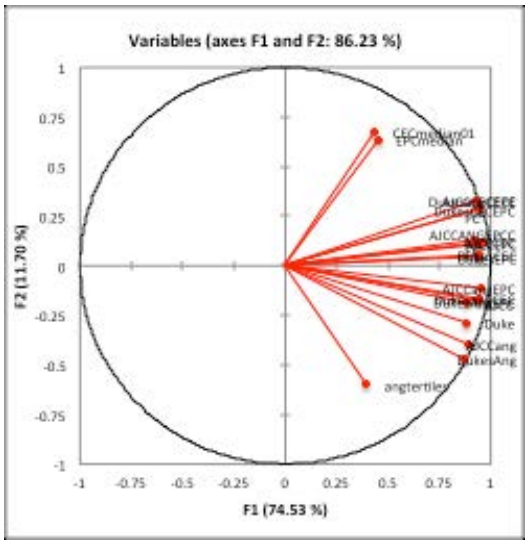
MODEL	r [A]	p [A]	OR [B]	p [B]
Time to Progression (TTP)				
Model1				
Dukes'	0.611	<0.001	0.5 (0.1-2.1)	0.371
mAJCC	0.631	<0.001	**4.3 (1.7-10.8)**	**0.002**
Dukes'+CEC[1]	0.632	<0.001	2.0 (0.7-5.6)	0.189
Dukes'+EPC[2]	0.618	<0.001	2.3 (0.7-5.3)	0.116
Dukes'+CEC[1]+EPC[2]	0.608	<0.001	**2.1 (1.2-3.8)**	**0.010**
Model 2				
mAJCC	0.631	<0.001	2.0 (1.1-5.8)	0.210
Dukes'	0.612	<0.001	**2.5 (1.0-5.6)**	**0.031**
mAJCC+ CEC[1]	0.630	<0.001	2.0 (0.7-5.7)	0.189
mAJCC+ EPC[2]	0.636	<0.001	2.2 (0.8-6.3)	0.116
mAJCC+ CEC[1]+EPC[2]	0.612	<0.001	**2.1 (1.1-3.8)**	**0.010**
Progression Free Survival (PFS)				
Dukes'	0.584	<0.001	1.7 (0.5-6.6)	0.421
mAJCC	0.598	<0.001	**3.5 (1.5-7.9)**	**0.003**
Dukes'+Ang[3]	0.545	<0.001	1.2 (0.5-2.9)	0.686
mAJCC+Ang[3]	0.571	<0.001	1.5 (0.5-3.8)	0.203

[A] Spearman's correlation for r and p values. [B] Binary logistic regression for Odds Ratio (OR) and p values. [1] CECs>12 /ml. [2] EPCs >22 ml. [3] Angiogenin >350ng/ml.

As with disease recurrence, mAJCC was more predictive of TTP and PFS. However the model incorporating thresholds of CECs and EPCs (i.e. a score of 0 if <median or 1 if >median) to the stage (1 to 4) was more predictive of TTP (p=.010 versus p=0.210 of mAJCC only). The Dukes' model that combined the same thresholds did not perform (p=0.010) as well Dukes' stage only (p=0.003).

However on principal component analysis, those with above median values of CECs and EPCs, above upper tertiles of angiogenin and with poorer mAJCC stage had poorer PFS and TTP within 2 years than mAJCC or Dukes' stage alone (figure 48).

Figure 49: prinicipal component analysis of CRC recurrence in 2 years and the mathematical models of EPCs, CECs and angiogenin with CRC stage



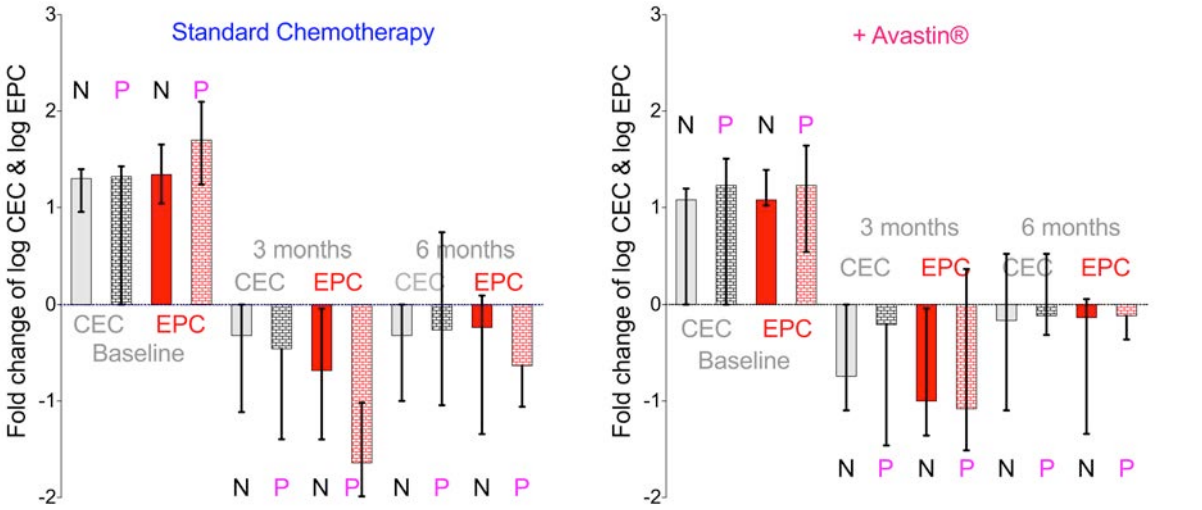
	Eigenvector	Correlation
mAJCC+EPC+CEC (F2)	0.261	0.957
mAJCC+EPC+CEC+Ang (F1)	0.263	0.962
mAJCC	0.241	0.884

Scores of the indices (0 or 1) are added to the mAJCC stage (1 to 4). Score of 1 if angiogenin>upper tertile; 1 if CEC > median level; and 1 if EPC >median values.

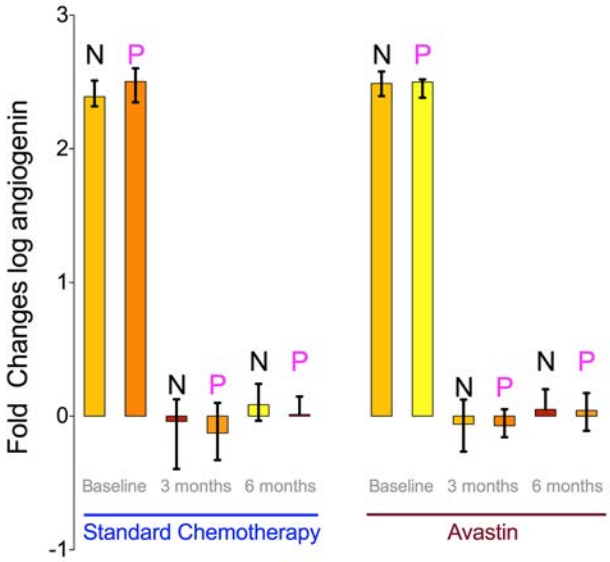
Unfortunately the level of the score for any of these models with potentially informative outcomes could not be analysed as the number of subgroups (6 in total, but derived from 16 permutations) of these ‘mathematical stages’ were inadequately powered ($r>0.40$) and potentially open to significant type 1 or 2 errors. Also, a point component analysis did not add any further information to the regression analysis.

The temporal relationship of CECs and EPCs with TTP, and angiogenin with PFS was examined by the treatment modality. The numbers showing progression within 2 years were generally small and therefore open to type 1 error. The results were however included as studies in the published literature also involved small but similar sized groups undergoing adjuvant treatment by standard chemotherapy with or without Avastin®. Generally there were no differences between the progression and non-progression groups (see figure 49). For progression in those who underwent Avastin® therapy, the fold changes of EPCs were significantly lower at 6 than 3 months when compared to baseline ($p=0.039$). This was not found on standard chemotherapy. Therefore, the changes were influenced by anti-VEGF treatment. The relationship of PFS with tertiles of angiogenin was not reproduced on ratio analysis.

Figure 50: CECs and EPCs in TTP, and angiogenin in PFS for adjuvant chemotherapy with and without Avastin® (Bevacizumab).



P= progression & N= no progression, within 2 years. Median is shown as box, and IQR as interval bar. Only EPCs were higher with progression (TTP) while on avastin (p=0.039 by Mann Whitney). Angiogenin showed no fold change patterns in PFS in either groups.



Discussion

The measures of the endotheliome and angiome in my study were hypothesised to predict outcomes when tested before surgery. Having found ordered trends with Dukes' and mAJCC stages, CECs, EPCs and angiogenin were further hypothesised to be markers of prognosis. A number of definitions of outcomes were reported for cancer therefore analysis was performed on several levels: progression (TTP), PFS, and OS. Oncologists often utilise the prognostic score put forward by Köhne et al for clinical trials to select patients for aggressive therapeutic strategies. The score incorporates the performance status [PS], number of metastatic sites, alkaline phosphatase [ALP] level, and white cell [WCC] count (472). This was not calculated in my study as participants with WCCs above $14x10^9$ cells/L were excluded to avoid missed EC events on flow cytometry. That is, the assay was more likely to reach a target of 1 million with a highly populated specimen and less likely to analyse a sufficient volume to find rare events. Disease progression, proposed by Parulekar and Eisenhauer (382), and later revised under the RECIST guidelines (381), was defined as evidence (on radiology or histology) after resection (with curative intent) of recurrence or metastasis. To my knowledge my thesis was the first to show a correlation of CECs and EPCs with 2-year progression for CRC at any stage, although it was less predictive than Dukes' or mAJCC staging systems.

Published studies on cellular markers were mainly in metastatic CRC, and comparisons with my cohort were limited. Understandably with better long-term outcomes from CRC treatment (5 year survival improved by 10% over two decade), there was a clear attempt in the published literature to use EC markers to improve

predictions of and guidance on chemotherapy (3, 11, 14, 15). Lin et al promoted an indirect measure of early circulating endothelial progenitors (CEPs, not EPCs) by real time PCR (RT-PCR) measurement of CD133 mRNA in peripheral blood monocytes to replace cumbersome cell sorting techniques by IMS (351). While FC superseded RT-PCR over the years, theirs was the first to measure markers of CECs/CEPs relative to CRC stage and survival outcomes. Although they could not estimate the number of CEPs from CD133 mRNA quantification, they showed poorer survival and recurrent disease (ROC area 0.81, $p<0.05$) when levels were above the median threshold. This was a relatively small study and not powered to test its performance against each Dukes' stage, though a significant difference of the combined Dukes' A+B was found against C+D, (OR 17 [1.8-154.1]). Mehra et al found similar correlations with CD133 mRNA, and not CD146 mRNA, positive monocytes (CD146 being the better marker of ECs), to metastasis and survival (473). Critically neither study measured the degree of CD133 loss in their monocyte fraction, if at all, as it was known to quickly downgrade with EPC maturity in the circulation (169). Assuming a correlation of the CD133 cellular expression with its mRNA precursor (though not proven by the authors) false positives may have arisen from tumour cells or, as with errors of CEC identification, from EC carcasses and/or circulating microparticles (391, 474).

The effect of standard chemotherapy on the cellular markers and outcomes was also not previously reported. Goon et al found correlations with prognostic indices of non-metastatic breast cancer, the highest CECs found in the poor prognostic group (PPG), though long-term outcomes were not reported (24). My study confirmed that while CECs and EPCs were significantly elevated before surgery, Dukes' and the

mAJCC staging system remained the best predictors of outcomes as measured by TTP, PFS and OS. There were no publications of this kind to corroborate the findings, as most were limited to the temporal relationship before and during chemotherapy, specifically anti-VEGF, for mCRC only (28, 201). Understandably the traditional endpoints by CT scan to monitor response to chemotherapy both routinely and in the clinical trial setting could not differentiate responders until after treatment was initiated, and typically many months later. Anti-VEGF is more cytostatic than cytotoxic, but had a noticeable impact on progression and survival. CT scan findings of non-progressive disease alone would not discriminate those with better PFS (201, 468). Studies on CECs and EPCs as surrogate markers of responders to chemotherapy were encouraging. For mCRC, longer PFS was found in participants with lower CECs (< 40 cells/ml) before receiving the combination of first line chemotherapy (5-FU with folinic acid) and bevacizumab (201). A fall in CEPs below 0.04 % after day 4 of Bevacizumab correlated to longer PFS ($p<0.001$) and OS (0.002), a valuable change that may identify better responders to anti-VEGF (28).

Willet et al reported the first phase II study on adjuvant Bevacizumab for localised non-metastatic rectal cancer and found that higher CECs after treatment were significantly associated with poorer outcomes (475). A higher number of CECs was also noted with residual disease and with the inflammatory marker IL-6. The authors used a novel technique to measure intra-tumour interstitial pressures with a needle connected to the tubing of an endoscope channel, though the methodology required further evaluation. In theory this tested the leakiness of the EC and/or the disordered architecture in which higher pressures were found, a factor associated with poorer tumours (476). However, unlike their study, I found, along with EPCs, correlations

with VEGF continued from baseline to 3 months after surgery and again after adjuvant treatment. The complete results of QUASAR 2 trial on the effect of Bevacizumab to 5-FU in the adjuvant setting were not available at submission of my thesis (477). However preliminary reports were of better PFS for stage II CRC. This contrasted with the AVANT and NSABP C-08 trials, as both showed no benefit of Bevacizumab with FOLFOX regimes for stage II and III CRC (477-479).

The FNCLCC ACCORD trial of combining first-line chemotherapy with Bevacizumab for mCRC reported that higher CECs at baseline and after one cycle correlated to better 6-month PFS (for CEC> 23/ml log rank p=0.02) (349). Objective response rate (ORR i.e. at least 2 independent CT reports of reponse) also improved. ORR was not explored in my study, as it was not the policy of the NHS hospitals to examine CT scans by 2 or more radiologists. I found that only progression (TTP), not PFS (progression and/or mortality) associated with higher CECs across all 4 stages. The PFS analysis with CECs and EPCs in Dukes' D only was not as reliable given the small subgroups involved (15 cases of good PFS versus 17 with poor PFS: p=0.282 for CECs and 0.120 for EPCs by Mann Whitney).

I would agree with Malka et al (349) and Willet et al (475) of the need for larger in-depth studies. However, my findings suggested some value in measuring CECs and EPCs to identify those at risk of recurrent disease within 2 years after surgery (with curative intent) followed by adjuvant chemotherapy (for CECs ROC area=0.638, p=0.017 and EPCs ROC=0.867, p<0.001). Specifically, as preoperative test, above median levels may be valuable indicators for adjuvant therapy in those without poor

histological features such as lymph node spread, poor differentiation, vascular invasion or local invasion (section 1.4.2).

I was unable to reproduce the relationship between VEGF and disease progression described by Poon et al, and could not offer plausible reasons for the differences (366). There may be more value in measuring tissue expression rather than serum concentration as proposed in the first meta-analysis of its role as a marker in CRC (284). Liu et al reported on their angiogenic assessment of mCRC by a panel of EC markers, including sE-selectin and vWf, which stratified survivors into low- and high-risk groups before and after various chemotherapy regimes (470). Though not the authors' intention, the study gave further evidence to the predictive value of measuring the endotheliome and angiome. Damin et al also reported links between baseline vWf to OS and PFS (16). I also found very high levels of vWf in Dukes' D stage only but outcome analyses with or without treatment were unyielding. While there was growing evidence of the role of sE-selectin in promoting haematogenous metastasis via EC recognition of carbohydrate ligands and cancer survival by death receptor-3 mediated pathways, circulating levels had limited prognostic value (369, 480). sE-selectin fell, though not significantly, after surgery, but was unpredictable after adjuvant therapy and as reported by Uner et al (78), not correlated to OS. The findings of Ito et al as a biomarker of CRC were not reproduced in my study (369).

As shown in section 3.1.4, angiogenin was highly predictive of CRC and rose significantly with disease stage on trend analysis. Shimoyama et al showed sera levels of angiogenin reflected its distribution and genetic expression in CRC tissues (364). The mean serum angiogenin was significantly higher in CRC patients versus

healthy controls and significantly higher in mCRC. Though a small study it showed a correlation with high serum angiogenin and worse DFS at 5 years [p=0.03]. I also found a similar correlation with tertile levels above 350 ng/mL (p=0.002) but for PFS within 2 years, a finding not previously reported. Like EPCs and CECs, angiogenin were not better than Dukes' or mAJCC at predicting TTP, PFS or OS.

Overall, the markers chosen were not correlated to prognosis, which brought into question either the choice of markers or the value of multi-parameter assessment of EC activity. The recent study of Liu et al may offer a better panel including VCAM-1, TSP-1, IL-8, MMP-2 and angiogenin-2 for OS, and vWf with angiogenin-2 for PFS (470). It was however limited to mCRC and did not include cellular markers. In my study, combining EPCs and CECs with mAJCC, was better than mAJCC alone at predicting progression (by TTP) on mathematical modelling. That is, restaging by adding to the mAJCC stage (1-4) a score of 1 for CECs>12/mL and 1 for EPCs>22/mL, [OR (CI) of 2.1(1.1-3.8), p=0.010] performed slightly better against the mAJCC only [OR (CI) of 2.5(1.0-5.6), p=0.031]. When principal component analysis was applied to the mathematical models, the combination of mAJCC with tertiles of CECs, EPCs and angiogenin correlated to TTP and PFS. While the findings were promising, my study was not powered to determine the predictive value of this restaged model as it was scored out of 6 and derived from 16 possible permutations.

In summary, CECs and EPCs were associated with poor TTP and angiogenin with poor PFS. Mathematical models combining angiogenin, CECs and EPCs, with the mAJCC may better predict TTP and PFS than mAJCC alone.

Chapter 4

Conclusions

4.1 Summary of findings

In section 3.1.1, I found that circulating endothelial and progenitor cells were elevated in CRC regardless of its stage. Although CECs strongly correlated to EPCs and were comparable to the stable coronary artery disease (SCAD) group, EPCs were higher in CRC only and therefore a better biomarker of the disease.

In section 3.1.2, I found that cellular markers had prognostic but not predictive value for disease severity as CECs and EPCs showed positive trends with increasing cancer differentiation, Dukes' stage and mAJCC classification.

In section 3.1.3, the relationship of the markers of endotheliome and angiome were analysed. VEGF and angiogenin were higher in CRC compared to controls. Only VEGF was linked to CEC and EPC levels. Angiogenin, more so than VEGF and EPCs, was the best biomarker of CRC. Angiogenin, independent of CECs and EPCs, had a positive trend with stage. Though the markers were not predictive of the stage, the relationship of angiogenin with Dukes' remained after ordinal regression.

In section 3.1.4, all measured indices fell after surgery (3 months) but some rose unpredictably at 6 months following adjuvant therapy. CECs and EPCs rose regardless of the adjuvant regime. Standard chemotherapy, but not Bevacizumab, increased VEGF above post-surgery levels. Correlations between these three indices

at 6 months suggested disturbances to EC activity unrelated to tumour activity, assuming complete response to treatment. This may therefore have been the consequence of the adjuvant chemotherapy or unresponsive and undetected cancer.

In section 3.1.5, pre-treatment indices were of some value in predicting outcomes. Higher pre-surgery levels of CECs, EPCs and angiogenin were associated with poorer time to progression (TTP) and progression free survival (PFS) at 2 years. However Dukes' and more so mAJCC stages were the best independent predictors of both outcomes. Nevertheless, a mathematical model incorporating scores for the mAJCC stage, CECs and EPCs (above the median levels of this study) and angiogenin (above the upper tertile levels) may predict TTP and PFS within 2 years. For those on adjuvant treatment, there were no significant fold changes of markers that distinguished participants with poorer outcomes.

4.2 Recommendations for the future

Over the last few decades, studies on CECs and EPCs contributed significantly to the understanding of angiogenesis in cancer and consequently their clinical value as measures of treatment outcomes. Yet, there was no consensus on their phenotypic identity. To move forward, and determine the importance of CECs and EPCs beyond the findings of my thesis, I propose my choices, as supported by other authors, to be the best definitions for studies using multi-channel flow cytometry (24, 139, 140).

In this thesis I demonstrated the importance of the endotheliome and angiome in the vascular biology of CRC staging and, potentially, of its prognosis. Angiogenin may hold diagnostic value, while high levels of vWf, EPCs and CECs may reflect metastastic disease. However, these markers may have been influenced by cardiovascular disorders, such as coronary artery disease (CAD), though not significant on regression analysis. Both CRC and coronary disease occurred in patients at a similar age range, and may be concurrent, but not always detected. While I did not find any influence of CAD on the markers in CRC, a powered study of risk factors for CAD would better determine the influence of confounders on the measured markers. Though not initially intended in my study, CD34+CD45- cells overall were higher in SCAD than CRC, which may have both diagnostic and prognostic importance, and again require a large-scaled study. Further in vitro characterisation and differential content of these cells, other than EPCs, will support their distinct roles, if any, in SCAD as well as CRC.

Given I found increased levels in an ordered trend with CRC differentiation and stage, EPC may hold therapeutic advantages. Conceptually, vessel heterogeneity from disordered angiogenesis, promoted by hypoxia in rapidly growing tumour cells, effectively hampered drug delivery (481). Normalising these vessels, as seen with anti-angiogenic therapy, may overcome this problem (482). Therapeutic EPCs, given their role in vasculogenesis and potentially for vessel repair in CAD (483), may aid the normalisation process and therefore deserves further in vitro investigation. Alternatively, their affinity to tumour vasculature (209) may deliver targeted therapy, once engineered as vectors of anti-angiogenic drugs (484).

I did not have the power to investigate the role of the endotheliome and angiome in predicting outcomes of the numerous modalities of adjuvant therapy. This, as well as the value of measuring levels before surgery versus at several time points during or after adjuvant therapy warrants a large-scaled comparative cohort study. Modifications to the array of markers may yield more information. For example, other EC-specific markers associated with CRC outcomes, could replace sE-selectin, such as NO or eNOS. Akbulut et al proposed an angiogenic index of VEGF and NO independently predicted survival and better DFS outcomes for operable CRC (49).

Angiogenin, EPCs and CECs may determine long-term outcomes beyond 2 years. Their importance, particularly in the mathematical model incorporating their pre-treatment levels with mAJCC stage, also need further evaluation, ideally by 5-year endpoints of progression (TTP, PFS) and survival (OS).

4.3 Conclusion

The investigations of the chosen markers of endothelial activity further contributed to the understanding of the vascular biology of colorectal cancer and its staging. It may inform on concurrent dysfunction of the EC in undetected cardiovascular disease. Angiogenin was diagnostic of the CRC but required further evaluation against other cancers. EPCs, given the relationship to tumour differentiation and stage, may have therapeutic implications in anti-angiogenesis. Both, along with CECs and the mAJCC stage, predicted cancer progression within 2 years. The prognostic value may improve by expanding the panel to include other EC biomarkers, such as nitric oxide. Therefore the endotheliome and angiome is worthy of further study.

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Appendix 1: QUASAR 2 Trial

A multicentre international study of capecitabine ± bevacizumab as adjuvant treatment of colorectal cancer.

EudraCT number: 2005-000629-32 **Sponsor: University of Oxford**

Chief Investigator: Prof David Kerr

This trial summary was downloaded from the The Oncology Clinical Trials Office (OCTO) website, Oxford. http://www.octo-oxford.org.uk.

QUASAR 2 is a study comparing 'standard' chemotherapy using capecitabine, against capecitabine + Avastin® (bevacizumab) with the expectation that adding bevacizumab to capecitabine may have the potential for improved relapse free and overall survival compared to capecitabine alone. Initial results from the Cancer Research UK website suggested benefit to disease free survival to stage II disease.

STUDY STATUS

Recruitment into QUASAR 2 is now closed.

Primary endpoint (3Y DFS stage II & III) target of 1892 patients: met 26 May 2010.

Secondary endpoint (3Y DFS stage III only) target of 1411 stage III patients: 1206 randomised by close of recruitment (Oct 2010).

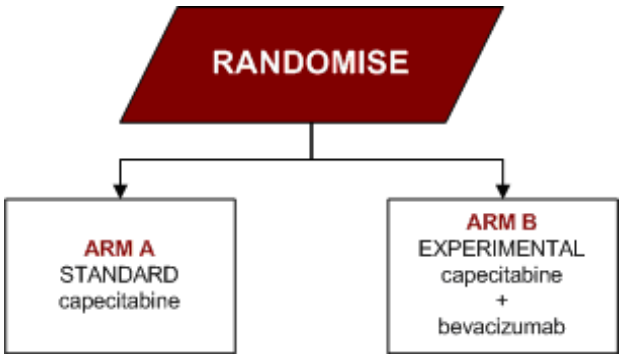
Number of active sites: UK - 123, Non-UK - 61 (Sep 2010)

KEY DATES

Accrual completed 13th October 2010

Planned study completion: September 2014

STUDY SCHEMA



INCLUSION CRITERIA

- Histologically proven stage III (stage T2, T3 or T4) and stage II (any one or more of the following – stage T4, lymphatic invasion, vascular invasion, peritoneal involvement, poor differentiation, obstruction and perforation of the primary tumour during the pre-operative period) colorectal cancer (expected ratio 70%:30%). N.B Patients can be Stage II, T3 as long as they have one of the other poor prognostic features. For the purposes of stratification, rectal cancers will be anything below the peritoneal reflection.
- Patients must have undergone complete resection of the primary tumour without evidence of residual disease.
- Patients must be randomised to start treatment a minimum of 28 days and maximum of 70 days* after surgery. [If a subject has had a major surgical

procedure, open biopsy, or significant traumatic injury within 28 days prior to study treatment start, or there is the anticipated need for major surgical procedure during the course of the study they are not eligible].

- WHO Performance Status 0 or 1.
- Male or female outpatients age >= 18 years.
- Written informed consent given.
- Life expectancy of >=5 years, in terms of non-cancer-related morbidity.

EXCLUSION CRITERIA

- Previous chemotherapy, immunotherapy or infra-diaphragmatic radiotherapy; or patients who are expected to require radiotherapy to these sites within the next 12 months, for any reason.
- Received any investigational drug or agent/procedure, (i.e. participation in another treatment trial) within 4 weeks of randomisation.
- Moderate or severe renal impairment [creatinine clearance <30ml/min (calculated according to Cockroft-Gault formula–see Appendix 4).
- Any of the following laboratory values (tests must not have been carried out more than 2 weeks prior to randomisation):
    a. Absolute neutrophil count (ANC) <1.5 x 109/L
    b. Platelet count < 100 x 109/L
    c. Total bilirubin > 1.5 ULN
    d. ALT, AST > 2.5 x ULN
    e. Alkaline phosphatase > 2.5 x ULN (ULN = Upper Limit of Normal)

- Patients requiring chronic use of full dose oral or parenteral anticoagulants, high dose aspirin (>325mg/day), anti-platelet drugs or known bleeding diathesis. Low dose aspirin is allowed. Low dose clopidogrel (≤75mg) is allowed.
- Proteinuria > 500 mg/24 hours.
- Known coagulopathy.
- Clinically significant cardiovascular disease [i.e. active; or <12 months since e.g. cerebrovascular accident, myocardial infarction, unstable angina, New York Heart Association (NYHA) grade II (see Appendix 14) or greater congestive heart failure, serious cardiac arrhythmia requiring medication; or uncontrolled hypertension].

  Concomitant treatment with sorivudine or its chemically related analogues such as brivudine.
- Pregnant (positive pregnancy test within 7 days of starting treatment), or lactating women.
- Sexually active patients of childbearing potential not using adequate contraception (male and female).
- Previous malignancies other than adequately treated in situ carcinoma of the uterine cervix or basal or squamous cell carcinoma of the skin, unless there has been a disease free interval of at least 10 years.
- Lack of physical integrity of the upper gastrointestinal tract, malabsorption syndrome or inability to take oral medication.
- Chronic inflammatory bowel disease and/or bowel obstruction and/or active peptic ulcer, either of which have been active or required medication in the last 2 years.

- History of uncontrolled seizures, central nervous system disorders or psychiatric disability judged by the investigator to be clinically significant precluding informed consent or interfering with compliance for oral drug intake.
- Patients with known allergy to Chinese hamster ovary cell proteins or other recombinant human or humanized antibodies or to any excipients of bevacizumab formulation; or to any other study drugs.

* Calculation of these dates is based on date of surgery being day 1. ** Women of childbearing potential randomised to receive bevacizumab are required to have a serum pregnancy test at baseline (i.e. prior to starting treatment). Postmenopausal women must have been amenorrheic for at least 12 months to be considered of non-childbearing potential.

STUDY OBJECTIVES

Primary: Disease free survival (DFS)

Secondary: Overall survival (OS) & side effect profile

KEY DATES

Planned accrual completion: September 2010

Planned study completion: December 2013

Appendix 2: Diagnosis and Staging of CRC

CRC is identified by tumour biopsy at colonoscopy or sigmoidoscopy (285). Along with a physical examination, therapy is guided by staging with colonoscopy (after sigmoidoscopy) for synchronous tumours, and/or radiology (CT, MRI, PET) to identify metastatic disease (314). Staging is an estimate of the penetration into the bowel wall and distant spread to determine the best method of treatment. It determines the extent of local invasion, lymph nodes involved and metastasis, if any (285). It may require abdominal ultrasound, PET scanning, and, in the case of rectal tumours, MRI and endo-anal ultrasound (314). MRI gave good global staging of rectal cancers, margin involvement, extramural venous invasion, indication for radiation treatment and pelvic node spread (286). Though few contraindications, there were limitations when correlating radiological with pathological findings postoperatively. Endorectal ultrasound has similar advantages except that it is user-dependent, gives little information on node status and cannot assess nodal or vascular invasion. CT and PET may determine distant spread to other organs specifically lymph nodes, liver and lungs (294). The future of the non-invasive assessment of angiogenesis/tumour vasculature probably lies with dynamic MRI with tumour EC specific contrast but the parameters to be assessed are still undecided (315). It would therefore follow that definitive staging can only be achieved after excision and with histopathology. The exceptions were endoscopic excision of a polyp cancer, and advanced disease not suitable for surgery (314). The two key methods of staging are the Dukes' and AJCC-TNM systems, as shown below.

Dukes' system

The classification, described in 1932 by Dr. Cuthbert Dukes, identified 4 stages (316) and was the forerunner to TMN. It is reported routinely in the UK as it informed prognosis and the need for further oncological treatment.

Dukes' Classification of CRC Stage based on histological staging

Stage	Description
A	Tumour confined to the intestinal wall
B	Tumour invading through the intestinal wall
C	With lymph node(s) involvement. This is further subdivided into:
	C1 - apical node is not involved
	C2 - apical lymph node is involved
D	With distant metastasis

This has stood the test of time as stage correlated with 5-year survival (table 13).

TNM system

The most common staging system is the TNM (for Tumours/Nodes/Metastases) system, from the American Joint Committee on Cancer (AJCC), shown below.

TNM Classification of Colorectal Cancer

T		**N**		**M**	
Level of invasion of the bowel wall		**Nodes involved**		**Metastases**	
T0	no evidence of tumour	**N0**	No Nodes	**M0**	No metastases
Tis	Cancer in situ tumour without invasion	**N1**	1 to 3 Nodes	**M1**	Metastases
T1	Invasion through muscularis mucosa into submucosa	**N2**	4 or more		
T2	Invasion through the muscularis propria into subserosa but not to neighbouring tissues				
T3	Invasion through the muscularis propria into subserosa but not to neighbouring tissues				
T4	Invasion of surrounding structures or on the free external surface of the bowel				

TNM is usually quoted as a stage I, II, III and IV, roughly equivalent to Dukes' A, B, C, and D respectively. The higher the stage the more advanced the CRC and, therefore, more likely have a poorer outcome or 5 year survival (below).

Dukes', AJCC-TNM Stage and 5-Year Survival Rates

Dukes'	Dukes' 5 yr survival %	AJCC Stage	T	N	M	AJCC 5 yr survival %
na	na	0	Tis	N0	M0	—
A	93	I	T1, T2	N0	M0	97
B	77	IIA	T3	N0	M0	88
		IIB	T4	N0	M0	72
C	47	IIIA	T1, T2	N1	M0	88
		IIIB	T1, T2	N2	M0	68
		IIIB	T3	N1	M0	69
		IIIC	T3	N2	M0	47
		IIIC	T4	N1	M0	51
		IIIC	T4	N2	M0	27
D	8	IV	Any T	Any N	M1	8

The American Joint Committee on Cancer (AJCC) TNM staging for Tumour, Nodes, Metastases for colorectal cancer (2, 317)

Dataset for Colorectal Cancer (2nd Edition). Royal College of Pathologists (UK)

With permission from the Royal College of Pathologists
Reference: Williams GT, Quirke P, Shepherd NA on behalf of the RCPath Cancer Services Working Group. Dataset for colorectal cancer (2nd edition). London: The Royal College of Pathologists; 2007)

Surname: …… Forenames: …… Date of birth: ……

Hospital…… …….. Hospital no: …… NHS no: ……..…..

Date of receipt: …… Date of reporting: …….. Report no: ……

Pathologist: …… Surgeon: …… Sex: ……

Specimen type: Total colectomy / Right hemicolectomy / Left hemicolectomy / Sigmoid colectomy / Anterior resection / Abdominoperineal excision / Other (state) ……..

Gross description

Site of tumour …….

Maximum tumour diameter: ……..…..mm

Distance of tumour to nearer cut end ……mm

Tumour perforation (pT4) Yes ☐ No ☐

If yes, perforation is serosal ☐ retro/infra peritoneal ☐

For rectal tumours:

Relation of tumour to peritoneal reflection (tick one):

Above ☐ Astride ☐ Below ☐

Plane of surgical excision (tick one):

Mesorectal fascia ☐

Intramesorectal ☐

Muscularis propria ☐

For abdominoperineal resection specimens:

Distance of tumour from dentate line .....mm

Histology

Type

Adenocarcinoma Yes ☐ No ☐

If No, other type .....

Differentiation by predominant area

Well / moderate ☐ Poor ☐

Local invasion

No carcinoma identified (pT0) ☐

Submucosa (pT1) ☐

Muscularis propria (pT2) ☐

Beyond muscularis propria (pT3) ☐

Tumour invades adjacent organs (pT4a) ☐

AND/OR

Tumour cells have breached the serosa (pT4b) ☐

Maximum distance of spread beyond muscularis propria ……..mm

Response to neoadjuvant therapy

Neoadjuvant therapy given Yes ☐ No ☐ NK ☐

If yes:

No residual tumour cells / mucus lakes only ☐

Minimal residual tumour ☐

No marked regression ☐

Tumour involvement of margins

	N/A	Yes	No
Doughnuts	☐	☐	☐
Margin (cut end)	☐	☐	☐
Non-peritonealised 'circumferential' margin	☐	☐	☐

Histological measurement from tumour to non-peritonealised margin ..... mm

Metastatic spread

No of lymph nodes present .....

No of involved lymph nodes .....

(pN1 1–3 nodes, pN2 4+ nodes involved)

Highest node involved (Dukes C2) Yes ☐ No ☐

Extramural venous invasion Yes ☐ No ☐

Histologically confirmed distant metastases (pM1):

Yes ☐ No ☐ If yes, site: ……..…

Background abnormalities: Yes ☐ No ☐

If yes, type: (delete as appropriate)

Adenoma(s) (state number ……..…)

Familial adenomatous polyposis / Ulcerative colitis / Crohn's disease / Diverticulosis / Synchronous carcinoma(s) (complete a separate form for each cancer)

Other ..... …….

Pathological staging

Complete resection at all surgical margins

Yes (R0) ☐ No (R1 or R2) ☐

TNM (5th edition)

(y) pT …….. (y) pN ……..(y) pM ……..

Dukes

Dukes A ☐ (Tumour limited to wall, nodes negative)

Dukes B ☐ (Tumour beyond M. propria, nodes negative)

Dukes C1 ☐ (Nodes positive and apical node negative)

Dukes C2 ☐ (Apical node involved)

Signature: …… **Date** …../…../……. **SNOMED Codes T…….. / M……**

Appendix 3: Standard Operating Procedure for the enumeration of EPCs and CECs

A. Introduction

This method described the enumeration of:

- KDR+, CD34+, CD45- cells for **endothelial progenitor cells**, and
- CD146+, CD34+, CD45- cells for **circulating endothelial cells**.

This enumeration demanded an accurate WCC from the Bayer Advia® Analyser.

B. Materials (and Supplier):

1. BD “FACS Flow” Running solution. (Becton Dickinson, UK)
2. BD “FACS Clean” Cleaning Solution (Becton Dickinson, UK)
3. 3 ml BD Falcon tubes (Becton Dickinson, UK)
4. BD Lysing solution (Becton Dickinson, UK)
5. Sterile Phosphate Buffered Saline solution, 0.5L bottles (Gibco)
6. CD45 -FITC conjugated monoclonal antibody (Becton Dickinson, UK)
7. CD146 – PE conjugated monoclonal antibody (Becton Dickinson, UK)
8. CD34 –PerCP conjugated monoclonal antibody (Becton Dickinson, UK)
9. VEGFR2 (KDR)-APC conjugated monoclonal antibody (R&D Systems)
10. Clear pipette tips (Alpha Laboratories Limited**)**
11. Yellow pipette tips (Alpha Laboratories Limited).

C. Health and Safety / COSHH (**C**ontrol of **S**ubstances **H**azardous to **H**ealth) **issues**

- Lasers
- Danger of electric shocks
- Assume bloods may be biohazardous

D. Detailed method

A full blood count (Bayer Advia) was obtained on the same sample of blood to be used for flow cytometry to back-calculate CEC/EPC numbers to whole blood.

a. Lysing/fixative solution.

This was made with 50mls of 10x concentrated FACS Lysing Solution (containing formaldehyde, stored at room temperature). It was diluted with 450ml distilled water in a 500ml bottle and was not used if older than a month.

b. Blood sample preparation

2. The vacutainer of EDTA or citrate blood sample was gently vortexed. With yellow tip pipette 0.2mL of blood was added to a 3 mL BD Falcon tube.
3. Using a different micropipette each time to avoid cross-contamination, 10µL each of CD45, CD146, CD34, KDR fluorochrome labelled antibodies with a yellow tip micropipette was added. This was flushed into and out of pipette tip to ensure thorough mixing and then gently vortexed. The mixture was incubated in the dark at room temperature for 20 minutes.

4. Using a 1ml pipette 3 ml of pre-diluted BD lysing/fixing solution was added. The solution was incubated for 15 minutes in the dark (e.g. inside a box) and then centrifuged at 200g for 5 minutes.
5. The supernatant was decanted and 3 ml of PBS solution is added. With gentle vortex the pellet was re-suspended and the solution centrifuged once again at 200g for 5 minutes. The supernatant was then discarded.
6. The pellet was re-suspended with 0.5 ml of PBS solution by flushing into and out of the clear pipette tip and by gentle vortex to ensure it was thoroughly mixed. The sample was stored in dark at 4^{o}C until ready to be analysed (note sample must be processed within three days).

c. Running blood sample.

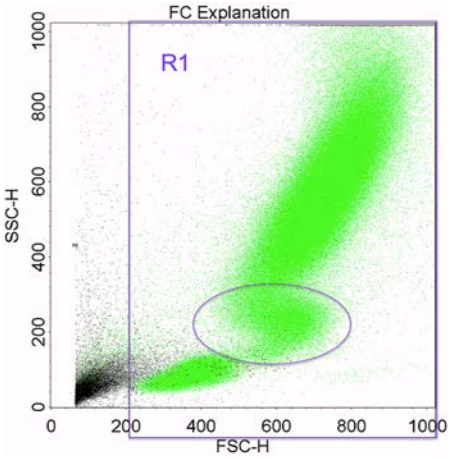
1. The CellQuest Pro software was opened and the required settings were applied.
2. The link to the BD FACSCalibur™ was established by clicking '**Connect to Cytometer**', located under the 'Acquire' menu. When completed proper shut down procedures of the computer and the BD FACSCalibur™, as per the manufacturers' guidance, were observed.
3. Under the '**Cytometer**' menu the '**Instrument Settings**' of the compensations and threshold were changed by clicking on the **open** icon of the window of the folders to set the appropriate instrument settings. The system was updated to the preferred settings for the acquisition, followed by **'Set'** and '**Done**'.
4. The '**Acquire'** menu was clicked once more followed by the '**show browser**'.
5. '**Change'** the directory was clicked to specify the location folder.

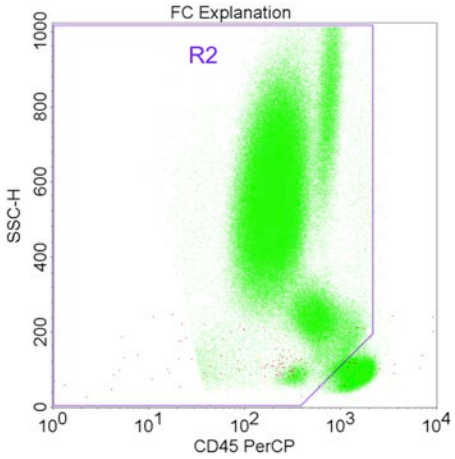
6. A '**new folder'** was created if needed.
7. The sample (in a 3 mL BD Falcon tube) was mixed thoroughly and positioned onto the SIP by opening swing arm at bottom right of the cytometer. The swing arm was replaced under the tube when ready.
8. The **setup** box was un-ticked. The "RUN" and "HIGH" was then clicked.
9. Both the '**Run**' and '**High**' on the control panel of the cytometer were set for a target of ≤ 1500 events per second adjusting to '**Med'** or '**Low'** as necessary.
10. On the browser menu, '**Acquire'** was clicked. Typically the sample ran for ~ 7 minutes, depending on the cell number. Total events and five windows of detected events were displayed on the screen throughout the process and ran until 1,000,000 events were achieved or no sample left was left.
11. The acquisition was observed closely for blockages (which happened very rarely) as suggested when plots did not show any progress and the counters did not display any events per second.
12. If a block was suspected the acquisition was paused and the sample from the SIP replaced with water and run for 5 minutes. Once resolved the acquisition with the sample on the sip was continued. If the problem persisted assistance was sought from the senior scientific staff or the manufacturer.
13. Once obtained both an electronic and a hard copy were obtained.
14. Step 11 was repeated for the next sample (again re-suspended with vortex).
15. The sheath and waste fluid level were checked regularly, refilled and emptied respectively as required.

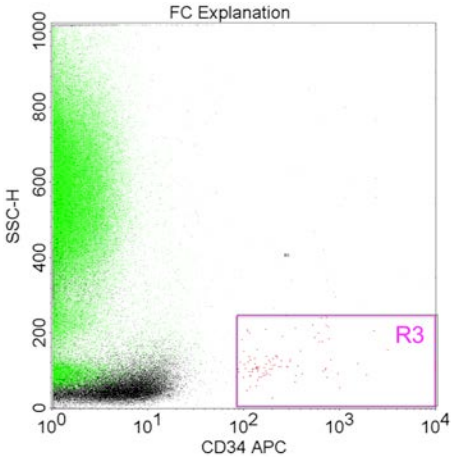
E. Interpretation (example given)

7. The WBC count by Bayer Advia was determined e.g. 4.71 x10^6/mL
8. The first showed the FSC/SSC plot, which gated almost all cells (R1).
9. The SSC/CD45 and SSC/CD34 plots determined R3, highly positive 'CD34 events' e.g. n=176 in the gate statistics out of 612,840 total WBC events on FC.

Gating for CEC and EPC enumeration







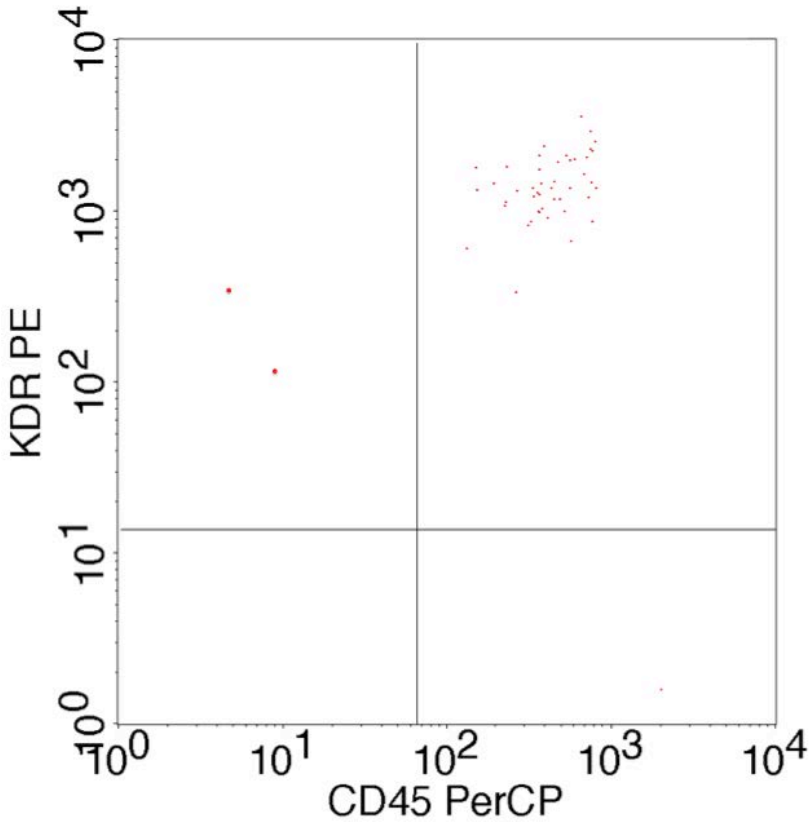
File: CRCT.004
Acquistion Date:29-Jun-10
X Parameter: FSH-H (Log)
Y Parameter: SSC-H (Log)

Gate	Events	% Gated	% Total
CD34 POS Events	176	0.02	0.02
CD34 Cells	114	0.02	0.02
Total WBC Events	612,840	92.50	92.50

10. The R2 of SSC/CD45 with R3 SSC/CD34 collected 'CD34 cells', i.e. n=114. Hence 114/612840 = 1.860 x 10^{-4} WBCs are CD34 cells. From the WBC of the Advia i.e. 4.71 x 10^{6} cells/ml, there were 876 CD34+ve cells/ml in the venous blood. These cells were called CD34+CD45- cells.

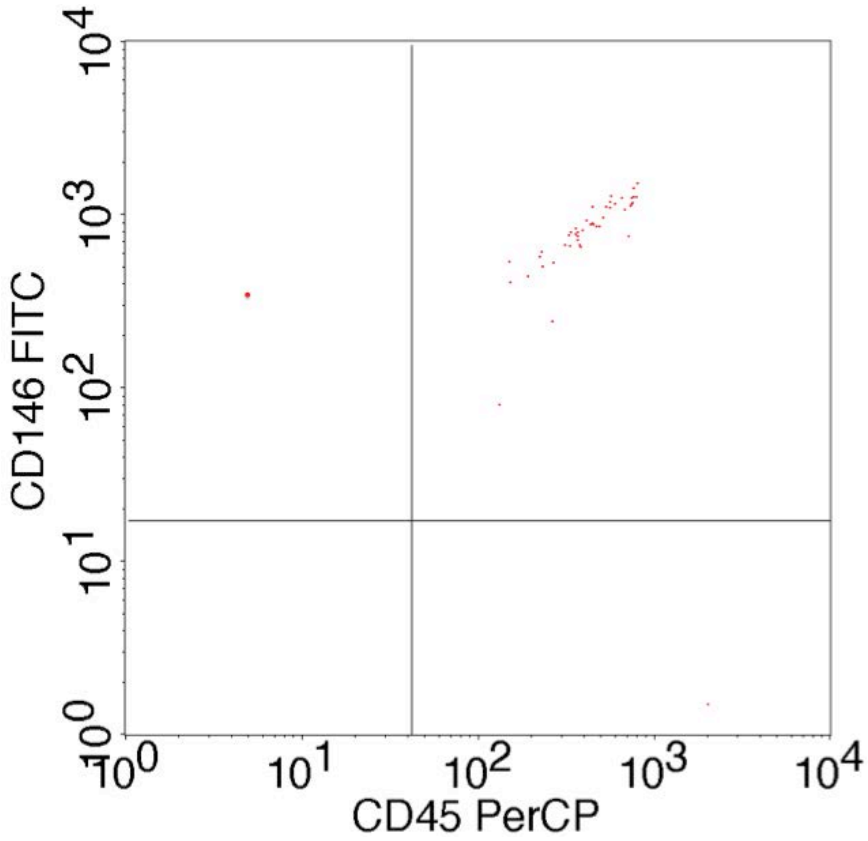
11. On the plot of KDR PE vs CD45 PerCP the CD34+ve events were scanned for KDR and CD45. The events, i.e. 2, in the upper left quadrant were KDR+ but CD45- and therefore were EPCs. Thus 2/114 of the CD34+ve cells were EPCs, (or 2/114th of 876) = 15 EPCs/mL.

Plot derived from R3 of KDR positive CD 45 negative events (EPC).



12. Similarly, on the plot of CD146 FITC vs CD 45PerCP, CECs were low for CD45 and high for CD146, i.e. 1 event. Therefore, 1/114 of the CD34+ve cells were CECs. That is 1/114 of 876 CD34+ cells/mL, or **8 CECs/mL**.

Plot derived from R3 of CD 146 positive and CD 45 negative events (CEC).



Instrument Settings for the FACSCalibur® Flow Cytometry Enumeration of CECs and EPCs

Cytometer Type: FACSCalibur

Detectors/Amps:

Param	Detector	Voltage	AmpGain	Mode
P1	FSC	E00	1.70	Lin
P2	SSC	457	1.00	Lin
P3	FL1	540	1.00	Log
P4	FL2	600	1.00	Log
P5	FL3	744	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL4	819		Log

Threshold:
Primary Parameter: FSC
Value: 0

Secondary Parameter: None

Compensation:
FL1 - 0.7 % FL2
FL2 - 34.0 % FL1
FL2 - 0.0 % FL3
FL3 - 27.5 % FL2
FL3 - 2.9 % FL4
FL4 - 3.8 % FL3

Appendix 4: Standard Operating Procedure for vWf ELISA Assay

A. Introduction

The method was modified from Short el al (375) and used by my colleagues in previous cancer and cardiovascular studies (24, 89, 375).

Synopsis

This was the standard ELISA for the measurement of von Willebrand factor (vWf) using commercial antisera from the Danish company Dako.

Brief Method

7. The microtitre plate was coated with 100 µl of diluted primary antiserum (30 µl in 20.5 ml coating buffer pH 9.6) at room temperature (RT) for >60 minutes.
8. The plate was washed 3 times in PBS/tween, 100 µl of 1/40 serum or plasma in pbs/tween was added along with the standards and incubated for >60 minutes.
9. The wash was repeated 3 times and 100 µl secondary antiserum was added i.e. the peroxidase-labelled conjugate (30 µl in 20.5 ml PBS/tween), and incubated for >45 minutes at room temperature.
10. The plate was washed and 100 µl substrate (OPD, hydrogen peroxide, pH 5 citrate buffer) was added. The colour developed almost immediately.
11. This was stopped with 50 µl acid, read at 492 nm and concentrations determined against the standard curve.

Expected values: in citrated normal plasma are in the region of a mean of 100 with a standard deviation 30 IU/dL. Typical values in stable atherosclerosis are 130 IU/dL and acute coronary syndromes often 150 IU/dL. Data is generally of normal distribution.

B. Materials

1. Coating Buffer

Reagents

- Sodium carbonate or Na_2CO_3 (Sigma).
- Sodium hydrogen carbonate ($NaHCO_{3)}$ also known as sodium bicarbonate (Sigma).
- Distilled water (Sigma-Aldrich).

Method

To 500 ml of distilled water 0.795 g (795 mg) sodium carbonate and 1.465 g (1,465 mg) sodium hydrogen carbonate were added and placed on rotary mixer with magnetic stir bar. At room temperature the salts took around 5 minutes to dissolve (as carbonates are relatively insoluble). Generally the pH was very stable when stored at 4^oC.

Biohazard/COSHH: There were no major concerns unless a few kilos/litres are consumed orally. Spills were washed off with water and good laboratory practice was maintained.

2. Primary Antiserum

This is rabbit anti-human vWf polyclonal antiserum stored at 4^{o}C (DakoCytomation).

3. Microtitre plates & Yellow and Blue Tips

Flat-bottomed 96 well microtitre high binding plates (Immunlon 2, Thermo Electron Corporation).

4. Wash Buffer

Reagents

- Phosphate buffer saline tablets (Sigma).
- Tween 20 (Sigma).
- Distilled water (Sigma-Aldrich).

Method

To one litre of water 5 tablets of buffer and 0.5 ml Tween were added. This was placed on rotamixer with bar and stirred until tablets dissolved. The solution was stored at room temperature.

Biohazard/COSHH: There were no major concerns unless a few kilos/litres are consumed orally. Spills with were washed off with water and good laboratory practice was maintained.

Secondary Antiserum

This is rabbit anti-human vWf polyclonal antiserum conjugated to horse radish peroxidise and stored at 4^{o}C (DakoCytomation).

Substrate

Reagents

- Citrate Phosphate Buffer
  - Citric acid (anhydrous), $C_3H_4OH(COOH)_3$ (Sigma C).
  - Sodium hydrogen phosphate (Na_2HPO_4) or anhydrous Sodium phosphate (Sigma S).
- Ortho-phenylene diamine (Sigma P).
- Hydrogen peroxide (Sigma H).
- Distilled water (Sigma-Aldrich).

Method for Substrate: citrate buffer plus OPD plus peroxide

To 500 ml distilled water 3.65 g citric acid and 4.73 g sodium hydrogen phosphate were added. If it did not dissolve immediately the pH of 5.3 was ensured and adjusted with concentrated acid or concentrated sodium hydroxide solution. The buffer was stored at 4^{o}C in the refrigerator.

To 20 ml citrate phosphate buffer one OPD tablet with 10 µL hydrogen peroxide was added and properly mixed (the tablet dissolve in a few minutes). The buffer when at room temperature aided more rapid colour development.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Good laboratory practice was maintained.

Stop Solution

Hydrochloric acid 1 mol/L (Sigma).

Biohazard/COSHH: **Considerable**. Contact with skin/clothes were avoided, spills washed off with water and good laboratory practice was maintained.

C. DETAILED METHOD

1. Coating microtitre plates

With a micropipette and a yellow tip 30 µL primary antibody was added to approximately 20.5 mls coating buffer, mixed well and poured into a trough. Using an eight-channel micropipette with yellow tips, 100 µL was applied to each well of two microtitre plates. This was incubated on the bench for at least 1 hour or in the refrigerator overnight.

2. Wash

Using the eight-channel washing manifold (Sigma), the unbound antiserum was washed 3 times with >250 µL PBS/tween. The plates were blotted out on tissue paper between steps.

3. Samples of plasma, serum or tissue culture fluid

The samples were thawed in warm water but never for long, to take the chill off (as small aliquots thawed quickly even from -70^{o}C).

vWf is extracted commercially from frozen plasma as cryoprecipitate and therefore undissolved precipitates were avoided.

A 1/40 dilution of plasma was made by adding 10 µL to 390 µL PBS/Tween or equivalent. Generally, it was best to lay out the 80 empty, screw-top vials in a rack and add 390 µL (blue tip) to each en masse. Then the 10 µl were individually added and mixed with up-and-down tip washing.

4. Standards

These standards were referenced against a WHO standard from the National Institute for Biological Standards and Controls (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QH. They provided a lyophilised ampoule of plasma with a defined mass of vWf that could be made up in water to a desired concentration e.g. 100 IU/dL.

These were stored in an Eppendorf tube at -70^{o}C. One vial was thawed in warm water and different aliquots were added to 780 µL of dilution buffer as follows.

a. The *Top Standard* was created by adding 90 µL of plasma to 780 µL of dilution buffer. This ultimately gave a result of 180 IU/dL
b. The *Second Standard* was made by adding 60 µL of plasma to 780 µL of dilution buffer. This ultimately gave a result of 130 IU/dL
c. The *Third Standard* was made by adding 30 µL of plasma to 780 µL of dilution buffer. This ultimately gave a result of 90 IU/dL

d. The *Fourth Standard*: 10 µL of plasma was added to 780 µL of dilution buffer to ultimately give a result of 65 IU/dL
e. The *Bottom Standard:* 5 µL of plasma was added to 780 µL of dilution buffer to ultimately give a result of 50 IU/dL
f. A *Blank* was made by adding 0 µL of plasma to 780 µL of dilution buffer to ultimately give a result of 0 IU/dL
g. Two wells were left empty (e.g. row D, columns 11 and 12) as blanks
h. 100 µL sample or standard was added to each well in duplicate.
i. The *Universal control*, were also placed in duplicate e.g. row E of columns 11 and 12.
j. The plates were incubated at room temperature for minimum of one hour.

5. Wash Again

The wash procedure was repeated as in step 2 above.

6. Conjugate

Using a micropipette and a yellow tip 35 µL of secondary peroxidase-conjugated antibody was added to approximately 20 mls wash buffer (e.g. PBS/tween, green Stago buffer) in a tube. This was mixed well and with an eight-channel micropipette with yellow tips 100 µL was added to each well of the two microtitre plates.

The plates were incubated at room temperature for minimum of 45 minutes.

NB: tips were immediately discarded, as a mere 1 molecule of contaminating enzyme would turn the substrate yellow prematurely.

7. Wash Again

As for step 2 but performed very thoroughly at this stage. Delays were avoided as the blanks may become positive giving high backgrounds.

8. Colour Development

100 µL of substrate was added in a controlled manner, and then, almost immediately, the 50 uL acid stop solution at the same speed in the same direction (i.e. both right to left). This was achieved by applying stop solution as soon as the substrate was added to the last wells of the second plate.

When to stop the reaction was a learned skill but generally determined when a gradient between all the standards could be differentiated and the blanks were still blank. Delays of more than 30 seconds for the colour to develop may have caused the enzyme to quickly consume all the substrate and produce a plateau of the

dose/response curve. If the reaction was slow the substrate buffer was most likely not of the correct pH and adjusted accordingly as already described.

9. Reading and Calculation

This was performed on an ELISA reader after start-up procedures were observed and read at a wavelength of 492 nm. A curve from the standards (IU/dL) was plotted from which the optical densities was determined.

Appendix 5: Standard Operating Procedure ELISA for soluble e-selectin

A. Introduction

Synopsis

This was the standard ELISA for measurement of soluble E-Selectin\CD62E (sE-Sel) using the duoset from R&D. The method and quantities described were for two plates (~75 samples), which required one day for completion. My colleagues in cardiovascular disease and breast cancer studies have used it extensively. (22, 24).

Brief method

7. The microtitre plates were coated with 112ul of capture primary antibody in PBS buffer and incubated in the fridge (4^{o}C) overnight or 1.5 hours at room temperature (RT).
8. The plates were washed, 100 ul serum/plasma (diluted 1/5 i.e. 20 plasma plus 80 blue or pbs-tween buffer) with standards (top 'prepared' at 50 ng/mL) were added and incubated for 1.5 hours at room temperature.

   The 'Universal' plasma was also prepared.
9. The plates were washed again, 112 ul detection antibody (one vial in 20 mls 1% BSA PBS for two plates) was added and incubated for 1.5 hours at RT.
10. Again the plates were washed, 100 ul Streptavidin-HRP conjugate (diluted 1/200 in PBS, i.e. 100 ul plus 20 mls) was added and incubated for a minimum of 20 minutes at room temperature in the dark**.**

11. The plate was washed again and 100ul of substrate (made up from equal volumes of reagents A and B) was added. This went blue after 3 – 5 minutes. The key definition was a clear gradation of blue colour from the top to the blank.
12. The reaction was stopped with 75ul Acid (a yellow reaction) and the optical density was read at 450 nm. A curve of standards was generated and used to determine the concentrations.
13. The expected values were about 20-40ng/mL and the Universal at 40 ng/mL.

B. Materials

1. Wash Buffer

Reagents

- Phosphate buffer saline tablets (Sigma)
- Tween 20 (Sigma)
- Distilled water (Sigma- Aldrich)

Method

To one litre of water 5 tablets and 0.5 ml Tween with a pastette was added and placed on rotamixer with stir bar until tablets dissolved. This was stored at room temperature.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Spills were washed off with water and good laboratory practice was maintained.

2. Primary capture antibody:

One aliquot (obtained from R&D systems) was defrosted and diluted in a total volume 20ml PBS buffer to make a working solution for 2 plates.

3. Recombinant standard:

Each vial in the DuoSet contained 190ng/ml when reconstituted with 0.5ml of 1% BSA PBS. To the vial 500 ul of 1% BSA PBS was added, vortexed gently and incubated at room temperature for 15 minutes. The required top standard for an ideal standard curve was 10ng/ml prepared with 52.6ul from a standard's vial and 947.4ul of 1% BSA PBS.

The standard was double diluted in 1% BSA PBS in the wells of the plate as follows:

(i) 200 ul (of 10ng/ml) was placed in each of wells 11 and 12 of row A.

(ii) 100 ul in wells of rows B to H of columns 11 and 12.

(iii) Wells A to G were double diluted down; the leftover 100 ul was discarded.

(iv) It followed that 11H and 12H were blanks.

4. Controls

- *Internal control (Universal Plasma):* This was a pool of plasma aliquots of 60 ul and 500 ul volumes. This was treated as one of the plasma samples i.e. dilution of 20 ul with 80 ul blue or pbs-tween buffer.
- *Control 1 (8ng/ml):* This was prepared fresh from the standard vial to verify the accuracy of the points on the standard curve obtained by double dilution. From the standard vial 42.1ul was added to 957.9ul of 1% BSA PBS.
- *Control 2 (1ng/ml):* From the standard vial 5.2 ul was added to 994.8ul of 1% BSA PBS.
- *Control 3 (0.5ng/ml):* From the standard vial 2.6ul was added to 994.8ul of 1% BSA PBS.

The controls were placed above the blank and the universal below the blank.

5. Secondary detection antibody:

For this assay, one aliquot (R&D System) was defrosted to make a 20ml in 1% BSA PBS for two plates.

6. Streptavidin HRP conjugate

This was stored at 4^{o}C and diluted to 1/1000 with PBS e.g. 100 ul to 20ml PBS for two plates.

7. Substrate

   The R&D System substrate reagent pack was stored at 4^{o}C, contained two different reagents in glass bottles, A and B, and mixed in equal amounts immediately before use.

8. Stop Solution:

   Hydrochloric acid 1 mol/L (Sigma).

   Biohazard/COSHH: Considerable. Contact with skin/clothes was avoided, spills washed off with water and good laboratory practice was maintained.

C. Detailed Methods

1. Using a micropipette and a yellow tip, one aliquot of defrosted primary antibody to approximately 20 mls PBS buffer was added. Using an eight-channel micropipette with yellow tips, 100 µL was added to each well of two microtitre plates. These were placed in covered boxes, refrigerated overnight OR incubated on the bench for two hours.
2. With the eight-channel washing manifold (Sigma), unbound antiserum was washed out with three lots of >250 µL PBS/tween and blotted between steps.
3. The 34 plasma samples per plate (in duplicates) along with the universal were defrosted and left in fridge overnight.
4. Into the plate wells 80 uL of blue PBS buffer and 20 ul of plasma were placed.
5. Top Standard (10ng/ml): rEsel were prepared as above and 200 ul added to wells of columns 11 and 12. To wells B-H columns 11 and 12 was added 100 ul

buffer. The standards were double dilute in 100 ul volumes down the plate leaving wells 11H and 12 H as blanks.

6. The plates were rested for a minute to enable homogenisation.
7. The plates were incubated for 2 hours at room temperature and washed again.
8. One aliquot of secondary antibody was defrosted and made to 20ml solution in 1%BSA PBS for two plates. To each well 100 ul of the detection antibody was added.
9. The plates were incubated for 2 hours at room temperature in the lunch box and washed.
10. To each well 100ul of streptavidin-HRP diluted 1/200 in PBS was added. Generally this was 100 ul + 20mls buffer. From the fridge reagents A and B were brought to room temperature on the bench.
11. The plates were incubated for 30 minutes at room temperature in the dark.
12. Excess enzymes were thoroughly washed with THREE cycles.
13. Equal volumes of substrate components A+B in were placed on a tray and 100 ul added to each well. The blue colour developed quite rapidly.
14. After 3 - 5 minutes, a clear gradient between the top and bottom standard was established. The stop reagent i.e. 75 uL acid, was added to each well. The blue colour went lemon yellow. Using the ELISA plate reader at 450 nm a standard curve was generated from which concentrations were determined.

Appendix 6: Standard Operating Procedure ELISA for VEGF

A. Introduction

Synopsis

Vascular endothelial growth factor (VEGF) is a multi-functional peptide capable of inducing angiogenesis and may have effects on endothelial integrity. It has been implicated in neovascularisation in adult pathophysiology. This ELISA uses commercial antibody and used by my colleagues in previous studies (24, 485, 486).

Brief Method:

8. Microtitre plates were coated with 100µl of primary antisera (40µl of 40µg/ml in 10ml coating buffer for 1 plate) and stored overnight in the fridge.
9. Plates were washed and blocked with 100µl /well of 5% Marvel (1g in 20mls PBS-T for 2 plates) for 1 hour at room temperature.
10. Plates were washed, 100µl of neat plasma and recombinant standards added and incubated for 2 hours at room temperature. Standards were diluted tenfold with fresh tips for each sample.
11. Plates were washed; 100µl of 500ng/ml of biotinylated anti-human VEGF antibody (100µl of 5 µg/ml in 10ml PBS) was added and incubated for 2 hours at RT.
12. Plates were washed; extravidin peroxidase (100µl/well) was added and incubated for 45 minutes at room temperature.

13. tes were washed; 100µl substrate (Solutions A and B) was added and incubated for 30 minutes at room temperature. The blue colour developed.
14. Reaction was stopped with 50µl/well acid, the colour changed yellow and the concentration after reading at 450 nm.

Expected values: Data was usually non-parametrically distributed. Controls generally had median values of about 30-50pg/ml (but wide IQRs, at times exceeding 200pg/ml). Patients' median values generally were 100 to over 200pg/ml.

B. Materials

1. Coating Buffer

Reagents

- Sodium carbonate [Na_2CO_3] (Sigma).
- Sodium hydrogen carbonate ($NaHCO_3$), or sodium bicarbonate (Sigma).

  NB: both these are anhydrous - ensure you do not order a hydrated species.
- Distilled water (Sigma-Aldrich).

Method

To 500 ml distilled water 0.795 g (=795 mg) sodium carbonate and 1.465 g (=1,465 mg) sodium hydrogen carbonate) was added and placed on rotary mixer with magnetic stir bar. At room temperature the salts took ~5 minutes or so to dissolve (carbonates are relatively insoluble). The solution was stored at 4^oC.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Good laboratory practice was maintained.

2. Primary Antiserum

The primary antiserum is goat anti-human VEGF antibody (R&D systems). A vial was reconstituted with 1ml PBS (gave a stock of 1mg/ml) and 40µl aliquots (gives 40µg) made and stored at –70^{o}C freezer until used. To this 10mls of coating buffer (carbonate) was added to give a 4µg/ml solution, i.e. the working concentration and enough for 1 plate.

3. Microtitre plates; Yellow and Clear Tips

Flat-bottomed 96 well microtitre plates (Immunlon 2).

4. Wash Buffer

Reagents

- Phosphate buffer saline tablets (Sigma)
- Tween 20 (Sigma).
- Distilled water (Sigma- Aldrich)

Method

To one litre of water, add 5 tablets and 0.5 ml Tween (with a pastette) was added, placed on rotamixer with stir bar and mixed until tablets dissolved. This was stored at room temperature.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Good laboratory practice was maintained.

5. Secondary Antiserum

The secondary antiserum is biotinylated goat anti-human VEGF polyclonal antiserum i.e. conjugated to biotin (R&D systems).

The vial was reconstituted with 1ml of PBS-0.1% BSA (which gave stock of 50µg/ml), portioned into 100µl aliquots (giving 5000ng) and stored at –70^{o}C freezer until used.

To get a 5000ng/10mls or 500ng/ml solution 10mls of PBS-T was added.

6. Steptavidin-HRP conjugate

Streptavidin binds to biotin, thus amplifying the signal. HRP = horse radish peroxidase (R&D Systems). Alternatively extravidin peroxidase may be use (Sigma). It was stored at 4 ^{0}C.

7. Substrate

This was formed by mixing solutions A and B from R&D Systems.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Good laboratory practice was maintained.

8. Standards

Standards were obtained from R&D systems. It was present in 100 ul aliquots each containing 1000 ng of protein. To get a 200 ng/ml concentration 400 ul of PBS tween was added. This was further diluted for the final concentration of 100 ng/mL.

9. Stop Solution

Hydrochloric acid 1 mol/L (Sigma).

Biohazard/COSHH: Considerable. Contact with skin/clothes was avoided, spills washed off with water and good laboratory practice was maintained.

C. Detailed Method

1. Coating microtitre plates: With a micropipette and a yellow tip, 40 µl of primary antibody aliquots was transferred to 10 mls coating buffer. Using an eight-channel micropipette with yellow tips 100 µL was placed in each well of the microtitre plate. This was incubated at 4^{o}C overnight.
2. Wash: Using the eight-channel washing manifold (Sigma), the unbound antiserum was washed with three lots of >250 µL PBS/tween. Plates were blot out on tissue paper between each step.
3. Samples: Plasma AND standards were thawed in warm water, but never for long, just to take the chill off (from –70^{o}C small aliquots quickly thawed). To each well 100µl of neat plasma was added and tips changed with each sample.
4. The Standard Curve: The standard consisted of a small amount of pre-aliquoted rVEGF and stored at -70^{o}C. For four plates 2 mls of 3000 pg/mL was used.
    a. From the standard, 1ml was added to 2 ml of PBS/tween to make 3mls at 1000 pg/mL.
    b. From a, 1 mL was added to 2 ml of PBS tween to make 3 mls at 333 pg/mL.
    c. From b, 1 mL was added to 2 ml of PBS tween to make 3 mls at 111 pg/mL.
    d. From c, 1 mL was added to 2 ml of PBS tween to make 3 mls at 37 pg/mL.
    e. From d, 1 mL was added to 2 ml of PBS tween to make 3 mls at 12 pg/mL.
    f. From e, 1 mL was added to 2 ml of PBS tween to make 3 mls at 4 pg/mL.
    g. From f, 1 mL was added to 2 ml of PBS tween to make 3 mls at 1 pg/mL.

Thus this gave eight tubes; 100 µl was added to each of rows A – H, columns 11 and 12 for the standard curve. The plates were placed in a covered box as before and incubated for 2 hours at room temperature.

5. Wash and secondary detection antibody: Wash was performed as above. The secondary Ab was pre- aliquoted and stored at -70^{o}C. This was transferred to 10mls of PBS-T, mixed well and 100µl was added to each well with an 8-channel pipette. The plates were incubated at room temperature for 2 hours.

6. Wash: as above.

7. Peroxidase: With a micropipette and a yellow tip, 10µl of extravidin peroxidase was added to approximately 10mls of PBS-T and, using an eight-channel micropipette with yellow tips, 100 µL were put into each well. The plates were incubated in a covered box and incubated on the bench for 45 minutes. All tips were discarded immediately as a mere 1 molecule of contaminating enzyme will activate the substrate.

8. Colour Development: The R&D Systems reagents A and B were used at room temperature for quicker development. Once mixed 100 µL was added in a controlled manner, and then the acid at the same speed in the same direction (eg. both right to left). This was achieved by applying stop solution as soon as the substrate was added to the last wells of the second plate. When to stop the reaction was a learned skill but generally determined when a gradient between all the standards could be differentiated and the blanks were still blank. Delays of longer than 30 seconds for the colour to develop were avoided as the enzyme would have quickly consumed all the substrate and given a plateau of the

dose/response curve. The initial blue colour turned yellow with the acid. Slow reactions suggested that the substrate buffer was not of the correct pH and this was adjusted accordingly.

9. Reading and Calculation: The wavelength of 450 nm on optical reader was used and a standard curve using 4-6 log versus optical densities was constructed.

Appendix 7: Standard Operating Procedure ELISA for Angiogenin

A. Introduction

Synopsis:

Angiogenin is a potent angiogenic protein whose concentration in serum is elevated in patients affected by various types of cancers. Mechanisms by which angiogenin induces neovascularization are yet to be elucidated, though this protein interacts with endothelial and smooth muscle cells, stimulates a second messenger cascade, induces cell proliferation, mediates cell adhesion, activates proteases and induces cell invasion. This SOP has been developed and results reported by my colleagues in breast cancer and cardiovascular disease (24, 378, 487).

Brief Method:

7. Microtitre plate wells were coated with 100µl of primary antiserum at room temperature (RT) for 90 minutes or at 4^{o}C overnight.
8. Plates were washed, plasma added and recombinant standards diluted in PBS/tween for 90 minutes at room temperature.
9. Plates were washed again, 100µl of biotinylated anti-human angiogenin antibody (one vial for 10ml PBS-T) added to each well for 90 minutes at RT.
10. Plates were washed again, 100µl/well of streptavidin-HRP (50µl strep-HRP in 10mls of PBS-T for 1 plate) was added and incubated for at least 20 minutes at room temperature avoiding direct light.

11. Plates were washed again, 100µl warm substrate solution (5mls A + 5mls B for 1 plate) were added. The colour developed in less than 5 minutes.
12. The reaction was stopped with 50µl/well of acid and read at 450 nm.

Expected values:

Data was non-parametric, controls generally had median values of about 5mcg/ml.

B. MATERIALS

1. Primary Antiserum

The primary antiserum was mouse anti-human Angiogenin antibody (R&D systems). A vial was reconstituted with 1ml of PBS (to give 360 µg/ml) and divided into 18 aliquots of 55µl or 20 µg and stored at -70^{0}C until used. To get 2 µg/ml (enough for 1 plate), 10mls of PBS-T was added.

2. Coating Buffer

Reagents

- Sodium carbonate [Na_2CO_3] (Sigma).
- Sodium hydrogen carbonate ($NaHCO_3$), also known as sodium bicarbonate (Sigma).
- Distilled water (Sigma-Aldrich).

Method

To 500 ml distilled water 0.795 g (=795 mg) sodium carbonate and 1.465 g (=1,465 mg) sodium hydrogen carbonate) was added and placed on rotary mixer

with magnetic stir bar. At room temperature the salts took ~5 minutes or so to dissolve (carbonates are relatively insoluble). The solution was stored at 4^{o}C.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Spills were washed off with water and good laboratory practice was maintained.

3. Microtitre plates; Yellow and Clear Tips

Flat-bottomed 96 well microtitre plates (Immunlon 2).

4. Wash Buffer

Reagents

- Phosphate buffer saline tablets (Sigma)
- Tween 20 (Sigma).
- Distilled water (Sigma- Aldrich)

Method

To one litre of water, add 5 tablets and 0.5 ml Tween (with a pastette) was added, placed on rotamixer with stir bar and mixed until tablets dissolved. This was stored at room temperature.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Spills were washed off with water and good laboratory practice was maintained.

5. Secondary Antiserum

The secondary antiserum was biotinylated mouse anti-human Angiogenin antibody (R&D systems). One vial was reconstituted with 1ml of PBS 0.1% BSA (to give a stock of 50µg/ml), divided into aliquots of 100µl or 5000ng and stored at -70^{o}C freezer until used. To get 500ng/ml (enough for 1 plate) 10mls of PBS-T was added.

6. Steptavidin-HRP conjugate

To 100µl of this conjugate 20mls of PBS-T was added. This gave enough for 2 plates or 50µl into 10mls of PBS-T for 1 plate. This was stored at 4 ^{0}C.

7. Substrate

This was formed by mixing solutions A and B from R&D Systems.

Biohazard/COSHH: There were no major concerns unless a few litres were consumed orally. Good laboratory practice was maintained.

8. Standards

Standards were obtained from R&D systems. To one vial 500µl of PBS was added to give stock of 50ng/ml (or 25 000pg in 500µl). This was divided into 20 µl aliquots containing 1000pg each and stored at -70^{0}C until used. Adding 1980µl of PBS-T to each aliquot (i.e.: 1 in 10 dilution) gave a working top standard concentration of 500pg/ml (4 plates in duplicates).

9. Stop Solution

Hydrochloric acid 1 mol/L (Sigma).

Biohazard/COSHH: Considerable. Contact with skin/clothes was avoided, spills washed off with water and good laboratory practice was maintained.

C. METHOD

1. Coating microtitre plates: With a micropipette and a yellow tip, 55 µl of primary antibody aliquots was mixed with approximately 10 mls of PBS. Using an eight-channel micropipette with yellow tips, 100 µL was placed into each well of the microtitre plate. Plates were incubated in a covered box overnight at 4^{o}C.
2. Wash: Using the eight-channel washing manifold (Sigma), the unbound antiserum was washed with three lots of >250 µL PBS/tween and blot out on tissue paper between steps.
3. Samples: i.e. plasma and standards were thawed in warm water but never for long, just to take the chill off. The plasma was diluted to 1:2010 as follows: 10µl of serum/plasma was mixed with 2000µl of PBS-T (i.e. a 1:201 dilution) in a small screw-top plastic tube; with the lid secure it was mixed well by inversion. From

this diluted plasma 10µl was placed into the each of **three** wells of a microtitre plate and 90µl of PBS-T to give a final dilution of 1:2010.

4. Standards: The Standard Angiogenin is kept in the -70^{0}C.

   (a) To one vial 1980 µl of PBS tween was added.

   (b) From this 200 µl was placed in wells of columns 11 and 12 of row A.

   (c) 100 µl of PBS-tween was placed in wells of columns 11 and 12, rows B to H.

   (d) Column 11 and 12 were double diluted in 100 µl aliquots from rows A to F.

   (e) It follows that wells 11 and 12 in rows G and H were blanks.

   (f) The universal control was placed in one of the empty wells.

   The plates were replaced in the covered box as before and incubated for at least 1.5 hours at room temperature. All leftover tubes, vials and tips were discarded.

5. Wash and Secondary (Detection) Antibody: The plates were washed as above (C2). The 55µl aliquots of secondary Ab was transferred to 10mls of PBS-T, mixed, 100µl put into each well with an 8-channel pipette and incubated at room temperature for 2 hours.

6. Wash and Conjugate: The plates were washed as above (C2). Using a micropipette and a yellow tip, 50µl of streptavidin-HRP was added to 10mls of PBS-T. With an eight-channel micropipette with yellow tips, 100 µL was placed to each well. The plates were incubated in the covered box on the bench for a minimum of 20 minutes avoiding direct light. The tips were discarded immediately as a mere 1 molecule of contaminating enzyme will activate the substrate.

7. Wash: This was performed FOUR times and thoroughly to avoid positive blanks.

8. Colour Development:

(i) The substrate (equal amounts of Solution A and B) was mixed and brought to room temperature. The acid stop solution was placed into its own trough.

(ii) In a controlled manner 100µl of substrate was added from right to left. That is standards developed first. Exactly when to stop the reaction was a learned skill. Generally, it was when a gradient was differentiated between all the standards and the blanks were still blank.

(iii) When there was a good gradient down the standard, the stop acid was added at the same speed in the same direction (e.g. both right to left). This often meant the reaction was stopped on completing the addition of the substrate to the second plate.

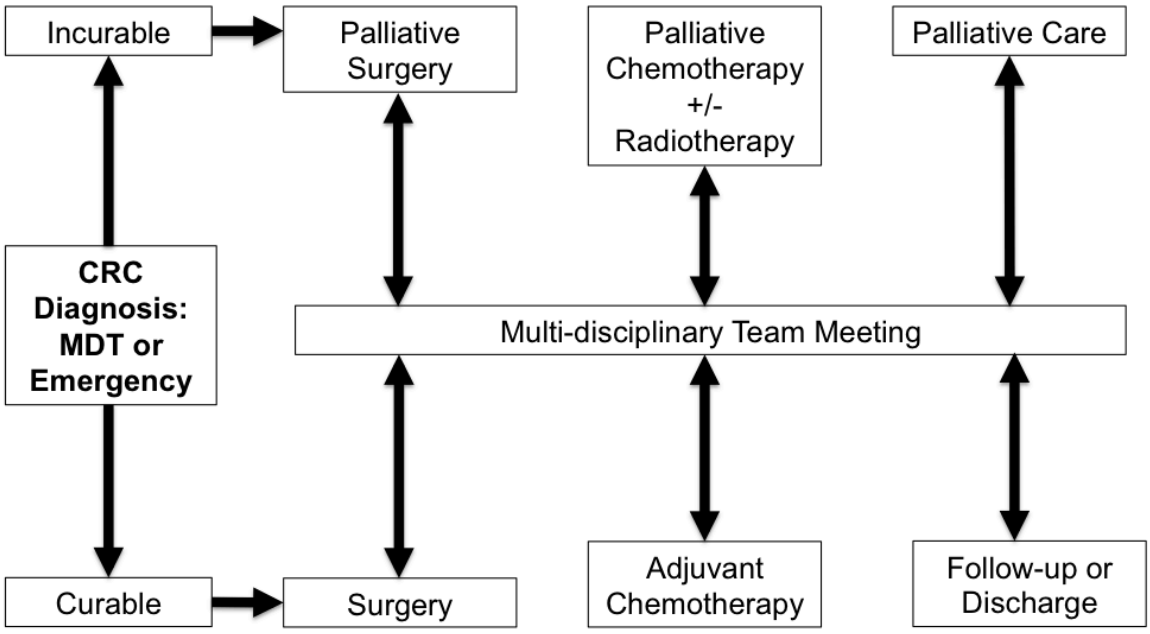
(iv) To stop the reaction 50µl of concentrated was added which changed the blue colour to bright yellow.

9. Reading and Calculation: The ELISA reader the wavelength was set to 450 nm and a standard curve was generated to determine the concentrations.

Appendix 8: Treatment of CRC

A summary of the treatment strategy for CRC is shown below and adapted from guidelines of National Institute for Clinical excellence [NICE] and Association of Coloproctology of Great Britain and Ireland [ACPGBI 2007] (285, 318) .

Summary of the Treatment Strategy of Colorectal Cancer



MDT- all patients are discussed in the Multi-Disciplinary Team (primarily of Oncologists, Radiologists, Surgeons, Histopathologists, Gastroenterologists, Nurse Specialist) weekly meeting to determine their best therapeutic strategy at all stages of the patient's treatment.

Surgery

Surgery, whether laparoscopic or open, is the cornerstone for cure in localised disease (285). Curative resections were based on histological and surgical confirmation of complete excision as follows (319-321):

- Distal margins of 5 cm, 2cm for rectal cancers
- At least 10 to 12 lymph node
- En-bloc resection of involved organs with no microscopic cancer at the margins
- For rectal cancer, total excision of the mesorectum (TME) with inferior mesenteric nodes and clear circumferential and distal margins.

The liver, more so than the lung, was the most common site of relapse after surgery. Resection of liver and lung metastases may substantially improve 5- and 10-year survival rates. Improvements in liver surgery along with chemotherapy before and after surgery versus surgery alone reduced the risk of relapse. Chemotherapy for inoperable liver metastases may render them resectable (322, 323).

Palliative resections may be required to relieve obstruction or improve quality of life for advanced and debilitating disease (318).

Neoadjuvant treatment

This refers to treatment before surgery and typically applies to rectal cancers. The meta-analysis of the Colorectal Cancer Collaborative Group concluded both preoperative (from 22.5% to 12.5%; p<0.001) and postoperative radiotherapy (25.8% to 16.7% p=0.001) reduced local recurrence but the benefit to overall survival was marginal (62% vs 63% deaths; p=0.06) (314). Analysing a number of trials in the last decade, the Association of Coloproctologists of Great Britain & Ireland recommend two strategies: preoperative short course radiotherapy [SCRT] followed by Total Mesorectal Excision (TME) surgery or preoperative long course chemotherapy and radiotherapy followed thereafter by surgery (285).

Poor results (i.e. local recurrence rates > 10%) are seen with surgery alone, or long course postoperative radiotherapy alone (314). The best results were from short course preoperative radiotherapy and high quality TME (285). However the long-term complications from radiotherapy against the benefits of reducing local recurrence and the marginal effect on disease free and overall survival were uncertain.

Adjuvant treatment

There are well-established predictive factors for local recurrence after resection and hence the need for adjuvant chemotherapy i.e. therapy after surgery (285, 318):

- indeterminate or positive resection margin.
- lymph node spread.
- extramural vascular invasion.
- bowel obstruction at presentation.
- poor differentiation.
- less than 10 lymph nodes in the resected specimen.
- CT,PET, or US scan evidence of metastases.

Ideally radiotherapy is offered preoperatively for rectal cancers but if omitted postoperative long course treatment is combined with chemotherapy. Adjuvant fluoropyrimidine (Capecitibine or 5-FU) alone or oxaliplatin with 5-FU and folinic acid, are offered for of node-positive disease following putatively curative surgery (294).

In node positive disease (Dukes' C or stage III) 6 months of 5-FU or capecitibine reduces mortality by 30% i.e. a 10-15% survival gain. However, beyond this many adjuvant trials have not matched the overall survival (OS) benefit at 5 years with the 3 year disease free survival [DFS] (324).

Adjuvant chemotherapy for Dukes' B or stage II CRC is controversial given the small gains in survival when studies combined stages II and III. However high-risk patients

are offered treatment based on the QUASAR study in which OS improved for those ≤70 years of age [risk reduction of 18% (stage III) and 3·6% (stage II)] (325, 326).

The addition of adjuvant biological or immunotherapy with either edrecolomab (monoclonal antibody to epithelial cell adhesion molecule) or bevacizumab (humanised monoclonal antibody against VEGF) to chemotherapy did not improve DFS of stage II or III disease (327). The QUASAR 2 trial (Bevacuzimab with capecitabine vs capecitabine alone for stage II and III disease) conducted in the UK has recently closed and the results pending (Appendix 1, page 257). Full details of all chemotherapy regimes are tabulated on page 311.

Relapse following surgery, regardless of adjuvant chemotherapy, occurs within 2 years (328, 329). Intensive follow-up improves survival in a third of patients by detecting and radically treating metastases (commonly liver or lung) (314). Surveillance involved clinical review, CT scan every year for the first 3 years, and colonoscopy a year after surgery and then 3 to 5 yearly (285, 306). Monitoring CEA was not routine as it was neither sensitive for nor specific to CRC (314).

Adjunct Chemotherapy

This therapy is either part of a trial or for palliation (306). Palliative chemotherapy may improve survival, symptoms, quality of life, and resectability of liver or lung metastases (294). 5-FU with irinotecan, oxaliplatin, and targeted regimes has increased survival from 1 to roughly 2 years (330, 331). For survival, the sequence of the regime, either for initial therapy or for progression of the disease, was not as crucial as treatment with all active agents at any given period (332).

The main advance in managing mCRC is the addition of targeted therapies (333). Though shown to have relatively little survival benefit, they include Cetuximab [human EGFR monoclonal antibody], bevacizumab [human VEGF monoclonal antibody], and panitumumab [human EGFR monoclonal antibody], (294). Bevacizumab theoretically is a modifier of the endotheliome and angiome, as it 'normalises' the vasculature and hence improves drug delivery to the growing tumours (334). When administered with irinotecan and fluorouracil, the OS in the responders of untreated mCRC improved by 4·7 months (333, 335, 336).

In summary, the staging of CRC has improved with radiological techniques but, in the absence of obvious distant spread, is only accurate after resection. Adjuvant and adjunct treatment is also monitored radiologically (CT/PET/MRI). However, the sensitivity for diagnosing recurrence and/or metastasis on CT is only 70% (specificity of 94%). At present invasion into blood vessels, described histologically, is the only 'vascular marker' of prognostic value and identified post-resection.

Chemotherapy regimes

Dukes	Standard Chemotherapy Regimes
A	None
B	Modified de Gromant regime (488) Leucovorin [folinic acid] + 5-fluorouracil [5-FU] (2). 5-FU 400mg/m^2 IV bolus, followed by 5-FU 1,200mg/m^2/day IV x 2 days (total 2,400mg/m^2) as a 46–48 hr continuous infusion. Repeat every 2 weeks equivalent to 12 cycles or 6 months.
or	Capecitibine [Xeloda] - rarely used by the hospitals in my study. Days 1–14: Capecitabine 1,250mg/m^2 orally twice daily. Repeat cycle every 3 weeks for 8 cycles over approximately 6 months.
C	FOLFOX (488-490) Leucovorin [folinic acid] + 5-fluorouracil [5-FU] + oxaliplatin [Eloxatin] Day 1: Oxaliplatin 85mg/m^2 IV over 2 hrs + leucovorin 400mg/m^2 IV over 2 hrs, followed by 5-FU 400mg/m^2 IV bolus, followed by 5-FU 1,200mg/m^2/day IV x 2 days (total 2,400mg/m^2) as a 46–48 hr continuous infusion. Repeat every 2 weeks. * FOLFOX is superior to 5-FU alone in Stage III patients.
or	CapeOX (490, 491) Capecitibine [Xeloda] + Oxaliplatin [Eloxatin] Capecitibine 1000 mg/m^2 PO BID on days 1-14. Oxaliplatin 130 mg/m^2 IV over 2 hours on day 1. Twenty one day cycle x 8 cycles.
or	As for Dukes' B depending on age and/or coomordities.
D	Modified de Gromant regime
or	FOLFOX
or	CapeOX
or	FOLFOXIRI FOLFOX and irinotecan Used by the oncologist if no response to the above regimes.

	Anti-VEGF Regimes
D	Modified de Gromant regime + Bevacuximab (Avastin®) (492) 5-FU 400mg/m^2 IV bolus, followed by 5-FU 1,200mg/m^2/day IV x 2 days (total 2,400mg/m^2) as a 46–48 hr continuous infusion. Bevacizumab 5mg/kg every 2 weeks. Repeat biweekly.
B or C	QUASAR 2 Trial: Capecitibine vs Capecitibine + Bevacuximab (Avastin®) Group 1 - capecitabine (equivalent to standard chemo). Capecitabine tablets twice a day (12 hours apart) for 2 weeks out of every 3. None given in the 3rd week. Each 3 week block is one cycle of chemotherapy or 8 cycles over about 6 months altogether. Group 2 **-** capecitabine and bevacizumab. capecitabine As for group 1. On day 1 of each 3 week cycle bevacizumab IV (5mg/kg). In total 8 cycles of capecitabine over about 6 months and 16 cycles of bevacizumab or about 12 months altogether.

Summary of Treatment of Operable Rectal Cancer

MRI/CT Stage	Histology	Pre-Surgery		Surgery	Post-Surgery		
		Chemo	Radio		Chemo	Radio	Adjunct
Stage I low risk • T1 or 2 • <3 cm • <30% of the bowel circumference • CRM clear	Moderately or Well differentiated	-	-	Local excision of tumour [TART, TEM]	-	-	-
Stage I high risk • not fulfilling low-risk criteria • CRM clear	Lymphatic +/or vascular invasion +/or poorly differentiated	-	SCRT	Rectal Resection	+/-	LCRT	-
Stage II to III • CRM clear AND • T3N0 OR • T4N0 OR • Any T,N1 or 2		+/-	SCRT	Rectal Resection	+/- CRT	+/- CRT	+/-
Stage IV • +/- CRM		CRT	CRT	Rectal Resection	CRT	CRT	+/-

MRI- Magnetic Resonance Imaging; Chemo- chemotherapy; Radio- Radiotherapy; TART- Transanal Resection of Tumour; TEM- Transanal Endoscopic Microsurgery; SCRT- short Course Radiotherapy; CRT- Chemo- Radio- therapy; CRM- Circumferential resection margin.

Appendix 9: Regression analysis of patient and tumour factors in CRC stage.

Table A: Univariate and Multivariate regression analysis of patient and tumour factors with Dukes' Stage.

	Univariate		Multivariate		
Versus Dukes' Stage	**R^2**	**p**	**OR**	**95 % CI**	**p**
CEC	0.091	0.159	**1.0**	**1.0 - 1.0**	**0.032**
EPC	-0.119	0.096	1.0	1.0 - 1.0	0.219
CD34+CD45- cells	-0.110	0.115	1.0	1.0 - 1.0	0.189
WCC	0.086	0.173	1.0	1.0 - 1.1	0.295
BMI	-0.131	0.074	1.0	0.9 - 1.0	0.244
Sex	0.097	0.143	1.1	0.8 - 1.6	0.408
Age	-0.018	0.424	1.0	1.0 - 1.0	0.712
Screen detected	0.009	0.461	1.0	0.6 - 1.8	0.929
Hypertension	-0.095	0.115	0.8	0.5 - 1.2	0.225
Diabetes Mellitus	-0.031	0.368	1.1	0.6 - 1.9	0.787
Ischemic heart disease	-0.098	0.114	0.6	0.4 - 1.0	0.065
First Degree Relative	0.057	0.268	1.1	0.7 - 1.6	0.791
ACE Inhibitor	-0.034	0.355	1.3	0.8 - 2.1	0.251
ARB	-0.051	0.288	1.0	0.5 - 2.2	0.996
Beta Blocker	-0.004	0.481	1.4	0.9 - 2.2	0.148
Calcium Channel Blocker	0.011	0.451	1.3	0.8 - 2.1	0.345
Systolic BP	-0.018	0.421	1.0	1.0 - 1.0	0.501
Heart Rate	-0.007	0.468	1.0	1.0 - 1.0	0.400
Saturation	0.020	0.614	1.1	1.0 - 1.3	0.133
Temperature	0.079	0.192	1.0	0.9 - 1.2	0.583
Tumour Diameter	0.126	0.084	1.0	1.0 - 1.0	0.635
Tumour Differentiation	0.092	0.156	1.0	0.8 - 1.3	0.697
Vascular Invasion	0.131	0.076	1.1	0.8 - 1.7	0.545
Tumour Site	-0.072	0.215	1.0	0.9 - 1.0	0.265
Number of nodes*	**0.376**	**0.001**	**1.2**	**1.1 - 1.3**	**0.001**

P values of <0.05. R^2 by Pearson's correlation. OR=Odds Ratio. Tumour perforation and involved resection margins were excluded given their very small numbers. Model fit was R2=0.334, p=.013. ARB- angiotensin receptor blocker. BMI- Body mass index. BP- blood pressure. * Number of involved nodes- correlation was likely as Dukes' stage C included positive nodes by location, not number involved. However, there was minimal difference when number of involved nodes was excluded.

Table B: Univariate and Multivariate regression analysis of patient and tumour factors with modified AJCC (mAJCC) Stage.

	Univariate		Multivariate				
Versus mAJCC Stage	**R^2**	**p**	**OR**	**95 % CI**			**p**
CECml	0.089	0.139	1.0	1.0		1.0	0.166
EPCml	-0.024	0.387	1.0	1.0	-	1.0	0.984
CD34+CD45- cells	-0.056	0.246	1.0	1.0	-	1.0	0.303
WCC	0.139	0.043	1.1	1.0	-	1.2	0.236
Sex	-0.027	0.373	1.0	0.7	-	1.4	0.936
Age	-0.026	0.375	1.0	1.0	-	1.0	0.877
BMI	**-0.136**	**0.047**	**0.9**	**0.9**	**-**	**1.0**	**0.049**
Screen detected	-0.062	0.223	0.8	0.4	-	1.6	0.597
Hypertension	-0.052	0.330	0.6	0.4	-	1.0	0.330
Ischaemic hear disease	-0.064	0.150	0.6	0.4	-	1.0	0.062
First Degree relative	0.019	0.407	0.9	0.6	-	1.4	0.770
ACE inhibitor	0.014	0.431	1.1	0.7	-	1.8	0.593
ARB	-0.040	0.314	1.0	0.5	-	2.2	0.958
Beta blocker	-0.026	0.374	1.4	0.8	-	2.2	0.198
Calcium channel blocker	0.097	0.106	1.8	1.1	-	3.0	0.325
Diabetes Mellitus	0.081	0.161	1.5	0.9	-	2.5	0.147
Systolic BP	0.001	0.497	1.0	1.0	-	1.0	0.602
HR	-0.056	0.245	1.0	1.0	-	1.0	0.346
Saturation	0.126	0.061	1.1	0.9	-	1.2	0.252
Temperature	0.123	0.066	1.2	1.0	-	1.4	0.096
Tumour Diameter	0.181	0.013	1.0	1.0	-	1.0	0.075
Differentiation	-0.020	0.406	0.9	0.7	-	1.2	0.422
Vascular Invasion	0.020	0.405	1.0	0.7	-	1.6	0.975
Tumour Site	-0.085	0.150	1.0	0.9	-	1.0	0.456

P values of <0.05. R^2 by Pearson's correlation. Model fit was R2=0.212, p=.108. Tumour perforation and involved resection margins were excluded given their very small numbers. ARB- angiotensin receptor blocker. BMI- Body mass index. BP- blood pressure. Number of nodes involved as nodal status is a factor of AJCC staging and were therefore excluded. AJCC was modified by pooling T4N2M0 with stage 4 disease (Stage 1-4 were equivalent to Dukes' A to D), as both had similar life-expectancies.

Appendix 10

Publications related to the thesis

1. Ramcharan KS, Lip GY, Stonelake PS, Blann AD. The endotheliome: a new concept in vascular biology. Thromb Res. 2011 Jul;128(1):1-7.

2. Blann AD, Ramcharan KS, Stonelake PS, Luesley D, Lip GY. The angiome: a new concept in cancer biology. J Clin Pathol. 2011 Jul;64(7):637-43.

3. Ramcharan KS, Lip GY, Stonelake PS, Blann AD. Angiogenin outperforms VEGF, EPCs and CECs in predicting Dukes' and AJCC stage in colorectal cancer. Eur J Clin Invest. 2013 Aug;43(8):801-8.

4. Ramcharan KS, Lip GY, Stonelake PS, Blann AD. Effect of standard chemotherapy and antiangiogenic therapy on plasma markers and endothelial cells in colorectal cancer. Br J Cancer. 2014 Oct 28;111(9):1742-9

5. Ramcharan KS, Lip GY, Stonelake PS, Blann AD. Increased pre-surgical numbers of endothelial progenitor cells and circulating endothelial cells in colorectal cancer fail to predict outcome. Int J Colorectal Dis. 2015 Jan 20.

Presentation of results at international meetings

1. The endotheliome and angiome: new concept in colorectal cancer biology. Association of Coloproctology of Great Britain and Ireland International Meeting, 20-23 June 2011, Birmingham, UK. Abstract published in Volume 13, Issue Supplement s4 Page 21. *(Shortlisted for short paper prize).*

2. CECs and EPCs in colorectal cancer staging.
   The International Surgical Congress of the Association of Surgeons of Great Britain and Ireland, 11–13 May 2011, Bournemouth, UK. Abstract published in British Journal of Surgery Volume 98, Issue S3, page 17.
   (Shortlisted for poster prize).

3. Angiogenin outperforms EPCs and CECs in predicting Dukes' stage.
   The Association of Coloproctology of Great Britain and Ireland Annual Meeting, 1-3 July 2012, Dublin, Ireland. Abstract published in Colorectal Disease Volume 14, Issue Supplement s1, pages 31, July 2012.