VASCULAR ENDOTHELIAL GROWTH FACTORS IN COLORECTAL CANCER

A Thesis Submitted to the University of Manchester

for the Degree of M.D.

in the Faculty of Medicine, Dentistry, Nursing and Pharmacy

2004

SARAH ELIZABETH DUFF BM, BCh, MA, FRCS

Laboratory Medicine Academic Group,
University of Manchester,
Stopford Building,
Oxford Road,
Manchester M13 9PT

Department of Surgery,
Christie Hospital NHS Trust,
Wilmslow Road,
Manchester M20 4BX

INDEX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5′-NA	5′-nucleotidase
5-FU	5-fluorouracil
AP-1 or -2	activator protein-1 or -2
APC	adenomatous polyposis coli
ASCO	American Society of Clinical Oncology
CAM	chorioallantoic membrane
CEA	carcinoembryonic antigen
CI	confidence interval
CRF	case record form
CSA	catalysed signal amplification
CT	computerised tomography
DAB	3′-diaminobenzidine
DCC	deleted in colon cancer
DNA	deoxyribonucleic acid
EC	endothelial cell
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EUA	examination under anaesthetic
FAP	familial adenomatous polyposis
FFPE	formalin fixed paraffin embedded
FGF	fibroblast growth factor
FIGF	*c-fos* induced growth factor
FITC	fluoroisothiocyanate
Flk	fetal liver kinase
Flt	fms-like tyrosine kinase
FOB	faecal occult blood
FS	flexible sigmoidoscopy
H & E	haematoxylin and eosin
HDMEC	human dermal microvascular endothelial cells
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HIF-PH	HIF-proyl hydroxylase
HNPCC	hereditary non-polyposis colon cancer
HR	hazard ratio
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
ICAM-1	intracellular adhesion molecule-1
ICG-HNPCC	International Collaborative Group for HNPCC
Ig	immunoglobulin
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
IMS	industrial methylated spirit
ISH	*in situ* hybridisation
J	junctional mucosa
kb/bp	kilobase pairs/base pairs
K_d	dissociation constant
KDR	kinase domain containing receptor
LN	lymph node
LVD	lymphatic vessel density
LYVE-1	lymphatic vessel hyaluronan receptor-1
MAPK	mitogen activated protein kinase
MMR	mismatch repair
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSI	microsatellite instability
MVD	microvessel density
N	number of cases
N	normal mucosa
NA	not assessed
NF-κB	nuclear factor kappa B
NICE	National Institute for Clinical Excellence
NK	not known
NO	nitrous oxide
NRP	neuropilin
NS	not significant
NSCLC	non-small cell lung cancer
OS	overall survival
OR	odds ratio
PA	plasminogen activator
PAI	plasminogen activator inhibitor

PC	protein convertase
PD-ECGF	platelet derived endothelial cell growth factor
PDGF	platelet derived growth factor
PET	positron emission tomography
PGE_2	prostaglandin E_2
PI3-K	phosphatidylinositol 3′-kinase
PlGF	placental growth factor
pM	picomolar
PMA	phorbol myristate 12,13-acetate
QUASAR	quick and simple and reliable
RER	replication error
RFS	relapse free survival
RIP	rat insulin promoter
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SLC	secondary lymphoid tissue chemokine
Sp1	specificity protein 1
sVEGFR1	soluble vascular endothelial growth factor receptor 1
TAM	tumour-assoicated macrophage
TBS	Tris buffered saline
TGF-α or -β	transforming growth factor-α or –β
TC	central tumour
TI	invasive edge of tumour
TNF-α	tumour necrosis factor-α
TNM	tumour/node/metastasis stage
TME	total mesorectal excision
TS	superficial tumour
tPA	tissue-type PA
uPA	urokinase-type PA
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VIP	very intense purple
VRP	VEGF-related protein
vWF (FVIII)	von Willebrand factor (factor VIII related antigen)

ABSTRACT

Background: Colorectal cancer is a common human malignancy with an incidence of approximately 35,000 new cases per year in the UK. Angiogenesis, the formation of new blood vessels, is important for the growth and metastasis of solid tumours and is influenced by the expression of growth factors, in particular by members of the vascular endothelial growth factor (VEGF) family. The occurrence of lymphangiogenesis and its relevance to metastasis in human cancer is debated and its role in colorectal cancer is not yet defined. VEGF-C and VEGF-D are members of the VEGF family with both angiogenic and lymphangiogenic actions. Altered expression of VEGF-A, VEGF-C and VEGF-D is noted in human malignancies with increased expression tending to correlate with negative clinicopathological features. Furthermore, VEGF receptors may be expressed by malignant epithelial cells in addition to endothelial cells. The hypothesis arises that the VEGF family is involved in the natural history of colorectal cancer by multiple routes and that the relative levels of expression of the growth factors and receptors influence tumour behaviour and metastatic potential.

Methodology: I) Immunohistochemical studies were performed to determine the expression and distribution of VEGF family members and receptors in primary and metastatic sites of colorectal cancer and to examine primary tumours for the presence of lymphatic vessels and determine lymphatic vessel density (LVD). II) Immunoassay experiments were performed to develop an enzyme-linked immunosorbent assay (ELISA) for the measurement of VEGF-C and assess its clinical potential in patients with colorectal cancer.

Results (I):

- The pattern of expression of the VEGF family members and VEGFR2 increased from normal tissue adjacent to colorectal cancer, throughout the tumour to a maximum at the invasive tumour edge.

- Expression of VEGF-C and VEGFR2 correlated with one another and co-localised throughout colorectal cancer and in metastatic lymph nodes, whilst expression of VEGF-C and VEGFR2 at the invasive tumour edge correlated with metastatic lymph node expression of the same antigens.
- VEGF-C and VEGF-D were expressed in the liver metastases of colorectal cancer. VEGFR2 expression was reduced in liver metastases in comparison to expression at the invasive edge of the primary tumour.
- The presence of deep intra-tumoural lymphatic vessels was associated with an altered balance of VEGF-C and VEGF-D at the invasive tumour edge.
- Although LVD could be measured there was no association with clinicopathological stage.

Results (II):

- Plasma VEGF-C levels could be measured using the ELISA developed.
- VEGF-C levels did not predict clinicopathological stage.

Conclusions: The variety of VEGF family expression in primary colorectal cancer suggests a role for autocrine and paracrine interaction between VEGF family members and VEGFR2. VEGF-C/VEGFR2 interaction is noted in the presence of lymph node metastasis and may offer an opportunity for targeted anti-VEGF treatment.

DECLARATION

I declare that no portion of the work referred to in this thesis bas been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Sarah Duff FRCS

NOTES ON COPYRIGHT AND THE OWNERSHIP OF INTELLECTUAL PROPERTY RIGHTS

1) Copyright in text of this thesis rests with the Author. Copies (by any process) either in full, or of extracts, may be made **only** in accordance with instructions given by the Author and lodged in the John Rylands University Library of Manchester. Details may be obtained from the librarian. This page must form part of any such copies made. Further copies (by any process) of copies made in accordance with such instructions may not be made without the permission (in writing) of the Author.
2) The ownership of any intellectual property rights which may be described in this thesis is vested in the University of Manchester, subject to any prior agreement to the contrary, and may not be made available for use by third parties without the written permission of the University, which will prescribe the terms and conditions of any such agreement.
3) Further information on the conditions under which disclosures and exploitation may take place is available from the Head of the Department of Medicine, Dentistry, Nursing and Pharmacy.

DEDICATION

This thesis is dedicated to my husband, Christopher, with thanks for his continual good-humour, and unfailing support throughout the duration of this research period and to my parents, Ian and Heather Rankine, with many thanks for the love, encouragement and opportunities they have provided me with throughout my life.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Dr Gordon Jayson and Miss Sarah O'Dwyer, for their enthusiasm, support and advice.

I am also indebted to many colleagues and friends in the Christie Hospital, the University of Manchester and elsewhere for their advice, training and support. Special thanks to: Dr Maria Jeziorska, for her friendship and immunohistochemical expertise; Dr Cheng Li, St Mary's Hospital; Dr Najib Haboubi, Pathology Department, Trafford General Hospital; Dr David Jackson, University of Oxford; Dr David Ryder, Medical Statistics Department; Dr Philip Wilson, St Mary's Hospital; Drs Christine Davies, Mei Lin and Kelly Warrington, University of Manchester; and to Professor Shant Kumar, in whose laboratory the work described in this thesis was performed.

1. INTRODUCTION

1.1. CLINICAL BACKGROUND

1.1.1. Incidence of colorectal carcinoma

Colorectal carcinoma is a major public health problem in the Western world. In the United Kingdom, the incidence of colorectal cancer was 78.7 cases per 100,000 population in 1997 (Commission for Health Improvement/Audit Commission, 2001) and 34,600 new cases were diagnosed in 1998 (Cancer Research UK, 2002). In the year 2000, it accounted for 16,250 deaths and the UK five-year survival rate is currently in the region of 40% (Commission for Health Improvement/Audit Commission, 2001). It is the second most common cause of cancer death after lung cancer in the United Kingdom (Cancer Research UK, 2002).

The risk of developing colorectal cancer increases with age and is greatest in the seventh decade for both men and women. An increase in incidence has been reported, which is more pronounced in males: the incidence in men increasing from 45 per 100,000 in the late 1970s to over 55 per 100,000 in the late 1990s. The incidence in women has remained stable over the last thirty years at around 35 per 100,000 (Cancer Research UK, 2002).

1.1.2. Aetiology of colorectal carcinoma

Colorectal carcinogenesis is a multifactorial process involving genetic and environmental influences. The vast majority of colorectal cancers are sporadic with only 5% due to inherited conditions such as familial adenomatous polyposis (FAP), the

polyposis syndromes and hereditary non-polyposis colorectal cancer (HNPCC). In 15-20% of sporadic cases, hereditary factors may contribute to susceptibility to malignancy (Fearnhead et al., 2002). The inherited colorectal cancer syndromes, together with an easily identifiable precursor lesion, the adenomatous polyp, have aided research into the genetic mechanisms responsible for colorectal carcinogenesis.

The majority of colorectal cancers are thought to arise from pre-existing adenomatous polyps. The progressive genetic transformation from normal colonic epithelium, to adenoma, dysplasia and carcinoma, was first described by Fearon & Vogelstein in 1990 (Fearon & Vogelstein, 1990). This adenoma-carcinoma sequence is a multistep process characterised by a random sequential acquisition of genetic somatic mutations offering a survival advantage and clonal expansion of the mutated cell population. Neoplastic (mutated) cells are not constrained by the normal control mechanisms for cell-cell/cell-extracellular matrix interactions, cell proliferation, growth, differentiation and apoptosis. This results in a cell population with progressively altered genetic material and increasing malignant features.

Key genetic regulators include proto-oncogenes, tumour suppressor genes and the mis-match repair genes (Fearnhead et al., 2002). Proto-oncogenes eg. K-ras, are involved in cell proliferation mechanisms: abnormal activation results in uncontrolled cellular proliferation. Tumour suppressor genes eg. adenomatous polyposis coli (APC), deleted in colon cancer (DCC) and the p53 gene, are involved in the regulation of normal cell functions, are recessive, and require loss or inactivation of both alleles for neoplastic progression. The mis-match repair genes eg. MLH1, MSH2, correct cellular genetic abnormalities by binding mis-matched nucleotides. Mutations of these genes allow genomic genetic alterations to accumulate and instability to develop.

Discovery of the more frequent genetic alterations has been aided by study of the autosomal dominantly inherited cancer syndromes, FAP and HNPCC.

FAP accounts for less than 1% of colorectal cancers and is characterised by the development of multiple ($>10^{2-3}$) colorectal adenomatous polyps. It is now known that autosomal dominant mutation of the APC gene on chromosome 5q is responsible for the development of FAP. The site of the gene mutation influences the disease phenotype (Caspari et al., 1994), which ranges from a severe to an attenutated phenotype. Inter-individual variation in severity is also seen for the identical genetic site mutations suggesting that additional modifying genes and environmental influences can alter disease expression (Paul et al., 1993). The APC gene product is a protein involved in many cellular processes, including migration, adhesion and proliferation. The APC protein is vital for the maintenance of cytoplasmic β-catenin levels, which is achieved through the formation of complexes to target β-catenin for degradation via the ubiquitin pathway. In the absence of functional APC protein, β-catenin escapes degradation and translocates to the nucleus causing transcriptional upregulation of various genes including cyclin D1 (Shtutman et al., 1999) and the oncogene *c-myc* (He et al., 1998), promoting progression and development of colorectal cancer. APC mutations have been identified in adenomas (Powell et al., 1992) and in dysplastic aberrant crypt foci (precursors of adenomas) (Smith et al., 1994) supporting the proposal that this genetic alteration is one of the earliest events in the adenoma-carcinoma sequence.

HNPCC accounts for up to 5% of colorectal cancers (Vasen et al., 1991). This family of diseases is characterised by colorectal tumours occuring at a relatively young age (<45 years), more commonly affecting the right side of the colon. Polyps are frequently multiple and are associated with multiple invasive cancers (both synchronous and metachronous). Histopathological characteristics include mucinous and poorly differentiated tumours, with signet ring cells and marked lymphocytic infiltration. Individuals affected have an improved survival in comparison with equivalent sporadic colorectal carcinomas. HNPCC can occur with (Lynch type II) or without (Lynch type

I) extracolonic carcinomas of the ovary, endometrium, renal pelvis, ureter, small bowel and stomach (Lynch et al., 1988). The Amsterdam II criteria produced by the International Collaborative Group for HNPCC (ICG-HNPCC) are designed to predict families at risk of HNPCC (Vasen et al., 1999) (**Table 1**), in whom 50-60% have identifiable genetic mutations (Fearnhead et al., 2002).

Table 1: Amsterdam II criteria for the identification of HNPCC families

At least 3 relatives with an HNPCC-associated cancer (colorectal, endometrial, small bowel, ureter, renal pelvis), one of whom should be a 1st degree relative of the other two
At least 2 successive generations should be affected
At least 1 should be diagnosed before the age of 50 years
FAP should be excluded in the colorectal cancer case(s) if any
Tumours should be verified by pathological examination

Mutations in the mismatch repair genes (MMR) that correct errors in base pair matching during DNA replication are responsible for the development of HNPCC. The most commonly affected MMR genes are *hMLH1, hMSH2* and *hMSH6.* Correlations between genotype and phenotype have been recognised, for example, extracolonic tumours are more commonly found in association with *hMSH2* mutations whilst women with *hMSH6* mutations are more likely to develop double primary cancers of the colorectum and endometrium (Charames et al., 2000). However, as in FAP, identical genotypic abnormalities do not always produce identical phenotypes.

Individuals in HNPCC families are heterozygous for mismatch repair gene mutations in the germline, hence a further somatic mutation is required in the normal allele before gene function is lost [a phenomenon described in Knudson's 'two hit' hypothesis (1971)]. Lack of DNA mismatch repair is seen particularly in poly-oligo tracts and base

pair repeat areas in the genome known as microsatellites, which are predominantly found in intronic (non-coding) areas. Disruption of microsatellite sequences is seen in more than 90% of colorectal cancers in HNPCC patients, and is known as microsatellite instability (MSI) or replication error (RER^+) (Liu et al., 1996). MSI is also seen in about 15% of sporadic colorectal cancers (Ionov et al., 1993; Lothe et al., 1993; Liu et al., 1995).

Identification of individuals with HNPCC can be difficult due to the large number of mutations described. Individuals and families fulfilling the Amsterdam II criteria (**Table 1**) can be tested for common HNPCC associated mutations. An alternative approach is to test colorectal cancers for microsatellite instability (bearing in mind the fact that 15% of sporadic tumours exhibit MSI) and proceed to germline testing in patients with marked MSI (MSI-H) and a family history. This approach is described in the Bethesda guidelines and is summarised in **Table 2** (Rodriguez-Bigas et al., 1997).

Table 2: The Bethesda criteria for testing colorectal cancer for microsatellite instability

• Individuals with a family history that fulfils the Amsterdam criteria
• Individuals with 2 or more HNPCC-related cancers, including synchronous and metachronous colorectal cancer or associated extracolonic cancers
• Individuals with colorectal cancer and a 1st degree relative with colorectal cancer +/- HNPCC-related extracolonic cancer +/- a colorectal adenoma: one of the cancers diagnosed at age <45 years and the adenoma at age <40 years
• Individuals with colorectal cancer or endometrial cancer diagnosed <45 years of age
• Individuals with undifferentiated or right-sided colorectal cancer at <45 years of age
• Individuals with signet-ring type colorectal cancer at <45 years of age
• Individuals with colorectal adenomata diagnosed <40 years of age

Sporadic colorectal cancer

In sporadic colorectal cancers, both APC and mismatch repair gene mutations can also arise (Mulcahy et al., 1997). The frequency of the commonly occurring genetic alterations are indicated in **Table 3**. Other important mutations include those in the K-ras gene, which results in the stimulation of cell growth and occurs in large adenomas and carcinomas. Mutations in this gene are a later event than APC mutations. In addition, p53 mutations are commonly seen in colorectal cancer as late events and are associated with the invasive phenotype, these mutations are rarely seen in adenomas. The majority of patients with sporadic carcinomas have multiple genetic abnormalities within the cancer cells and no one event is seen in all tumours.

Table 3: Common genetic alterations in sporadic colorectal cancer

Pathway	Gene (location)	% of cases with mutation
Adenoma-carcinoma sequence	p53 (17p3)	70
	DCC (18q21)	65
	APC (5q21)	60
	K-*ras* (12p12)	50
	Nm23 (17q21)	25
RER pathway	Mismatch repair genes	15

Data from (Mulcahy et al., 1997)

Environmental factors and predisposing conditions

Since Burkitt's observations of a low incidence of bowel cancer in South African populations (Burkitt et al., 1972), environmental factors have been thought to influence the progression of colorectal carcinogenesis and the expression of disease. These influences have centred around a protective effect of fibre and vegetables and a harmful effect of animal fat, although early epidemiological studies were methodologically flawed (Stubbs, 1983) and some of the evidence is inconsistent (Potter, 1999). The reported inconsistencies may be a result of the variable susceptibility of individuals to

environmental influences as a consequence of genotype (Potter, 1999). The preventive role of fibre in particular is controversial. A recent population based study showed no evidence for lowered colorectal cancer risk with high cereal fibre intake (Terry et al., 2001) and a Cochrane review of dietary fibre supplementation showed no benefit in terms of reduction in adenoma or carcinoma development (Asano & McLeod, 2002). Two large population based papers, however, have challenged this position, by demonstrating that dietary fibre consumption is inversely related to colorectal cancer risk (Bingham et al., 2003; Peters et al., 2003).

Predisposing conditions that increase the risk of colorectal cancer include long-standing inflammatory bowel disease (IBD), particularly ulcerative colitis, where the risk of carcinoma increases more with the severity and extent than duration of the disease (Ekbom et al., 1990a, b; Lennard-Jones et al., 1990).

1.1.3. Staging of colorectal cancer

Staging is the process by which objective data are assembled to try to define the progression of a disease (Northover, 1997). The overall prognosis for colorectal cancer is poor. Almost half the patients present with locally advanced or disseminated disease, but even in patients undergoing a curative resection, approximately half will die of a cancer-related death within five years (McArdle et al., 1990; Commission for Health Improvement/Audit Commission, 2001). The prognosis is influenced by numerous factors including age at presentation, gender, duration of symptoms, the presence of bowel obstruction, perforation and tumour location (Fielding et al., 1986). Tumour characteristics such as vascular and lymphatic invasion, DNA ploidy, and differentiation may also have prognostic significance. However, the simplest and most consistently reliable prognostic indicator is the presence or absence of lymph node

metastasis in the surgical specimen (ie. the Dukes' classification; see **Pathological staging, page 22**).

Preoperative staging

Staging systems have traditionally been pathological in nature but the acquisition of preoperative staging information allows planning of appropriate primary treatment. Accurate preoperative staging of colorectal cancer allows patient selection for neo-adjuvant therapies, surgical interventions (including avoidance of surgery in patients with advanced disease) and adequate patient counselling concerning treatment options and probable outcomes. For example, preoperative assessment of T (Tumour) stage in rectal cancer patients allows selection for different regimens of neoadjuvant radiotherapy. Preoperative staging relies on a combination of modalities (Northover, 1997):

- **clinical** – history, examination, endoscopic assessment, laparoscopy and examination under anaesthetic (EUA)
- **radiological** – various imaging modalites are used for preoperative staging (reviewed in O'Dwyer et al., 2001), these include contrast studies, ultrasound scanning, cross-sectional imaging including computed tomography (CT) scanning and magnetic resonance imaging (MRI) (particularly for rectal cancer and recurrent disease). Functional imaging using modalities such as positron emission tomography (PET) is increasingly used for evaluation of cases where traditional imaging is equivocal.

Pathological staging

Pathological staging of colorectal cancer is crucial for predicting prognosis and planning further treatment. The well-established Dukes' classification was described in

a series of 215 rectal cancer cases in 1932 (Dukes, 1932). The progression of disease noted was from an initial mucosal lesion, to local invasion and then lymph node metastases. Originally, a three-level classification was adopted:

- Dukes' A - growth confined to the rectum, no extra-rectal spread, no lymphatic metastases
- Dukes' B - spread into extra-rectal tissues by direct continuity, no lymphatic metastases
- Dukes' C- lymphatic metastases present

Subsequent reports demonstrated significant survival differences between groups: patients with Dukes' A rectal tumours had a 5-year survival rate of 98% compared with 77% for Dukes' B and 32% for Dukes C (Dukes & Bussey, 1958).

Modifications were made by Dukes based on the division of stage C into C1 ('upward (lymph node) spread has not yet reached the glands at the point of ligature of the blood vessels') and C2 ('nodal spread up to the level of the point of ligature') first described by Gabriel et al. (1935). A fourth stage of metastatic spread was mentioned initially by Dukes in 1949 (Dukes, 1949) but only named as Dukes' D by Turnbull many years later (1967). Kirklin (1949) included colonic tumours and increased prognostic accuracy by dividing Dukes' B into B1 and B2, where B1 tumours were contained within the muscularis propria while the B2 lesions extended through this layer. Astler and Coller (1954) used Kirklin's modification of Dukes' stage, but restricted stage A tumours to those involving the mucosa only and further subdivided Dukes' stage C into C1 and C2, on a similar basis to Kirklin's B1 and B2 in lymph node positive tumours. This subdivision gave additional prognostic information. The Dukes' classification accurately predicts patient prognosis in large studies (Kune et al., 1990) and has the advantages of being simple and reproducible (**Table 4**). Multiple alterations to the original classification have been proposed in a bid to improve its prognostic accuracy in

both colon and rectal cancer (Northover, 1997). Selection of patients for adjuvant treatment by Dukes' stage still has limitations as it can only give a probability of survival for any given stage after surgical resection. Alternative systems to determine which patients would most benefit from adjuvant non-surgical treatments have been adopted over the last 2 decades.

The TNM system for cancer staging is widely used (UICC, 2002) (**Table 4**). This classification defines the tumour size and depth of penetration ('T'), nodal involvement ('N') and the presence of distant metastases ('M'). It is applicable to tumours at all sites, is reproducible and in colorectal cancer can be paralleled with Dukes' staging to facilitate comparison between studies (**Table 5**). A further advantage of the TNM system is its versatility by separate categorisation of each of the primary, locoregional and distant components of the disease.

Other classifications eg. the Jass classification (1987) have not improved significantly on the Dukes' or TNM classification (Deans et al., 1994). Both Dukes' and TNM staging systems are dependent on the extent to which lymph nodes are searched for and examined. For example, the increased yield of lymph nodes from specimens using fat clearance techniques may result in stage migration, whereby patients who would have been classified conventionally as Dukes' B are reclassified as Dukes' C (Cawthorn et al., 1986; Haboubi et al., 1992; Haboubi et al., 1998; Marks et al., 2000). The histopathological techniques employed and stage definitions have to be taken into consideration when comparing studies that address outcome.

Table 4: Clinicopathological staging of colorectal cancer

Dukes' staging (pathological examination of specimen)
A – Invasive carcinoma not breaching muscularis propria **B** – Invasive carcinoma breaching muscularis propria, but not involving regional LN **C1** – Invasive carcinoma involving regional LN (apical node negative) **C2** – Invasive carcinoma involving regional LN (apical node positive) **D** – Presence of distant metastases
TNM clinicopathological staging
T – primary tumour **TX** – Primary tumour cannot be assessed **TO** – No evidence of primary tumour **Tis** – Carcinoma *in situ*: intraepithelial or invasion of the lamina propria **T1** – Carcinoma invades submucosa **T2** – Carcinoma invades muscularis propria **T3** – Carcinoma invades through muscularis propria into subserosa or into non-peritonealised peri-colic or peri-rectal tissues **T4** – Carcinoma perforates the visceral peritoneum &/or directly invades other organs or structures (including other segments of the colorectum via the serosa)
N – regional lymph nodes **NX** – Regional LN cannot be assessed **N0** – No regional LN metastasis **N1** – Metastasis in 1 to 3 regional LN **N2** – Metastasis in 4 or more regional LN (A tumour nodule >3mm diameter in perirectal or pericolic fat without histological evidence of a residual LN in the nodule is classified as regional LN metastasis. A tumour nodule <3mm diameter is classified in the T category as discontinuous extension ie. T3)
M – distant metastases **MX** – Distant metastasis cannot be assessed **MO** – No distant metastasis **M1** – Distant metastasis

Table 5: Stage grouping of colorectal cancer

	T	**N**	**M**	**Dukes**
Stage 0	Tis	N0	M0	
Stage I	T1 or T2	N0	M0	A
Stage IIA	T3	N0	M0	B*
Stage IIB	T4	N0	M0	
Stage IIIA	T1 or T2	N1	M0	C*
Stage IIIB	T3 or T4	N1	M0	
Stage IIIC	Any T	N2	M0	
Stage IV	Any T	Any N	M1	(D)

* Dukes B is a composite of better (T3N0M0) and worse (T4N0M0) prognostic groups, as is Dukes C (any TN1M0 and anyTN2M0)

Despite the widespread use of clinicopathological staging systems to predict prognosis and select patients for adjuvant treatments, there remain subsets of patients with apparently early stage disease who develop recurrence and/or metastasis and others with locally advanced disease who are cured by resection. For example, Moertel et al. (1995) reported the results of 929 patients with Stage III colon cancer undergoing surgical resection alone or surgery and chemotherapy with 5-fluorouracil (5-FU)/levamisole. Of the surgically treated patients, 45% were alive and disease-free at 5 years, compared with 65% treated with surgery and chemotherapy. This illustrates that, in retrospect, 45% of the patients did not require chemotherapy, and that 35% died despite chemotherapy. The QUASAR (Quick And Simple And Reliable) study has reported similar results in 4927 colorectal cancer patients treated postoperatively with 5-FU and folinic acid. The three-year survival rates of 70% in patients treated with chemotherapy imply that 30% still die despite adjuvant treatment (QUASAR Collaborative Group, 2000). Consequently, the use of clinicopathological staging systems to select patients for adjuvant therapy runs the risk of overtreating a significant proportion of patients and undertreating others. With improvements in adjuvant treatment and the introduction of newer agents there is a need for better prognostic markers to select patients at risk of recurrence and metastatic disease. The last decade has seen an explosion in studies exploring the potential of prognostic markers.

1.1.4. Prognostic factors and tumour markers

A prognostic factor is a variable that provides prospective information on patient outcome complementing the histopathology data and assisting future therapeutic decisions (McLeod & Murray, 1999). In order for a marker to be of widespread use, it must be reliable, valid, simple to apply and easy to assess. Numerous markers have been described and evaluated including oncogenes, tumour-suppressor genes, markers of

apoptosis, cell proliferation and angiogenesis (McLeod & Murray, 1999; Diep et al., 2003).

Tumour markers differ from prognostic factors. A tumour marker gives an indication of the presence or progression of disease and may be used to monitor for disease recurrence and treatment response. Many tumour markers have been evaluated for utility as prognostic factors, but have generally failed to show significant advantage over conventional components of staging systems. In 1998, the American Society of Clinical Oncology (ASCO) produced guidelines on the use of tumour markers for prevention, diagnosis, screening, treatment and surveillance in colorectal cancer (American Society of Clinical Oncology, 1998). These highlight a lack of solid evidence for the general use of tumour markers, with only carcinoembryonic antigen (CEA) measurements recommended for use in the detection of recurrence and/or metastasis and in the assessment of treatment response. More recently, the updated guidelines (Bast et al., 2001) have identified potential markers currently being investigated but not yet recommended for routine use, including serum interleukin (IL)-6, intra-tumoural expression of $p27^{Kip1}$ (a cell cycle inhibitor), the DCC gene, MSI and the enzyme thymidylate synthase whose expression/upregulation may be associated with response to 5-FU.

1.1.5. Treatment and prevention

Surgery

Surgical resection is the mainstay of treatment for tumours of the colon and rectum. The anatomical challenges to the surgeon differ in the two sites. Oncological principles for colonic resection have remained similar for many years and involve ligation of the major vascular pedicle supplying the colonic segment for resection, wide excision of the lymphatic drainage, excision with tumour-free margins and en-bloc resection of organs

involved in contiguous tumour spread. The surgical approach to rectal cancer has changed in the last 15 years with the adoption of anatomically based surgical resection in the form of total mesorectal excision (TME) (Heald et al., 1982; MacFarlane et al., 1993) and recognition of the importance of circumferential margin clearance to achieve low rates of local recurrence (Quirke et al., 1986). Widespread use of TME is associated with improved outcomes both in terms of local recurrence rates and long-term survival (Kapiteijn et al., 2002).

Radiotherapy

A role for radiotherapy in the treatment of rectal cancer has evolved over the last 15-20 years. A systematic overview including 8507 rectal cancer patients in 22 randomised trials of adjuvant radiotherapy (delivered both pre- and post-operatively) demonstrated a reduction in local recurrence for both methods of delivery and a marginal improvement in survival (Colorectal Cancer Colloborative Group, 2001). However, the trials included in this overview were performed prior to recognition of the importance of TME surgery. Confirmation of the continued benefit of radiotherapy in the TME era has been demonstrated in a large randomised trial by the Dutch Colorectal Cancer Group (Kapiteijn et al., 2001). TME surgery alone (937 patients) was compared with TME and adjuvant pre-operative radiotherapy (924 patients). The combined-treatment group had a reduced risk of local recurrence: 8.2% in the surgery alone group vs. 2.4% in the surgery and radiotherapy group ($p<0.001$), although no differences in survival at 2 years were observed (Kapiteijn et al., 2001). However, the local recurrence rate in the surgery alone arm of the Dutch study was substantially higher than that achievable by surgery alone in specialist centres (MacFarlane et al., 1993).

Chemotherapy

Chemotherapy has a place in neoadjuvant and adjuvant colorectal cancer treatment and in the management of advanced disease. The adjuvant use of 5-FU and its modulator leucovorin is established in Stage III/Dukes' C disease (Moertel et al., 1995; QUASAR Collaborative Group, 2000). The size of the survival benefit in this group is estimated to be around 6% (The Association of Coloproctology of Great Britain and Ireland, 2001). The uncertain arm of the QUASAR study will define the benefits of 5-FU based chemotherapy for patients with Stage II/Dukes' B disease.

Newer chemotherapeutic drugs that have demonstrated improvements in survival in the setting of advanced disease include the thymidylate synthase modulators (capecitabine and ralitrexed), the topoisomerase-I inhibitor, irinotecan and oxaliplatin. Equivalent survival benefits to 5-FU regimens or improved survival in combination with 5-FU or in 5-FU refractory disease have been demonstrated. Irinotecan in combination with 5-FU is associated with a significant survival benefit when used as 1st-line treatment for metastatic colorectal cancer (Douillard et al., 2000; Saltz et al., 2000). In the UK, the National Institute of Clinical Excellence (NICE) have only recommended irinotecan monotherapy in patients with advanced colorectal cancer who have failed to respond to 5-FU treatment (NICE, 2002) – although this has been questioned by a number of UK oncologists (Saunders & Valle, 2002).

Screening

Colorectal cancer fulfils the criteria for suitability of a disease for screening in that the disease is a major public health problem, the natural history of the condition is known, effective screening tests exist and early treatment of disease is associated with a better prognosis. Screening strategies evaluated in the UK include faecal occult blood testing (FOBT) and flexible sigmoidoscopy (FS). FOBT is aimed mainly at the detection of

asymptomatic small cancers whereas FS has the advantage of detection and removal of pre-malignant lesions ie. adenomatous polyps with the potential for reducing future cancer incidence in the screened population (Atkin et al., 1993).

Randomised trials of FOBT screening to identify individuals for colonoscopic investigation have demonstrated a 15-30% reduction in colorectal cancer-related mortality (Mandel et al., 1993; Hardcastle et al., 1996; Kronborg et al., 1996). Consequently, large-scale pilot studies of FOBT screening have been undertaken in Coventry and the Grampian region in the UK. Data from the pilot sites were presented at the Annual Meeting of the Association of Coloproctology of Great Britain and Ireland in 2003. These data illustrated an uptake of 56.8% of the invited population (approximately 1 million people per site) and 2% test positivity. Of individuals with positive FOBT, 12% had cancer (552 cancers including 92 polyp cancers) and 30% had adenomatous polyps. The distribution of the cancers detected was 33% Dukes' A, 32% Dukes' B and 34% Dukes' C. The preliminary conclusions were that FOBT was feasible although uptake needed to be increased and that a shift in stage distribution to earlier cancers had been noted.

An alternative approach to screening is reflected in the UK Flexible Sigmoidoscopy Screening Trial, which is examining the hypothesis that a single FS at 55-60 years, with polypectomy and colonoscopy for those with high-risk polyps (Atkin et al., 1992), can reduce the incidence and mortality of colorectal cancer (Atkin et al., 1993). The study commenced in 1996, it is anticipated that follow-up at 10 years will show a difference in colorectal cancer incidence and that 15 years will be required to demonstrate a reduction in mortality in the screened populations. Recent results demonstrate the safety, acceptability and feasibility of this technique (UK Flexible Sigmoidoscopy Screening Trial Investigators, 2002). Compliance rates of 71% were achieved. Distal adenomas were detected in 12.1% and distal cancer in 0.3%, 5% of the screened

population went on to undergo colonoscopy at which 18.8% had proximal adenomas and 0.4% had proximal cancers. Of the cancers detected, 62% were Dukes' A. Continuing follow-up will further define the effect of this screening intervention on incidence and mortality from colorectal cancer.

1.2. ANGIOGENESIS

Angiogenesis is the development of new blood vessels from the existing vasculature. Together with vasculogenesis ie. the formation of the primary vascular network in the embryo, it is a fundamental requirement for normal development. Angiogenesis occurs in the adult in both physiological eg. female menstrual cycle and pathological settings eg. tumour growth, wound healing.

The natural history of tumour development is of a stepwise progression of events from local tumour growth, to invasion and distant metastases. A central process in this chain of events is the development of tumour blood vessels. The growth of primary tumours and their metastases is dependent on angiogenesis, without which tumour foci are unable to grow larger than 1-2mm in diameter (Folkman, 1995). The acquisition of an angiogenic phenotype by a tumour is referred to as the 'angiogenic switch' (Hanahan & Folkman, 1996). This switch in tumour behaviour occurs as a result of increased production of angiogenic promoters and/or reduction of angiogenic inhibitors. Angiogenesis is a complex process involving degradation of pre-existing vessel basement membrane, endothelial cell proliferation, sprouting and migration, vessel lumen and loop formation, re-establishment of blood flow, in parallel with maturation and stabilisation of new vessels (Yancopoulos et al., 2000). This process supports tumour growth and haematogenous metastasis.

Multiple growth factors, receptors and pathways are known to be important in the angiogenic process, including acidic and basic fibroblast growth factor (FGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGB-β), hepatocyte growth factor (HGF) and tumour necrosis factor-α (TNF-α) but the most studied and best understood is the vascular endothelial growth factor (VEGF) family (see **sections 1.4 & 1.5, pages 47 & 68**).

1.2.1. Assessment of microvascular density (MVD)

Since the ability of a tumour to induce angiogenesis is a crucial step in the successful establishment of metastases, measurements of tumour neovascularisation have been evaluated as prognostic indicators in a number of tumour types. First reports describing MVD as a prognostic indicator in malignant melanoma and breast cancer were published over a decade ago (Srivastava et al., 1988; Weidner et al., 1991). Unfortunately, numerous endothelial markers and scientific methods have led to confusing results when reporting MVD in different tumour types. An international consensus document discussed the methods and evaluation criteria for achieving accurate and reproducible MVD scores (Vermeulen et al., 1996). The proposed standard described the optimum methods of tissue processing, immunostaining, selection of quantification fields and scoring and also highlighted unresolved areas for further investigation. However, the continued proliferation of studies using various markers and methods of MVD measurement implies a lack of widespread use of this document, which is now seven years old.

1.2.2. MVD in colorectal cancer

Numerous studies have investigated the role of MVD in colorectal cancer with different conclusions (**Table 6**). The conflicting results reported may be explained by the use of

different methodologies (including antibodies directed against a variety of antigens, different immunostaining and counting techniques) and varying specimen types (frozen tissue or formalin-fixed paraffin-embedded blocks; colonic or rectal cancer alone or both tumour types together; specific disease stages or all stages encompassed; polypoid or ulcerated tumours; different levels of inflammation in/around the tumour).

Few studies have addressed the optimum method of determining MVD in colorectal cancer specifically. Abdalla et al. (1996) investigated the use of different pre-treatment methods and different antibodies [directed against CD31, CD34 and von Willebrand factor (vWF)] for immunostaining blood vessels in paraffin embedded tissues. The authors concluded that in colorectal cancer, CD34 immunostaining of microwaved tissues produced intense, specific staining of endothelial cells and was the most sensitive method. However, other authors disagree; Tomisaki et al. (1996) found that although CD34 immunostaining on trypsinised tissues resulted in higher MVD counts compared to scores obtained with vWF staining, vWF staining was a more specific marker for microvessels. The use of pan-endothelial markers for the assessment of MVD has been questioned. Markers directed against activated endothelial cells, such as CD105, may provide a better prognostic indicator than standard measures (Akagi et al., 2002; Li et al., 2003). The best quantification method for MVD in colorectal cancer has also not been established. In breast cancer, Chalkley point counts are known to correlate closely with MVD hotspot counts, giving objective, rapid and reproducible prognostic information (Fox et al., 1995). This has been confirmed in colorectal cancer by Abdalla et al. (1996), whilst White et al. (2002) have demonstrated that Chalkley counts also correlate well with other MVD parameters.

The prognostic significance of MVD in colorectal cancer remains controversial with different studies demonstrating opposite prognostic correlation (**Table 6**).

Table 6: Summary of microvessel density (MVD) studies in colorectal cancer

Author	*N*	*Dukes'/ disease stage*	*Tissue type*	*Marker*	*Magnification*	*No. of fields assessed*	*Correlations*
Saclarides et al., 1994	48	A-D	FFPE	FVIII	x100 & x200	3	Increased MVD correlated with T stage (p=0.002) and poorer survival (p=0.034)
Bossi et al., 1995	178	A-D	FFPE	CD31	x200	3	Increased MVD with progression from normal to adenoma (p<0.001) to carcinoma (p<0.0001). No association of MVD with metastases, disease stage or survival
Frank et al., 1995	105	A-B	FFPE	FVIII	x100	3	Increased MVD correlated with recurrence (p=0.004) and survival <5 years (p=0.0004)
Takahashi et al., 1995	52	A-D	FFPE	FVIII	x200	1	Increased MVD correlated with increased Dukes' stage (p<0.001) and VEGF-A expression within tumour and at the invading edge (p<0.001, p<0.05)
Engel et al., 1996	35	A-C	FFPE	CD31	x400	3	Increased MVD correlated with recurrence (p=0.017) and predicted recurrence and survival in univariate analysis (p=0.006, p=0.006) but not on multivariate analysis
Lindmark et al., 1996	212	A-D	Frozen	FVIII	x125	5	Increased MVD correlated with longer survival (p=0.007) No correlation with tumour differentiation or Dukes' stage
Takebayashi et al., 1996	166	A-D	FFPE	FVIII	x400	NK	Increased MVD correlated with tumour size, invasion depth, LN status, lymphatic and venous invasion, Dukes' stage and risk of relapse (all p<0.001) MVD above the median correlated with poor prognosis (p=0.03) and was an independent prognostic indicator (p=0.007)
Tomisaki et al., 1996	175	A-D	FFPE	CD34 or FVIII	x200	5	Increased MVD correlated with lymphatic (p=0.004) and venous invasion (p=0.049), liver metastasis (p=0.012) and Dukes' stage (p<0.05) No association with increased MVD and survival
Amaya et al., 1997	107	A-D	FFPE	CD34	x200	5	Increased MVD was associated with reduced OS and RFS (both p<0.0001) Increased MVD correlated with VEGF-A, but not KDR expression

N, number of cases; NK, not known; MVD, microvessel density; RFS, relapse-free survival; OS, overall survival; FVIII, factor VIII related antigen (von Willebrand factor); FFPE, formalin fixed paraffin embedded tissue; VEGF-A, vascular endothelial growth factor A; LN, lymph node; KDR, kinase domain containing receptor.

Table 6 continued:

Author	*N*	*Dukes'/ disease stage*	*Tissue type*	*Marker*	*Magnification*	*No. of fields assessed*	*Correlations*
Takahashi et al., 1997	27	B	FFPE	FVIII	x200	1	Increased MVD correlated with metastasis ($p<0.001$) and was an independent prognostic factor for time to recurrence
Tanigawa et al., 1997	133	A-D	FFPE	CD34	x200	5	Increased MVD correlated with haematogenous metastasis, grade, depth of invasion, lymphatic vessel invasion, LN metastasis and serosal penetration Increased MVD associated with death from recurrence ($p<0.0001$) and was an independent prognostic indicator
Banner et al., 1998	22	B	FFPE	FVIII	x400	10	No association between MVD and survival, trend towards increased MVD in long-term survivors
Choi et al., 1998	127	A-D	FFPE	FVIII	x200	3	Increased MVD with poorer histological grade, LN metastasis, lymphatic and venous invasion, Dukes' stage and liver metastasis. Independent prognostic predictor for survival in curatively resected patients.
Fox et al., 1998	36	T1-T4	FFPE	FVIII	x200	4	Increased MVD in tumour in comparison to adjacent normal mucosa in T1/2 tumours ($p=0.02$) but no difference in T3/4 tumours ($p=0.12$)
Takahashi et al., 1998	93	A-D	FFPE	FVIII	x200	1	Increased MVD from adenoma to nonmetastatic cancer, to metastatic cancer. Increased MVD with increased VEGF-A expression and p53 accumulation
Abdalla et al, 1999	111	A-D	FFPE	CD31	x400	5	Increased MVD associated with longer survival ($p<0.002$) and MVD was an independent prognostic indicator
Vermeulen et al, 1999b	145	A-D	FFPE	CD31	x200	5	For stages A-C disease, increased MVD associated with reduced survival ($p=0.005$) and haematogenous metastasis ($p=0.001$). Increased MVD was an independent prognostic indicator for survival ($p=0.006$)
Galindo Gallego et al., 2000	126	A-C	FFPE	CD34	x200	3	Increased MVD correlated with grade ($p=0.03$) and reduced RFS and OS ($p=0.001$, $p=0.01$) but lost significance on multivariate analysis

N, number of cases; MVD, microvessel density; RFS, relapse-free survival; OS, overall survival; FVIII, factor VIII related antigen (von Willebrand factor); FFPE, formalin fixed paraffin embedded tissue; VEGF, vascular endothelial growth factor; LN, lymph node.

Table 6 continued:

Author	*N*	*Dukes'/ disease stage*	*Tissue type*	*Marker*	*Magnification*	*No. of fields assessed*	*Correlations*
Oh-e et al., 2001	254	A	FFPE	CD34	x400	3	Increased MVD at deepest site of submucosal penetration was an independent prognostic factor for lymph node metastases (p=0.026)
Akagi et al., 2002	20	T1-T2	FFPE	CD34 & CD105	x200	3	Increased CD105 detected MVD in carcinomas in comparison to high-grade dysplastic adenomas (p<0.05), no differences in CD34 detected MVD
Barozzi et al., 2002	101	A-D	FFPE	CD34	Image analysis	20	MVD was not a significant predictor of the risk of developing metastases
Nanni et al., 2002	263	B-C	FFPE	FVIII	x200	NK	Increased MVD correlated inversely with grade (p<0.001). MVD had no association with RFS or OS, although there was a trend towards better outcome with increased MVD
White et al., 2002	84	A-C	FFPE	CD31, FVIII	x200	5	Increased MVD in cancers compared to normal mucosa and adenomas MVD above median associated with longer overall survival (p=0.03) Chalkley counts correlated well with MVD counting
Furodoi et al, 2002; Kaio et al., 2003	152	B-D	FFPE	CD34	x400	3	Increased MVD correlated with grade, invasion depth, lymphatic and venous invasion, LN and liver metastases, Dukes' stage and non-curative surgical resection. Also with expression of VEGF-A, VEGF-C and PD-ECGF at deepest invasive site Increased MVD was associated with poorer survival (p<0.05) but lost significance on multivariate analysis.
Li et al., 2003	111	A-D	FFPE	CD34, CD105	x100	4	Increased MVD determined by CD105, but not CD34, correlated with reduced survival (p=0.0001) and was an independent prognostic factor. No correlation between CD34 and CD105 determined MVD

N, number of cases; NK, not known; MVD, microvessel density; RFS, relapse-free survival; OS, overall survival; FVIII, factor VIII related antigen (von Willebrand factor); FFPE, formalin fixed paraffin embedded tissue; VEGF, vascular endothelial growth factor; LN, lymph node; PD-ECGF, platelet-derived endothelial cell growth factor

The majority of studies in colorectal carcinoma have found an association between higher MVD, more aggressive tumour behaviour and poorer clinicopathological variables (**Table 6**). In some studies, MVD was an independent prognostic indicator for survival (Takebayashi et al., 1996; Amaya et al., 1997; Tanigawa et al., 1997; Choi et al., 1998; Vermeulen et al., 1999b) or time to recurrence (Takahashi et al., 1997). In others, MVD was associated with poorer survival but lost significance in multivariate analysis (Engel et al., 1996; Galindo Gallego et al., 2000; Furodoi et al., 2002; Kaio et al., 2003).

Three studies have reported an association of increased MVD with better prognosis, which conflicts with most other publications including the majority of those on colorectal cancer (Lindmark et al., 1996; Abdalla et al., 1999; White et al., 2002). The earlier two of these studies included a large number of patients encompassing the full range of Dukes' stages and assessed blood vessels with antibodies directed against CD31. Lindmark et al. (1996) employed frozen tissue specimens, whereas Abdalla et al. (1999) used formalin-fixed paraffin-embedded blocks in common with most other studies. The authors of these studies postulated that their unexpected results could have been due to methodological differences, which included selection of the area of greatest microvascular density (the 'hotspot') for counting rather than simply areas at the invading edge and the use of a mean of 5 field scores rather than a single field (Lindmark et al., 1996). It was also suggested that local tissue ulceration, inflammation and repair are common findings in colorectal cancer that may markedly influence MVD and alter the relation between MVD and prognosis in this particular tumour type (Abdalla et al., 1999). The most recent of these three studies (White et al., 2002) used both anti-CD31 and anti-FVIII antibodies to stain separate tumour sections and scored slides with both MVD counts and Chalkley grid methods. Elevated MVD above the median was associated with longer overall survival times in 84 colorectal cancer

patients (White et al., 2002). A further report demonstrated a non-significant trend towards improved survival with increased MVD (Banner et al., 1998). Banner et al. (1998) assessed 9 short-term and 13 long-term survivors with Stage II colorectal cancer using anti-FVIII-related antigen as an endothelial marker. No differences in MVD were seen between the two different outcome groups in this small sample, although there was a trend towards improved survival with higher MVD.

Nanni et al. (2002) attempted to resolve the issue of prognostic use of MVD in colorectal cancer along with a number of other biological markers. This multicentre study used strict quality control measures for methodology, counting, scoring and patient follow-up, thus aiming to eliminate some of the recognised variables. The 263 patients included were all participating in one of two randomised controlled trials of 5-FU based chemotherapy regimens and were pathologically staged as having Dukes' B2, B3* or C tumours. MVD did not show any relationship with age, gender, tumour site, Dukes' stage, lymphocytic infiltration or DNA ploidy. Nor was any relation found between high MVD and four-year disease-free and overall survival. The authors concluded that MVD was not a useful biological marker for prediction of behaviour and outcome of colorectal cancer.

In summary, although questions remain about antibody choice, methods for immunostaining and MVD quantification, the majority of studies in colorectal cancer show MVD correlates with poorer outcome in univariate analysis but that MVD often loses prognostic value on multivariate analysis. The most comprehensive and controlled study reported by Nanni et al. (2002) failed to identify MVD as a useful indicator in colorectal cancer, hence there is a need to evaluate more specific angiogenic markers such as the VEGF family.

* B2 and B3 refer to Astler-Coller disease stages, where B2 is tumour that extends through the muscularis propria and B3 is tumour that invades into adjacent structures, both lymph node negative

1.2.3. MVD and VEGF-A in colorectal cancer

The relationship between VEGF-A expression and MVD in colorectal tumours is not clear cut. Takahashi et al. (1995) demonstrated a correlation between MVD and VEGF-A expression at the invading edge and within colorectal cancer. Further positive associations between VEGF-A tumour expression and MVD have been documented by others (Amaya et al., 1997; Kang et al., 1997b; Takahashi et al., 1997; Kondo et al., 2000a; Harada et al., 2001; Kaio et al., 2003), whereas two further groups have been unable to demonstrate such a relationship (Nanashima et al., 1998; Nanni et al., 2002). A possible reason underlying this discrepancy is that although VEGF-A is important for tumour angiogenesis, numerous additional angiogenic agents and pathways co-exist and vary in importance in differing circumstances, hence a specific relationship between VEGF-A expression and MVD is difficult to envisage.

1.3. VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS

The VEGF family is a group of related molecules [VEGF-A, placental growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E] that are crucial for vasculogenesis, angiogenesis and lymphangiogenesis [(Veikkola & Alitalo, 1999; Partanen & Paavonen, 2001) and **section 1.4, page 47**]. The family of VEGF receptors will be examined prior to further discussion of the ligands. VEGF-A, VEGF-C and VEGF-D are considered in detail in **sections 1.4 & 1.5, pages 47 & 68.**

All VEGF family members are ligands for tyrosine kinase receptors of the VEGF-receptor (VEGFR) family for which they have overlapping specificities and variable affinity (Joukov et al., 1997b; Karkkainen & Petrova, 2000). The receptor family is a

subgroup of the platelet derived growth factor (PDGF) receptor family. There are three main receptors, VEGFR1 (flt-1 or fms-like tyrosine kinase-1), VEGFR2 (KDR or kinase domain-containing receptor/flk-1 or fetal liver kinase-1) and VEGFR3 (flt-4) (Neufeld et al., 1999; Karkkainen & Petrova, 2000; Veikkola et al., 2000; Partanen & Paavonen, 2001) with differing sites of expression, functions and ligand specificity (**Table 7**). In the human foetus, expression of the three main VEGFRs show distinct but overlapping patterns of tissue expression suggesting that each have varying functions in the regulation of growth and differentiation of different vessel types (Kaipainen et al., 1993).

The three VEGFRs are similar in structure, with seven extracellular immunoglobulin (Ig) homology domains and a split intracellular tyrosine kinase domain. These seven Ig-like domains define the VEGFRs as a subgroup within the PDGF receptor family, in which other group members are characterised by only five Ig-like extracellular domains. The 2nd and 3rd Ig-like domains are responsible for ligand binding and the 4th Ig-like loop contains a receptor dimerization domain (Neufeld et al., 1999).

Additional endothelial cell VEGF receptors also exist, such as the neuropilins (NRP), which modulate ligand binding to the main receptors and are important in blood vessel development (Soker et al., 1996). NRP-1 acts as a co-receptor for $VEGF_{165}$ and PlGF-2 whilst NRP-2 is a co-receptor for $VEGF_{165}$. VEGFR2 binds $VEGF_{165}$ more efficiently in cells expressing NRP-1 and this is translated into an enhanced migratory response (Soker et al., 1998). Neuropilins are also expressed on malignant cells and may have important roles in mediating VEGF-induced autocrine survival functions (Bachelder et al., 2001). Another group of molecules to which some VEGF family members ($VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF\text{-}B_{167}$) bind are the heparan-sulfate proteoglycans (Neufeld et al., 1999).

Ligand binding to VEGFRs leads to receptor dimerization and transphosphorylation, with recruitment of diverse adaptor and signalling molecules, initiating a complex sequence of intracellular pathways, finally activating a cascade of angiogenic programs in endothelial cells. The complexity of signalling is enhanced by the existence of differently spliced ligands, proteolytic processing and receptor heterodimerization (Karkkainen & Petrova, 2000). VEGFR-expressing cell proliferation and survival further depends on interactions with the extracellular matrix, which occurs via members of the integrin family, in particular $\alpha_v\beta_5$ (Friedlander et al., 1995).

Table 7: VEGF receptor specificities

Type of VEGFR	Ligands	Sites of expression	Outcome of receptor activation
VEGFR1 (flt-1)	VEGF-A, VEGF-B, PIGF	Vascular EC Trophoblast cells, monocytes, renal mesangial cells, some types of tumour cells	• Production of proteases, NO, EC migration • Possible role as negative regulator of angiogenesis by sequestration of VEGF-A, preventing activation of VEGFR2 • Embryonic vascular development
VEGFR2 (KDR/flk-1)	VEGF-A, VEGF-C, VEGF-D, VEGF-E	Vascular EC Haemopoietic stem cells, megakaryocytes, retinal progenitor and some malignant cell types	• Main angiogenic responses - EC proliferation, migration & survival • Embryonic differentiation of haematopoietic and EC & vascular development
VEGFR3 (flt-4)	VEGF-C, VEGF-D	Lymphatic EC in adults EC of vascular and lymphatic systems in development. Upregulated on blood EC in some tumours, fenestrated endothelia and malignant cells.	• Lymphatic system development & function, lymphatic EC proliferation, migration & survival • Maintains integrity of EC lining during angiogenesis • Embryonic vascular system development
Neuropilin-1 (NRP-1)	VEGF-A ($_{165}$ form), PIGF-2, VEGF-B, VEGF-E	Vascular EC Some tumour cell types	• Co-receptor, potentiates effects of ligand binding to VEGFR1 and VEGFR2 • May mediate autocrine survival responses

Abbreviations : VEGF(R), vascular endothelial growth factor (receptor); EC, endothelial cell; flk, fetal liver kinase; flt, *fms*-like tyrosine kinase; KDR, kinase domain containing receptor; NRP, neuropilin; PIGF, placenta growth factor; NO, nitrous oxide.

1.3.1. VEGFR1

VEGFR1 is expressed mainly on endothelial cells but is also present in other cells including trophoblast, monocytes, renal mesangial cells and some tumour cell types (**Table 7**). VEGFR1 has the highest affinity for VEGF-A of the VEGFRs, with a dissociation constant (K_d) of approximately 10-20 pM (de Vries et al., 1992).

Signalling via VEGFR1 is not responsible for the main angiogenic effects of VEGF-A in vascular endothelial cells (Bernatchez et al., 1999), but is essential in embryogenesis. VEGFR1-null mice die *in utero* at embryonic day 8.5-9.5, when they possess abundant endothelial cells that fail to develop into an organised primary vascular network (Fong et al., 1995).

VEGF-A signalling via VEGFR1 stimulates the production of proteases, tissue factor and endothelial cell and monocyte migration responses, but not endothelial cell proliferation (Waltenberger et al., 1994; Clauss et al., 1996; Keyt et al., 1996; Gille et al., 2001). It is thought that VEGFR1 has a predominantly negative role, acting as a decoy receptor to sequester VEGF-A at the cell surface, regulating access to and thus suppressing signalling via the main signalling receptor, VEGFR2. Furthermore, a reciprocal relationship between VEGFR1 and VEGFR2 has been illustrated in a VEGFR-expressing epithelial cancer cell line, whereby VEGFR1 negatively regulates VEGFR2-mediated cellular proliferation through the production of nitrous oxide (NO) (Dunk & Ahmed, 2001). Additional evidence for the antagonistic role of VEGFR1 comes from the existence of a truncated soluble form of VEGFR1 (sVEGFR1) that results from alternative splicing (Kendall & Thomas, 1993). This sVEGFR1 lacks the 7^{th} immunoglobulin-like domain, transmembrane and intracellular parts of VEGFR1. It retains high affinity for VEGF-A binding and inhibits VEGF-A induced mitogenesis (Kendall & Thomas, 1993) and tube formation by human microvascular endothelial

cells (Koolwijk et al., 2001). This soluble form of VEGFR1 has recently been detected in the sera of some patients with malignancy but not normal controls (Kumar et al., 2002b).

The predominantly negative role of VEGFR1 has been called into doubt recently by the discovery that PlGF (but not VEGF-A) signalling via VEGFR1 amplifies VEGF-A-induced angiogenic responses via VEGFR2 (Autiero et al., 2003). PlGF stimulated phosphorylation of different receptor tyrosine residues and and transcription of differing genes than those observed with VEGF-A stimulation of VEGFR1. This has raised the intriguing possibility that VEGFR1 can act both negatively and positively to regulate the functions of structurally similar growth factors (Autiero et al., 2003). Further investigation into the roles and mechanism of action of PlGF and VEGFR1 may provide therapeutic targets for augmentation of angiogenesis.

1.3.2. VEGFR2

VEGFR2 is expressed mainly on vascular endothelial cells but is also expressed on other cell types including haematopoietic cells, megakaryocytes, retinal progenitor cells and is increasingly recognised on a variety of malignant cell types (**Table 7** and **section 1.4.3, page 57**). VEGFR2 has a lower affinity for VEGF-A than VEGFR1 (Terman et al., 1992).

Expression of and signalling via VEGFR2 is essential for the embryonic differentiation of endothelial and haematopoietic cells and blood vessel formation (reviewed in Veikkola et al., 2000). VEGFR2-null mice fail to develop a vasculature and have a reduced number of endothelial cells (Shalaby et al., 1995) and the temporal and spatial correlation of VEGF-A/VEGFR2 in embryonic mice illustrates the major role for the ligand/receptor complex in vasculogenesis and angiogenesis (Millauer et al., 1993).

VEGFR2 is the main signalling receptor for VEGF-A, responsible for angiogenic effects in endothelial cells, such as cell proliferation, tube formation, morphological changes, actin reorganisation, chemotaxis and vascular permeability (Waltenberger et al., 1994; Keyt et al., 1996; Bernatchez et al., 1999; Gille et al., 2001; Koolwijk et al., 2001; Tille et al., 2003). Transcription of VEGFR2 is upregulated by VEGF-A which triggers a positive feedback loop (Shen et al., 1998). Mature forms of VEGF-C and VEGF-D (see **section 1.5.3, page 74**) also activate VEGFR2 producing similar angiogenic responses to VEGF-A (Tille et al., 2003). Binding of $VEGF_{165}$ to VEGFR2 is potentiated by the co-receptor, NRP-1, a semaphorin receptor with a role in axonal guidance, that also potentiates the $VEGF_{165}$ induced endothelial cell migratory response.

Cellular proliferation, survival and motility due to VEGFR2 signalling are mediated via multiple intracellular pathways. These include the phosphatidylinositol 3`-kinase (PI3-K)/Akt pathway which is crucial for cell survival (Gerber et al., 1998). Interactions are also observed with protein kinase C, mitogen activated protein kinase (MAPK), NO synthase (Ziche et al., 1997), Src kinases, focal adhesion kinases (Slack et al., 2001; Eliceiri et al., 2002) and integrin $\alpha_v\beta_5$ (Friedlander et al., 1995), all of which are important for VEGF-A induced angiogenic responses.

In the context of malignancy, the VEGF-A/VEGFR2 signalling pathway has been assumed to aid tumour growth by a paracrine action on endothelial cells, promoting angiogenesis and thereby allowing a permissive increase in tumour size. The recent finding that VEGFR2 is expressed on various malignant cell types has raised the possibility that VEGF-A/VEGFR2 are involved in autocrine loops directly promoting malignant cell growth and survival (Masood et al., 2001; Harmey & Bouchier-Hayes, 2002) (see section **1.4.3, page 57**).

1.3.3. VEGFR3

VEGFR3 is crucial for embryonic vascular development. This is demonstrated by gene disruption studies in mice, where in the primary vascular network vascular sprouting and endothelial cell differentiation develop normally, but impaired remodelling of the primary vascular plexus, large vessel disorganisation, pericardial effusion and cardiovascular failure cause death at embryonic day 9.5 (Dumont et al., 1998). VEGFR3 is also important for the maintenance and function of the lymphatic system. In humans, inactivating mutations in the VEGFR3 gene, including mis-sense mutations and intragenic polymorphisms result in defective VEGFR3 signalling and are described in families suffering from primary lymphoedema (Irrthum et al., 2000; Karkkainen et al., 2000). Understanding these genetic abnormalities was achieved through the creation of murine lymphodema models by inactivating mutations of VEGFR3 (Karkkainen et al., 2001) and by the use of soluble VEGFR3 molecules to inhibit VEGFR3 signalling (Makinen et al., 2001a).

VEGFR3 is a highly glycosylated, relatively stable, cell surface associated kinase of approximately 180kDa. It is proteolytically cleaved in the fifth extracellular immunoglobulin-like domain and the resulting chains remain linked with disulfide bonds (Pajusola et al., 1994). Short and long isoforms of VEGFR3 are recognised, differing in length in the carboxyl terminus (Pajusola et al., 1993). The longer form predominates in human tissues and the two forms have different functional abilities, as only activation of the long form is able to sustain fibroblast cell growth in soft agar cultures (Borg et al., 1995). Downstream signalling from VEGFR3 is dependent on residues within the intracellular carboxyl tail. Site directed mutagenesis of tyrosine residues in the C-terminal of the long form of VEGFR3 abolishes this signalling capacity (Fournier et al., 1995).

VEGFR3 is expressed on vascular endothelium in early development, but later becomes restricted mainly to the lymphatic endothelium (Kaipainen et al., 1995; Kukk et al., 1996). This distinction may be used to define in culture subsets of endothelial cells as lymphatic in origin (Kriehuber et al., 2001). However, VEGFR3 is not solely expressed on lymphatic endothelium in all situations. Expression is increased on the endothelial cells of angiogenic vessels in and around tumours (Jussila et al., 1998; Valtola et al., 1999; Niki et al., 2001; Witmer et al., 2001) and is also seen in some fenestrated endothelia (Partanen et al., 2000). However, VEGFR3 does not seem to be upregulated on the neovasculature of the acute healing wound, although it is present at low levels on the granulation tissue vascular endothelium in chronic wounds (Paavonen et al., 2000). VEGFR3 may be required to maintain endothelial cell integrity in angiogenesis, as VEGFR3 blocking monoclonal antibodies can inhibit tumour angiogenesis (Kubo et al., 2000).

1.4. VASCULAR ENDOTHELIAL GROWTH FACTOR-A

VEGF-A (also known as VEGF) is the prototypic member of the VEGF family, it is one of the most potent angiogenic factors, strongly stimulating endothelial cell activity (Ferrara & Henzel, 1989; Dvorak et al., 1995; Ferrara & Davis-Smyth, 1997; Neufeld et al., 1999; Partanen & Paavonen, 2001; Dvorak, 2002). VEGF-A possesses numerous biological properties listed in **Table 8** (Senger et al., 1983; Ferrara & Davis-Smyth, 1997; Dvorak, 2002). The large spectrum of effects of VEGF-A illustrate that it is crucial at all stages of development, both in embryogenesis and in angiogenesis of later life.

Table 8: Principle biological effects of VEGF-A

Target organ	Effects of VEGF-A stimulation
Embryo	Vasculogenesis and angiogenesis. Heterozygotes die *in utero*, with multiple developmental abnormalities, including abnormal vasculature
Vascular endothelial cell	Increases vascular permeability, cellular proliferation and migration Alters cell morphology and cytoskeletal structure Increases expression of proteins involved in fibrinolysis (serine proteases urokinase-type and tissue-type plasminogen activator (PA) and PA-inhibitor-1 (PAI-1) and uPA receptor) Increases expression of tissue factor (procoagulant) and matrix metalloproteinases eg. collagenase Increases expression of the glucose transporter GLUT-1, NO synthase and integrin receptors eg. ICAM-1, VCAM-1 Survival factor (via upregulation of mitogens and anti-apoptotic proteins) Increases expression of VEGFRs
Antigen presenting cell	Inhibition of maturation and differentiation
Monocyte/macrophage	Chemotactic responses
Other cell types that express VEGFRs	Multifunctional effects on: immune, inflammatory and haematopoietic cells, retinal pigment epithelial cells, Schwann cells, mesangial cells, uterine smooth muscle and some VEGFR expressing tumour cells

1.4.1. VEGF-A genetics

The organisation of the VEGF-A gene provides a model for the other genes of the group. The gene for human VEGF-A is located on chromosome 6p21.3 (Vincenti et al., 1996; Wei et al., 1996). It consists of 8 exons separated by 7 introns, with a coding region spanning about 14 kb. Potential specificity protein (Sp) 1, activator protein (AP) -1 and AP-2 transcription factor binding sites are found near a single transcription start site in the VEGF-A gene promoter region (Tischer et al., 1991). Gene expression is regulated by multiple mechanisms, which include upregulation by hypoxia (Pugh & Ratcliffe, 2003), cytokine stimulation [by epidermal growth factor (EGF), TGF-β, keratinocyte growth factor, IL-1α, IL-6, prostaglandin E_2 (PGE_2), insulin-like growth

factor (IGF)-1 all of which increase VEGF-A expression in different cell types] and cell differentiation and transformation. Differentiation of cell types either increases or decreases VEGF-A production, whereas cell transformation in malignancy is associated with upregulation of VEGF-A expression as a result of oncogenic mutations (Ferrara & Davis-Smyth, 1997). Hypoxia is one of the most important stimuli for increased VEGF-A production. The main route to increased VEGF-A activity under hypoxic conditions is through hypoxia-inducible factor (HIF). In normoxia, HIF-α subunits are normally rapidly broken down by the oxygen-dependent activity of HIF-prolyl hydroxylase (HIF-PH) enzymes, which hydroxylate the subunit marking it for destruction by interaction with the Von Hippel Lindau ubiquitin ligase (Jaakkola et al., 2001). In hypoxia, oxygen becomes rate-limiting for the HIF-PH enzymes, the turnover of the HIF-α subunits is reduced, allowing the HIF-α and β subunits to associate and form the active transciption factor which leads to increased transcription of hypoxia-regulated genes including VEGF-A (Jaakkola et al., 2001). Further enhancement of VEGF-A activity in hypoxic conditions is achieved by interactions between different mechanisms and pathways, including stabilisation of mRNA via binding of proteins to specific motifs in the 3'-untranslated region, preservation of mRNA translation in the face of cellular hypoxia via internal ribosomal entry sites, and increased VEGFR expression (Pugh & Ratcliffe, 2003).

Alternative exon splicing of the single VEGF-A gene results in the production of different molecular forms of the protein, differing in the number of amino acids. Thus, loss of the residues encoded by exon 6 produces $VEGF_{165}$, while $VEGF_{121}$ lacks the residues encoded by both exons 6 and 7 (Tischer et al., 1991).

1.4.2. VEGF-A proteins

VEGF-A exhibits molecular heterogeneity, existing as one of a number of molecular species with different numbers of amino acids, generated by alternative splicing as described above. The predominant isoform is $VEGF_{165}$ which is a highly conserved, disulfide-bonded, basic, heparin-binding dimeric glycoprotein of 34-45kDa (Ferrara, 1999; Dvorak, 2002). VEGF-A shares sequence homology with PDGF, and characteristically for this group, has highly conserved cysteine residues that are responsible for inter-chain and intra-chain bonds and vital for molecular structure. The determinants of VEGF-A which mediate binding to VEGFR1 and VEGFR2 have been described. VEGFR1 binding depends on the negatively charged residues Asp^{63}, Glu^{64} and Glu^{67} whereas VEGFR2 binding requires Arg^{82}, Lys^{84} and His^{86} (Keyt et al., 1996). These binding domains are located at opposite ends of the VEGF monomer. In the VEGF-A dimer, the monomers are linked by disulfide bridges in a 'head-to-tail' fashion, so that the main binding domains for both VEGFR1 and VEGFR2 are at opposite ends of the molecule. This allows the dimeric form of VEGF-A to bind and link two VEGFRs together to form signalling homo- or heterodimers.

Other isoforms of VEGF-A include $VEGF_{121}$, $VEGF_{145}$, $VEGF_{189}$, and rarely, $VEGF_{206}$. The isoforms have different isoelectric points by virtue of their different amino acid compositions, which affects their biological availability. The shortest isoform is weakly acidic and non-heparin binding, whereas the longer isoforms are more basic and bind heparin strongly. These properties are important because they determine isoform tissue location and behaviour. $VEGF_{121}$ is a freely soluble protein, $VEGF_{165}$ is secreted but some remains bound at the cell surface and in the extracellular matrix, while the larger isoforms are almost fully sequestered in the extracellular matrix bound to proteoglycans containing heparin-like moieties. Cleavage of the longer forms of VEGF-A from the extracellular matrix can be achieved by plasmin, which may be particularly relevant in

the context of the tumour microenvironment where increased protease expression is found.

The biological activity of the isoforms varies. A reduction in mitogenic activity is associated with loss of heparin-binding ability, so that $VEGF_{165}$ has greater potency to induce endothelial cell growth in comparison with $VEGF_{121}$. This may be due to enhanced signalling efficiency as a result of the formation of VEGF-A/heparan sulfate/VEGFR complexes.

As a result of variable VEGF-A molecular size and biological properties, VEGFR distribution pattern and relative levels of cellular and soluble receptor expression, VEGF-A molecules are able to induce a huge range of graduated responses in VEGFR expressing cells. The complexity of this pathway is increased still further by novel family members, generated by alternative splicing, that are endogenous inhibitors of VEGF-A signalling. For example, $VEGF_{165}b$ is an endogenous antagonist of VEGF-A, inhibiting VEGF-A-induced endothelial cell proliferation, migration and haemodynamic effects, this isoform is expressed at high levels in the normal kidney but is down-regulated in renal cell carcinoma (Bates et al., 2002).

1.4.3. Expression of VEGF-A and VEGFRs in colorectal cancer

There is a large but contradictory published literature on the subject of VEGF-A and VEGFR expression in colorectal cancer. A wide range of detection techniques are now used including *in situ* hybridisation (ISH), reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC), making comparison between studies difficult. Furthermore, the existence of endogenous antagonistic forms of VEGF-A eg. $VEGF_{165}b$, complicates the assessment of VEGF-A mRNA and protein expression in clinical specimens. Current anti-VEGF-A antibodies detect all VEGF-A isoforms and fail to discriminate between agonist and antagonistic types of VEGF-A.

Consequently, relationships between the expression of VEGF-A proteins and clinicopathological factors may be obscured and observed associations confounded. An overview of current knowledge is summarised in order to determine those areas that require further clarification with relevance to the VEGF family interactions.

The contribution of VEGF-A to colorectal tumour progression has been thought to be due to its effect on endothelial cells and tumour angiogenesis rather than by direct action on the tumour cells themselves. This concept was supported by transfection studies of a human colon cancer cell line, LoVo, with $VEGF_{121}$. No increased cell proliferation was seen *in vitro*, but increased blood vessel development occurred in the animal tumours produced, thus leading indirectly to enhanced tumour growth and metastatic potential (Kondo et al., 2000b). More recently, the recognition of VEGFRs on cell types other than endothelial cells has opened wider debate on the influence of VEGF-A on tumour growth (Harmey & Bouchier-Hayes, 2002) (see **page 57**).

Role in the development and progression of colorectal cancer

Authors concur that VEGF-A expression is increased in the progression from normal colonic mucosa, to adenoma development and carcinoma. However, the stage at which upregulation of VEGF-A expression occurs is a subject of debate. Brown et al. (1993) argued that VEGF-A upregulation occurred after the premalignant stage, as VEGF-A mRNA was increased in colonic tumour epithelial cells but not adjacent normal epithelium or polyps. A further study also investigated mRNA expression in 72 paired colorectal cancers/normal colonic mucosa specimens and 6 adenomas. Expression of VEGF-A mRNA transcripts was increased by 1.4-fold in tumours compared to their adjacent normal mucosa and tumours at all stages exhibited higher mRNA levels than adenomas, without any change during neoplastic progression (Andre et al., 2000). Using RT-PCR methods, significantly elevated VEGF-A mRNA was also found in 70

colorectal cancers, but not in 20 adenomatous polyps compared to their corresponding normal mucosal specimens (George et al., 2001b).

Earlier upregulation of VEGF-A in the premalignant stage has been demonstrated in late adenomatous polyps by Kondo et al. (2000a), with increased VEGF-A mRNA and protein expression identified in severely dysplastic adenomas and early carcinomas. Mild to moderately differentiated polyps, however, did not express increased VEGF-A mRNA or protein (Kondo et al., 2000a). Further evidence for earlier upregulation of VEGF-A was found in a study by Wong et al. (1999). Using RT-PCR and ISH, Wong et al. found significantly increased VEGF-A mRNA expression in adenomatous polyps compared to normal colonic mucosa, but no difference between adenomas and carcinomas. *In situ* and invasive components of tumours had similar VEGF-A mRNA expression levels. Recently, using ribonuclease protection assay techniques, Hanrahan et al. (2003) have added further to the evidence for early upregulation of VEGF-A. VEGF-A transcripts were higher in adenomas and carcinomas than normal colonic mucosa.

Whether VEGF-A expression may be even further increased in advanced tumours is argued by different authors. Takahashi et al. (1995) found elevated VEGF-A protein expression in colorectal tumours that were metastatic in comparison to non-metastatic tumour and adenomas. Others however, have found similar mRNA transcript levels in colorectal tumours at different Dukes' stages (Andre et al., 2000; Hanrahan et al., 2003). The lack of consistency of these results may be explained partly by the existence of endogenous antagonistic VEGF-A isoforms and by individual tumour heterogeneity. Hypoxia is a major upregulator of VEGF-A expression, hence, hypoxic tumours will demonstrate increased VEGF-A expression at an earlier stage than those with lower levels of hypoxia. The level of hypoxia existing within a tumour is determined by many factors including the tumour size, site and relative expression of other angiogenic

agents. None of the reports have commented on whether the colorectal tumours assessed were ulcerative or polypoid in nature. The tissue microenvironment in these macroscopically different tumour types is likely to alter in terms of levels of hypoxia, inflammation and concurrently expressed growth factors. A 'switch' for increased VEGF-A expression seems to occur at a variable time point during the pre-invasive phase of tumour development, this level of expression may then increase further in advanced disease.

Stromal expression of VEGF-A in colorectal cancer

The importance of stromal expression of VEGF-A lies in the potential for its contribution to evasion of immunosurveillance by the tumour. Stromal cell expression of upregulated VEGF-A mRNA is seen in fibroblasts and smooth muscle cells (Brown et al., 1993), whilst tumour associated macrophages show both increased VEGF-A mRNA (Wong et al., 1999) and protein expression (Khorana et al., 2003).

VEGF-A inhibits the maturation of antigen-presenting cells, such as dendritic cells (Gabrilovich et al., 1998) whilst anti-VEGF-A treatment reverses this effect allowing enhanced responses to cancer immunotherapy and anti-angiogenic treatment in animal cancer models (Gabrilovich et al., 1999). In human gastric and non-small cell lung cancers (NSCLC), levels of dendritic cell infiltration and VEGF-A expression are inversely related and dendritic cell infiltration was an independent prognostic factor in both tumour types (Saito et al., 1998; Inoshima et al., 2002).

The presence of VEGF-A expressing tumour associated macrophages (TAM)/stroma in Stage II and III colon carcinoma patients (in 42% of 131 cases) was an independent predictor of improved survival (Khorana et al., 2003) which contradicts the above findings. This is likely to be due to an abundance of tumour-derived VEGF-A mediating macrophage chemotaxis and infiltration via VEGFR1. These activated macrophages are

capable of producing a variety of cytokines with both tumouricidal and angiogenic actions, hence the relationship between the presence of TAM and outcome is complex.

Expression in metastatic disease

The liver is the main site for colorectal cancer metastases. The liver is well oxygenated deriving its blood supply from the hepatic artery and portal vein, consequently the hypoxic stimulus for VEGF-A expression in liver metastases is not as pronounced as that of the primary site. That the liver metastases of colorectal cancer can express VEGF-A is not in doubt. Increased VEGF-A mRNA transcripts were found in neoplastic cells compared with surrounding normal liver parenchymal levels, although the levels varied between individuals and stromal cell expression was not seen (Warren et al., 1995). Protein expression of VEGF-A is also found in liver metastases at a higher level in neoplastic cells in comparison with normal hepatic parenchyma (Nanashima et al., 1998; Cascinu et al., 2001).

Local conditions of the tumour microenvironment can exert influence over tumour growth factor production so that metastases at different sites may express different levels of VEGF-A with implications for anti-VEGF therapies. In an animal tumour model of colorectal cancer with metastases at orthotopic (liver) and ectopic (subcutaneous) sites, VEGF-A mRNA was found at higher levels in subcutaneous metastases in comparison to metastases within the well-vascularized liver (Fukumura et al., 1997). Similar findings occur in human studies. Cascinu et al. (2001) have shown that intra-abdominal metastases are significantly more likely to express VEGF-A protein than liver metastases (68% vs. 21%, p=0.02). These findings can be accounted for by the differing hypoxic level of the microenvironment of the metastases, although tumour biology may also differ between the metastatic sites.

The influence of the tumour microenvironment is reflected again in the difference in VEGF-A expression levels between primary and metastatic tumour sites. Primary colorectal tumours express higher levels of VEGF-A protein than their corresponding liver metastases. This is likely to be due to the relative normoxic conditions found within the liver, resulting in a local down-regulation of VEGF-A expression (Berney et al., 1998). These findings have been contradicted in a study by Barozzi et al. (2002), who studied 49 liver metastases and primary tumours, finding no significant difference in VEGF-A protein expression between sites, although it was noted that the MVD in the liver metastases was lower than that of the primary in 39% of patients. The conflicting results of these studies may be explained by methodological differences, genuine biological differences of the tumours and potential confounding by endogenous antagonistic VEGF-A isoforms.

VEGF-A expression and prognosis

VEGF-A influences tumour progression indirectly by enhancing angiogenesis and thus allowing tumour growth but may also contribute in other ways, such as increasing production of molecules involved in tumour invasion and metastasis and evasion of tumour immunosurveillance. The use of tumour VEGF-A expression as a biological marker has been suggested by various authors. Reports frequently show association of increased VEGF-A expression with negative clinicopathological factors (Kang et al., 1997a; Kang et al., 1997b; Landriscina et al., 1998; Nakata et al., 1998; Lee et al., 2000a; Ono & Miki, 2000; Kaio et al., 2003) and risk of recurrence and overall survival (Kang et al., 1997a; Kang et al., 1997b; Ishigami et al., 1998; Tokunaga et al., 1998; Harada et al., 2001; Kaio et al., 2003). This is exemplified by Cascinu et al. (2000) who examined the VEGF-A expression status of 121 Stage II colon cancers, finding an increased recurrence rate in patients with VEGF-A positive tumours and increased

VEGF-A immunoreactivity. The authors concluded that VEGF-A status could be used as a marker to select Stage II patients at risk of relapse for consideration of chemotherapy. However, in direct contrast, a lack of association with clinicopathological factors (Nanashima et al., 1998) and lack of independent association of VEGF-A expression with risk of recurrence and overall survival has also been reported (Takahashi et al., 1997; Berney et al., 1998; Nanashima et al., 1998; Lee et al., 2000a; Barozzi et al., 2002; Khorana et al., 2003).

In an attempt to resolve this issue, a large study set out to define the clinical use of VEGF-A expression as a biological marker in colorectal cancer (Nanni et al., 2002). This prospective study included 263 patients, followed within the context of two randomised controlled trials of chemotherapy, wherein VEGF-A expression was determined using strict quality control measures and assessment criteria limiting variability and maximising reproducibility. VEGF-A expression showed no relationship to the clinicopathological markers, including MVD, disease-free and overall survival.

Different levels of VEGF-A expression (or different levels of agonist and antagonistic isoforms) may be partly responsible for the better prognosis associated with tumours expressing microsatellite instability. These tumours have a lower level of VEGF-A and p53 mutations in comparison with microsatellite-intermediate or microsatellite-stable tumours. These results are consistent with the role of wild-type p53 in downregulation of VEGF-A, although this does not fully explain the observed associations (Wynter et al., 1999).

Expression of VEGF receptors

Endothelial cells are the main sites of expression for the VEGFRs (**Table 7, page 42**) but increasingly, it is becoming apparent that all three of the principal VEGFRs can be expressed on malignant cell types, where they are found both on human cancer cell lines

in vitro and human malignant tissues *ex vivo*. Co-expression of functional VEGFRs with their corresponding ligands in tumours raises the possibility of autocrine loops, whereby a tumour is capable of stimulating its own growth, progression and survival.

Upregulated expression of all three main VEGFRs and evidence for autocrine paths via VEGF-A signalling has been reported in various human cancer cells. This includes increased VEGFR1 expression in VEGF-A producing malignant prostate cell lines (Soker et al., 2001), VEGFR1 and VEGFR2 in prostate cancer specimens (Jackson et al., 2002) and pancreatic cancer (von Marschall et al., 2000), VEGFR2 expression in a bladder cancer cell line (Wu et al., 2003), ovarian cancer (Boocock et al., 1995), melanoma (Gitay-Goren et al., 1993), renal cell carcinoma (Tsuchiya et al., 2001), neuroblastoma (Fukuzawa et al., 2002) and squamous cell carcinomas of the head and neck (Neuchrist et al., 2001). Increased VEGFR3 expression has been identified in NSCLC (Arinaga et al., 2003), endometrial carcinoma (Yokoyama et al., 2003) and head and neck squamous cell malignancies (Neuchrist et al., 2003). Upregulation of NRP receptors on malignant cells may also provide a route for autocrine signalling and cell survival (Miao et al., 2000; Bachelder et al., 2001). These findings have implications for the development of novel anti-VEGF and anti-VEGFR compounds that could have potential anti-angiogenic and direct anti-tumour effects in human malignancy (Harmey & Bouchier-Hayes, 2002)

In colorectal cancer, there is little evidence of tumour epithelial VEGFR expression. In animal tumour models of colorectal cancer liver metastasis, upregulation of VEGFR mRNA expression has been reported in endothelial but not neoplastic cells (Warren et al., 1995). Similar findings have been described in human primary tumours, with upregulated VEGFR1 and VEGFR2 mRNA in endothelial cells within colorectal cancer stroma, but not in endothelial cells distant from the tumour or in tumour epithelium (Brown et al., 1993). Studies examining human tumour specimens by RT-PCR, are

unable to determine the precise tissue source of increased mRNA expression. However, Andre et al. (2000) have shown general upregulation of colorectal tumour VEGFR1 mRNA without upregulation of VEGFR2 or VEGFR3 mRNA. Similarly, George et al. (2001b) demonstrated equivalent levels of VEGFR2 and VEGFR3 mRNA in colonic tumour epithelium and normal mucosa. More recently, using ribonuclease protection assays and RT-PCR, Hanrahan et al. (2003) have found increased VEGFR1 and VEGFR2 mRNA in adenomas and carcinomas in comparison with normal tissue, and a relative decrease in VEGFR3 mRNA in adenomas and carcinomas. This investigation concluded that VEGFR1 and VEGFR2 are upregulated at the same time as VEGF-A in the pre-malignant phase of colorectal tumourigenesis, but was unable to draw conclusions as to the precise site of receptor expression.

Using immunohistochemical techniques, enhanced VEGFR2 protein expression has been identified in tumour endothelial cells (Ono & Miki, 2000), particularly in VEGF-A expressing and metastatic tumours but was not found on tumour epithelial cells (Takahashi et al., 1995). One study has identified VEGFR2 protein expression in both vascular endothelial cells and colorectal tumour cells in 65% of 136 colorectal cancer specimens (Amaya et al., 1997). A study examining malignancy within gastrointestinal tract endocrine organs also reported VEGFR2 upregulation on tumour cells. Normal cells within endocrine organs of the gastrointestinal tract, particularly gastrin-producing cells and pancreatic polypeptide cells in the head of the pancreas, express VEGF-A, but not VEGFR2. Malignant endocrine cells in these organs upregulate VEGF-A and also express VEGFR2 *de novo* (La Rosa et al., 2003). Such observations indicate that VEGF-A/VEGFR2 interaction is important in normal endothelial cell function and influences tumourigenesis by paracrine/autocrine mechanisms (La Rosa et al., 2003).

The presence of VEGFR3 in colorectal cancer cell lines has been demonstrated by Western blot analysis. That this is also the case *in vivo* has been confirmed by

immunohistochemical staining of 56 colorectal cancer specimens, all of which expressed VEGF-C; 46 cases expressed VEGFR3 and expression in >25% of the cancer cells was associated with reduced overall survival (Witte et al., 2002). Similar upregulation of VEFGR3 protein expression on colorectal cancer cells has been demonstrated by Kawakami et al. (2003), in association with VEGFR3 and VEGF-C mRNA over-expression in quantitative RT-PCR testing. Co-expression of VEGFR3 and its specific ligands, VEGF-C and VEGF-D, within tumours raises the possibility of autocrine and paracrine circuits between these tumour-produced ligands and their receptors.

In summary, upregulation of all VEGFR types has been identified on cells of various malignancies. In gastrointestinal malignancy, there is evidence for VEGFR2 upregulation in endocrine cells (La Rosa et al., 2003) and some limited evidence for upregulation of VEGFR2 (Amaya et al., 1997) and VEGFR3 in colorectal adenocarcinoma cells (Witte et al., 2002; Kawakami et al., 2003). The expression of VEGFRs on tumour endothelial cells allows for the existence of paracrine circuits, whereby tumour and stroma derived VEGF family members can influence tumour angiogenesis. Tumour epithelial co-expression of VEGFRs and one or more of their ligands would also allow autocrine/paracrine enhancement of tumourigenesis.

1.4.4. Detection of circulating VEGF-A

The use of circulating growth factor levels as tumour and/or prognostic markers has been widely investigated (Poon et al., 2001). Circulating cytokine levels are detectable by immunoassay in various body fluids. The measurement of circulating VEGF-A for diagnostic and therapeutic use in a variety of cancers has revealed elevated levels in serum (Yamamoto et al., 1996; Dirix et al., 1997; Salven et al., 1997; Fujisaki et al., 1998; Kumar et al., 1998), plasma (Hyodo et al., 1998; Duque et al., 1999; Fuhrmann-

Benzakein et al., 2000; Tamura et al., 2001) and malignant effusions (Kraft et al., 1999). It is noteworthy that until very recently there had been no report of the measurement or implications of circulating VEGF-C (Tamura & Ohta, 2003) and a single comment on serum levels of VEGF-D (George et al., 2001b).

There are many difficulties associated with the use of circulating VEGF-A as a tumour marker (Jelkmann, 2001). Circulating VEGF-A is not only derived from the tumour, but from many blood components including platelets (Mohle et al., 1997; Verheul et al., 1997; Banks et al., 1998; Maloney et al., 1998; Wartiovaara et al., 1998; Nielsen et al., 1999; Salgado et al., 2001), megakaryocytes (Mohle et al., 1997; Banks et al., 1998), leucocytes (Gaudry et al., 1997; Salven et al., 1999), monocytes and lymphocytes (Wartiovaara et al., 1998). Platelet derived VEGF-A is localised to the α granules and is released on degranulation (Mohle et al., 1997; Wartiovaara et al., 1998). Consequently, in matched serum and plasma sample pairs of both patients and controls, serum VEGF-A levels are consistently higher than those in plasma (Verheul et al., 1997; Banks et al., 1998; Hyodo et al., 1998; Webb et al., 1998; George et al., 2000; Lee et al., 2000b; Salgado et al., 2001; Werther et al., 2002). It has also been noted that serum VEGF-A levels increase with clotting time (Hyodo et al., 1998; Webb et al., 1998) and are influenced by the platelet count (Verheul et al., 1997; Salgado et al., 1999; Vermeulen et al., 1999a). A wide range of inter-individual variation is seen in the ratio between serum VEGF-A and platelet count, whereas intra-individual variation is much lower (Vermeulen et al., 1999a) suggesting that platelet-derived VEGF-A varies between individuals. If platelets are scavengers of VEGF-A, endocytosis of tumour-derived circulating VEGF-A would result in elevated levels of intra-platelet VEGF-A in cancer patients and could localise and concentrate the angiogenic action of VEGF-A to sites of platelet activation such as tumours (Pinedo et al., 1998). Such an effect is supported by various authors having confirmed that the platelets of cancer patients do contain an

increased level of VEGF-A in comparison with normal controls (Salven et al., 1999; George et al., 2000; Salgado et al., 2001). Recently, Poon et al. (2003) have provided strong evidence for the central role of platelets in the storage of tumour-derived VEGF-A. In 60 patients undergoing resection of hepatocellular carcinoma, the platelet VEGF-A load correlated positively with tumour VEGF-A mRNA, cytosolic levels and protein expression (Poon et al., 2003).

Thrombocytosis is a common occurrence in cancer patients which contributes further to the increased serum VEGF-A levels observed. Tumour-produced IL-6 stimulates platelet production and is therefore partly responsible for cancer-related thrombocytosis (Ishibashi et al., 1989; Salgado et al., 1999; Salgado et al., 2000; Salgado et al., 2002). Increased circulating IL-6 levels and increased platelet VEGF-A load have been shown to have a significant negative impact on prognosis (O'Byrne et al., 2000; Bachelot et al., 2003). Platelet activation also releases thrombopoietin, which contributes to megakaryocyte stimulation resulting in increased platelet number thereby creating a vicious circle (Verheul & Pinedo, 2003).

Although extensive debate exists about the optimal sample and method for measuring circulating VEGF-A, no internationally accepted measure is agreed. For comparative studies, therefore, circulating VEGF-A offers little advance in diagnosis and prognosis. Individual researchers must standardise their methods for comparison within groups. In addition, similar problems apply to current ELISA systems for the detection of VEGF-A, namely that the antibodies utilised are unable to discriminate between agonist and antagonistic isoforms of VEGF-A.

Potential clinical uses for circulating VEGF-A as a tumour marker in colorectal cancer have been studied and a summary of current knowledge is provided in **Table 9**.

Table 9: Summary of studies examining circulating VEGF-A in colorectal cancer

Author	*Number of cases*	*Disease stage*	*Sample source*	*Principle findings*
Akbulut et al., 2002	52	Stage I-IV	Serum	Serum VEGF-A corrected for platelet level predicted survival on univariate analysis. Derived angiogenic index was an independent prognostic indicator for RFS and OS
Berglund et al., 2002	87	Metastatic	Serum	Serum VEGF-A levels reduced in 94% with previously elevated levels in response to chemotherapy, but levels did not correlate with survival or response to chemotherapy
Gunsilius et al., 2002	29	Dukes' A-D	Plasma	Efferent mesenteric plasma VEGF-A levels higher than peripheral blood level in patients with metastatic (p=0.003) or histologically aggressive (p=0.01) disease
Karayiannakis et al., 2002	67	Dukes' A-D	Serum	Serum VEGF-A levels were higher than controls (p<0.001), correlated with differentiation (p=0.004), Dukes' stage, T stage, LN and distant metastases (all p<0.0001) Serum VEGF-A was associated with reduced survival (p=0.0018) on univariate analysis
Kumar et al., 2002a	108	Not specified	Serum	Reduced serum VEGF-A levels at 6 hours postoperatively in curative resections but not palliative resections or resections for benign disease
Minagawa et al., 2002	31	Stage I-IV	Plasma	Plasma VEGF-A level was increased in patients with liver metastases (p=0.05). Plasma VEGF-A level correlated with tissue expression of VEGF-A (p=0.03)
Nakayama et al., 2002	33	Dukes' A-D	Plasma	Trend for increased plasma VEGF-A level with increasing disease stage (NS). Postoperative (6/12) levels were reduced in comparison to preop (NS)
Werther et al., 2002	524	Dukes' A-D	Serum and plasma	Plasma VEGF-A levels elevated in cancer patients (p=0.01) and increased with Dukes' stage (p=0.001). No significant difference in serum VEGF-A levels between patients and controls (p=0.3), serum VEGF-A level was higher in Dukes' D disease than A-C (p=0.001). Plasma and serum VEGF-A levels correlated (p<0.001) Primary colon tumours had higher plasma and serum VEGF-A levels than rectal tumours (both p=0.01). Multivariate analysis: elevated serum but not plasma VEGF-A predicted poor survival in colon cancer, neither were predictive for rectal cancer
Broll et al., 2001	122	Stage I-IV	Serum	Serum VEGF-A had low sensitivity as a diagnostic marker (36% cases had levels above cut-off). Increased sensitivity was achieved by combination with CEA. Serum VEGF-A was increased in patients compared with controls (p<0.0001) and in T2-4 vs. T1 (p=0.04) and with tumour size >5cm (p=0.04) Increased serum VEGF-A associated with reduced survival (p=0.03) on univariate analysis

VEGF, vascular endothelial growth factor; LN, lymph node; CEA, carcinoembryonic antigen; RFS, relapse free survival; OS, overall survival; NS, not significant.

Table 9 continued:

Author	*Number of cases*	*Disease stage*	*Sample source*	*Principle findings*
George et al., 2001a	89	Not specified	Serum and plasma	Serum VEGF-A/platelet levels correlated with MRI assessed tumour permeability Serum VEGF-A levels correlated with tumour VEGF-A mRNA level. No difference in pretreatment serum VEGF-A/platelet levels between responders/non-responders. Non-responders showed a rise in VEGF-A/platelet ratio after treatment. Plasma and serum VEGF-A levels were lower at 12 months in disease free patients compared with preoperative values. Plasma levels increased in patients with progressive or recurrent disease.
Chin et al., 2000	81	Dukes' A-C	Serum	Increased serum VEGF-A in patients who developed metastases at follow-up ($p<0.0001$) Serum VEGF-A was an independent prognostic factor for outcome ($p<0.00001$)
Davies et al., 2000	29	Liver metastases present	Plasma	Increased plasma VEGF-A in patients with liver metastases in comparison to controls ($p=0.03$) Plasma VEGF-A level correlated with volume of liver metastasis and tumour vessel count.
George et al., 2000	116	Dukes' A-D	Serum and plasma	Plasma and serum VEGF-A levels were higher in cancer patients than controls ($p<0.001$), correlated with one another in all ($p<0.0001$) and with platelet count in cancer patients Plasma, serum and VEGF-A level/platelet increased with advancing disease stage
Nakayama et al., 2000	26	Dukes' A-D	Plasma	Increased plasma VEGF-A in advanced cancer (NS) and negative correlation with IL-12 levels (NS)
Takeda et al., 2000	111	Stage I-IV	Serum	Increased serum VEGF-A in cancer patients compared to controls ($p<0.001$), increased with disease stage. Correlated with depth of invasion ($p<0.01$), liver metastasis ($p<0.001$), LN metastasis ($p=0.007$) and lymphatic invasion ($p=0.033$). Portal vein VEGF-A level higher than peripheral blood level in patients with liver metastases ($p=0.015$). No correlation with CEA or CA19-9

VEGF, vascular endothelial growth factor; LN, lymph node; CEA, carcinoembryonic antigen; RFS, relapse free survival; OS, overall survival; NS, not significant; MRI, magnetic resonance imaging.

Table 9 continued:

Author	*Number of cases*	*Disease stage*	*Sample source*	*Principle findings*
Werther et al., 2000, 2001	614	Dukes' A-D	Serum	Increased serum VEGF-A in cancer patients compared to controls ($p<0.0001$) and Dukes' D patients in comparison to A-C. Colonic primary tumours had higher serum VEGF levels than rectal tumours ($p<0.0001$) Reduced survival in colon cancer patients with elevated VEGF-A levels ($p<0.0001$) but not in rectal cancer patients Preoperative blood transfusion was associated with increased preoperative serum VEGF-A and reduced survival in Dukes' A-C rectal cancer patients (not colonic cancer)
Fujisaki et al., 1998	67	Dukes' A-D	Serum	Serum VEGF-A was increased in cancer patients compared to adenoma patients and controls ($p<0.01$). In cancer patients, levels correlated with CEA ($p<0.001$) & Dukes' stage ($p<0.01$), liver & LN metastases. Curative resection reduced serum VEGF-A levels
Kumar et al., 1998	108	Stage I-IV	Serum	Serum VEGF-A levels elevated in cancer patients in comparison to controls ($p<0.0005$), increased with disease stage and lymph node metastasis.
Landriscina et al, 1998	35	Dukes' B-D	Serum	Serum VEGF-A levels in peripheral and mesenteric blood correlated with disease stage (Dukes' or TNM). No difference in levels between peripheral and mesenteric blood in cancer patients or between peripheral blood and control blood.
Dirix et al., 1996	44	Advanced	Serum	Increased VEGF-A levels associated with short tumour doubling time & disease progression ($p=0.0002$)

VEGF, vascular endothelial growth factor; LN, lymph node; CEA, carcinoembryonic antigen; RFS, relapse free survival; OS, overall survival; MRI, magnetic resonance imaging

The potential clinical uses of circulating VEGF-A measurements have included use:

- as a diagnostic marker (Kumar et al., 1998; Broll et al., 2001)
- as a marker for disease extent, progression and to predict clinicopathological variables (Dirix et al., 1996; Fujisaki et al., 1998; Kumar et al., 1998; Landriscina et al., 1998; Chin et al., 2000; Davies et al., 2000; George et al., 2000; Takeda et al., 2000; Werther et al., 2000; Broll et al., 2001; George et al., 2001a; Werther et al., 2001; Gunsilius et al., 2002; Karayiannakis et al., 2002; Minagawa et al., 2002; Salgado et al., 2002; Werther et al., 2002)
- as a prognostic marker (Chin et al., 2000; Werther et al., 2000; Broll et al., 2001; Akbulut et al., 2002; Karayiannakis et al., 2002; Werther et al., 2002)
- to predict the completeness of surgical resection (Fujisaki et al., 1998; Karayiannakis et al., 2002; Kumar et al., 2002a)
- to predict VEGF-A expression in the primary tumour (George et al., 2001a; Minagawa et al., 2002)
- and as a predictor of the response to therapy (George et al., 2001a; Berglund et al., 2002)

In summary, multiple studies have investigated the use of serum or plasma VEGF-A measurements in examining the different facets of colorectal malignancy (**Table 9**). Diagnostic use of VEGF-A levels may be limited on a population scale due to the broad range of overlapping values between cancer patients and controls. However, intra-individual measurements on a longitudinal scale over time may have potential for monitoring the development of recurrence (Chin et al., 2000; George et al., 2001a). Use of elevated VEGF-A measurements to select patients for adjuvant treatments and measurement of postoperative levels do not seem to offer additional advantages over

conventional clinicopathological staging systems. In large analyses, elevated serum and plasma VEGF-A levels may give independent prognostic information, which seems to be more important in colon cancer as opposed to rectal cancer (Werther et al., 2002).

1.4.5. Anti-VEGF-A treatments in colorectal cancer

Anti-angiogenic treatment is a novel approach to control the growth of cancer. The advantages include applicability to a wide range of solid tumours, ease of access of endothelial cells to intravenously administered treatments, possible avoidance of the side effects of cytotoxic chemotherapy and the potential to reduce tumour growth to a state of dormancy. The genetic stability of endothelial cells would also avoid the problem of tumour heterogeneity, which results in acquisition of resistance to chemotherapy. A number of anti-angiogenic agents are currently being developed and are at various stages of clinical trials (Longo et al., 2002).

The efficacy of an anti-VEGF approach as an anti-angiogenic therapy was first demonstrated by Kim et al. (1993), who showed that anti-VEGF-A monoclonal antibodies inhibited the growth of human xenografts in nude mice by inhibiting tumour angiogenesis. In colorectal cancer, monoclonal antibodies directed against VEGF-A have been used in a mouse model to assess tumour growth in both subcutaneous sites and liver metastases created by VEGF-A expressing tumour cell lines. A dose-dependent reduction in subcutaneous tumour growth and a reduction in the number and size of liver metastases were observed in the anti-VEGF-A treated animals. The reduction in liver metastatic growth was due to almost complete inhibition of angiogenesis, 91% of the liver metastases remained smaller than 1mm in the antibody treated group (Warren et al., 1995). The production of a humanized anti-VEGF-A monoclonal antibody has allowed human trials to be initiated (Presta et al., 1997).

Recent results of a phase III trial using bevacizumab, a monoclonal antibody to VEGF-A, in metastatic colorectal cancer have now been reported (Hurwitz et al., 2003). In this large study, 815 patients with previously untreated metastatic disease were randomised to standard treatment (bolus irinotecan, 5-FU, leucovorin; n=412) and placebo or standard treatment plus bevacizumab (5mg/kg every two weeks; n=403). The addition of bevacizumab to chemotherapy resulted in a significant increase in median survival (20.3 months vs. 15.6 months, p=0.00003), progression-free survival (10.6 months vs. 6.24 months, p<0.00001), overall response rate (45% vs. 35%, p=0.0029) and duration of response (10.4 months vs. 7.1 months, p=0.0014). Increased side effects noted in the bevacizumab treated group over the controls were grade III hypertension (10.9% vs. 2.3 %, p<0.01) and gastrointestinal perforations [0 vs. 1.5% (n=6)], although the reason for these increased adverse effects was not clear. Despite these complications, this is the first phase III study of an anti-angiogenic strategy in human metastatic disease that has been associated with a survival advantage and has resulted in the fast-tracking of bevacizumab (Avastin™, Genentech) in the USA for development for use as first-line treatment in metastatic colorectal cancer.

1.5. VASCULAR ENDOTHELIAL GROWTH FACTORS -C & -D

Although the role of VEGF-A and its receptors in angiogenesis is now well described (Karkkainen & Petrova, 2000; Veikkola et al., 2000) much less is known about VEGF-C and VEGF-D.

1.5.1. Discovery of VEGF-C and VEGF-D

Appreciation of the importance of VEGF-A in angiogenesis and the lack of a known ligand for VEGFR3 led to the search for additional VEGF family members. VEGF-C was identified as a specific activator for VEGFR3 isolated from the conditioned media of the PC-3 human prostate cancer cell line and was also found to stimulate VEGFR2 by causing tyrosine autophosphorylation of the receptors (Joukov et al., 1996). At the same time, Lee et al. (1996) identified the identical growth factor from a cDNA library of the human glioma cell line G61, naming this protein vascular endothelial growth factor-related protein (VRP), which has since been renamed VEGF-C. The mouse equivalent of VEGF-D, *c-fos*-induced growth factor (FIGF), was discovered by differential mRNA screening of fibroblasts obtained from *c-fos* deficient and wild type mice (Orlandini et al., 1996). The human form of VEGF-D is 85% identical to murine FIGF and was identified by computer-based homology searching for VEGF-A-related sequences and is now known to be a ligand for VEGFR2 and VEGFR3 (Achen et al., 1998).

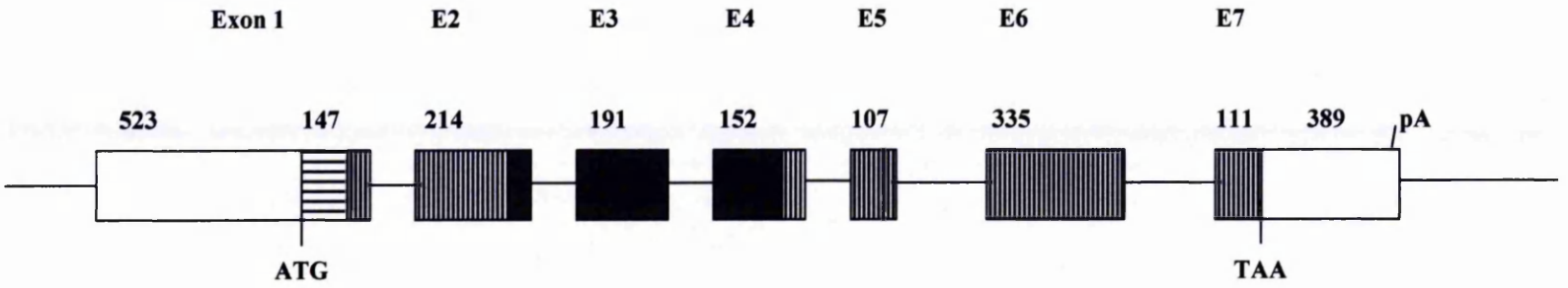
1.5.2. VEGF-C and VEGF-D genes

The gene for murine VEGF-C maps to chromosome 8 and in humans to chromosome 4q34 (Fitz et al., 1997). A high degree of conservation is seen in the organisation of the gene between mouse and man. The human gene is composed of more than 40 kb of genomic DNA and comprises seven exons, separated by introns varying in length from 301 base pairs to over 10 kb pairs. The intron-exon boundaries are highly conserved and all the exons contain coding sequences (Chilov et al., 1997) (**Figure 1**). Exons 2 – 4 code for a VEGF-A-homology domain.

Transcription of the VEGF-C gene begins 523 base pairs upstream from the translational start site, resulting in a long 5′-untranslated region of mRNA. The

promoter sequence contains putative Sp-1, AP-2 and nuclear factor-kappaB (NF-κB) transcription factor binding sites but no hypoxia-regulated element binding sites (Chilov et al., 1997). The lack of hypoxia-response elements in the gene promoter may explain why some authors have found that VEGF-C production, unlike VEGF-A, is unaffected by hypoxia (Enholm et al., 1997; Ristimaki et al., 1998). Recent work on seminoma cell lines in culture however, demonstrates upregulation of VEGF-C and VEGF-D gene expression in response to hypoxic culture by unexplained mechanisms (Fujii et al., 2002). The transcription factor NF-κB is induced by hypoxia and may be a potential contributor to this upregulation. The lack of a definite relationship between hypoxia and VEGF-C expression implies that VEGF-A and VEGF-C have distinct functions in the vascular system. The transcription factor NF-κB is also induced by various inflammatory stimuli and may partly account for the increase in VEGF-C production seen in response to inflammatory cytokines (Ristimaki et al., 1998). Recently, IGF-1 acting via the IGF-1 receptor has been shown to upregulate the expression of VEGF-C (Tang et al., 2003). Upregulation via the IGF-1/IGF-1R axis is likely to be mediated through the AP-2 and Sp1 transcription factors and depends on intracellular signalling through phosphatidylinositol 3′-kinase (PI3-K) and, to a lesser extent, mitogen-activated protein kinase (MAPK) (Tang et al., 2003). Other factors stimulating the production of VEGF-C include PDGF, epidermal growth factor (EGF) (both of which also activate PI3-K signalling), TGF-β, serum, the tumour promotor phorbol myristate 12,13-acetate (PMA) (Enholm et al., 1997) and FGF (Kubo et al., 2002).

Figure 1: Genomic organisation of human VEGF-C



The exons are represented by boxes E1-7, the numbers indicate the length of the exons in base pairs. The horizontal lines between the boxes represent the introns, which vary in length from 301 base pairs to >10 kilobase pairs. The start site for translation (ATG) and the termination codon (TAA) are indicated. pA represents the polyadenylation signal. White boxes, noncoding parts; horizontal lined boxes, signal sequence; vertical lined boxes, N- and C- terminal propeptides that are cleaved proteolytically during processing; grey boxes, sequences encoding the mature VEGF-C polypeptide (VEGF-A homology domain). Diagram adapted from Chilov et al. (1997).

The main form of human VEGF-C mRNA found in human tissues is 2.4 kb, although a 200-400 bp shorter minor mRNA species has been identified (Joukov et al., 1996; Chilov et al., 1997). The shorter species lacks the nucleotides of exon 4, which corresponds to the VEGF-homology domain. Consequently, the shorter transcript is unlikely to have biological function or relevance (Chilov et al., 1997). The 3′ region of human VEGF-C mRNA contains a single AUUUA motif, compared with 3 similar regions in murine VEGF-C mRNA. This motif is implicated in the rapid turnover of many cytokine mRNAs and the presence of only a single motif may explain the greater stability of VEGF-C mRNA, compared with that of VEGF-A.

The gene for VEGF-D maps to human chromosome Xp22 (Yamada et al., 1997; Rocchigiani et al., 1998), spans about 50 kb and possesses similar organisation to the VEGF-C gene with 7 exons and 6 introns. The promoter region has no TATA box but contains an AP-1 binding site at –54 from the translational start site. This site provides binding for members of the *c-jun/c-fos* family of transcription factors, consistent with evidence that mouse FIGF expression is stimulated by *c-fos* expression (Orlandini et al., 1996). Hypoxia may play a role in upregulation of VEGF-D promoter activity in rat pulmonary microvascular smooth-muscle cells, which is regulated within a 523-bp fragment of the rat VEGF-D promoter (Teng et al., 2002).

VEGF-D mRNA is a 2.3 kb transcript in the majority of human tissues and is most abundant in heart, lung, skeletal muscle, colon and small intestine. The mRNA is also found as an additional less abundant 2.8 kb transcript in skeletal muscle (Achen et al., 1998).

Despite their structural similarities, VEGF-C and VEGF-D expression are regulated differently. VEGF-D expression is not induced by cytokines but by activation of the proto-oncogene, *c-fos* (Orlandini et al., 1996) and VEGF-D mRNA expression is

induced in mouse fibroblasts by cell-cell contact mediated by cadherin-11 (Orlandini & Oliviero, 2001) (**Table 10**).

Table 10: Comparison between the molecular biology of VEGF-C and VEGF-D

	VEGF-C	**VEGF-D**
Chromosome	4q34	Xp22
Gene	40 kb, 7 exons AP-2, Sp-1, NK-κB binding sites	50 kb, 7 exons AP-1 binding sites
Upregulation	Inflammatory stimuli IGF, PDGF, EGF, TGF-β, FGF, serum, phorbol myrisate	*c-fos* expression Cell-cell contact mediated by cadherins ? hypoxia
mRNA	2.4 kb	2.3 and 2.8 kb
Protein	Produced as pre-proprotein and proteolytically processed ~30% and 61% identity to VEGF-A & VEGF-D respectively in VEGF homology domain -C terminal extension, 37% identical to VEGF-D -N terminal extension, 25% identical to VEGF-D 3 potential glycosylation sites, 2 of which are conserved with VEGF-D Open reading frame encodes a protein of 419 amino acids	Produced as pre-proprotein and proteolytically processed 31% and 61% identity to VEGF-A & VEGF-C respectively in VEGF homology domain -C terminal extension, 37% identical to VEGF-C -N terminal extension, 25% identical to VEGF-C 3 potential glycosylation sites, 2 of which are conserved with VEGF-C Open reading frame encodes a protein of 354 amino acids
Receptor binding	VEGFR3 in all forms VEGFR2 in proteolytically processed mature form	VEGFR3 in all forms VEGFR2 in proteolytically processed mature form

1.5.3. VEGF-C and VEGF-D proteins

Within the VEGF family, VEGF-C and VEGF-D form a subgroup because of their structural and functional similarity and receptor specificity (**Table 10 & Figure 2**). Unlike VEGF-A, no alternative splicing of VEGF-C or VEGF-D mRNA occurs to produce various molecular species of different molecular weights. However, both VEGF-C and VEGF-D are subject to extensive proteolytic processing which results in the production of proteins that vary in size (Joukov et al., 1997b; Stacker et al., 1999). Overall, VEGF-D is 48% identical to VEGF-C (Achen et al., 1998) and structurally, the subgroup is characterised by:

a) production as a pre-proprotein (Joukov et al., 1997b)

b) similarity to VEGF-A in the central part of the protein within the VEGF homology domain in which the cysteine knot structure typical to the family is conserved. Within the VEGF homology domain, VEGF-C is ~30% identical (Joukov et al., 1996; Lee et al., 1996) and VEGF-D is 31% identical to VEGF-A (Achen et al., 1998). The two cytokines are 61% identical to one another within this region (Achen et al., 1998).

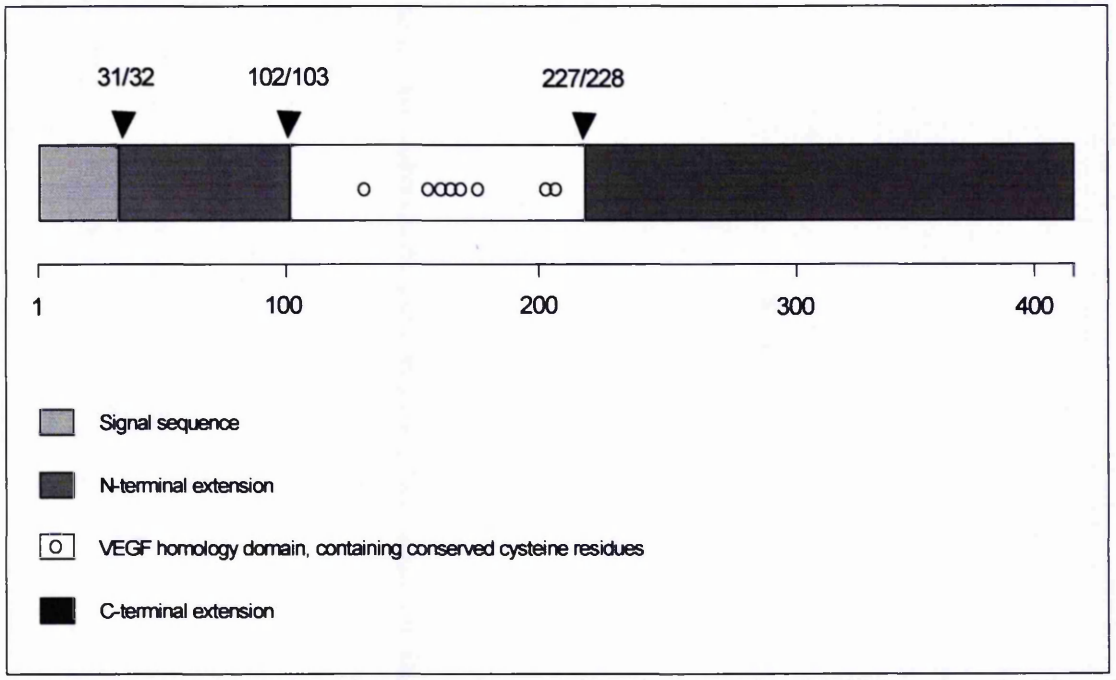
c) Amino (N) and carboxy (C) terminal extensions. The C terminal extension is unique in this subgroup of the VEGF family and rich in cysteine residues. The N and C terminal extensions are related in sequence between VEGF-C and –D, sharing 25% and 37% sequence identity respectively (Achen et al., 1998).

d) three potential N-linked glycosylation sites, 2 of which are conserved between VEGF-C and VEGF-D (Lee et al., 1996; Achen et al., 1998).

e) progressive proteolytic cleavage of the N and C terminal extensions to generate the mature forms of cytokine with increased receptor affinity (Joukov et al., 1997a; Joukov et al., 1997b). Proteolytic processing occurs both within and outside the secreting cell. The protein convertase (PC) group of enzymes, in

particular furin, PC5 and PC7, are responsible for some of the proteolytic processing of VEGF-C (Siegfried et al., 2003) and the serine protease plasmin is also implicated (McColl et al., 2003). Immature and partially proteolytically processed forms bind to VEGFR3 (K_d 130 pM) but full proteolytic cleavage is required for VEGFR2 activating ability (K_d 410 pM) (Joukov et al., 1997b; Stacker et al., 1999). Mature VEGF-C consists of the VEGF-homology domain, MW ~21kDa, dimerised with non-covalent bonds, whereas the partially processed forms have a wide variety of molecular masses (Joukov et al., 1997b). The open reading frame of VEGF-C encodes a protein of 419 amino acids, whereas VEGF-D is 354 amino acids in length.

Figure 2: VEGF-C protein structure



The VEGF-C protein consists of a signal sequence, N-terminal extension, VEGF homology domain and C-terminal extension. The cleavage sites are marked by arrowheads and the numbers of the flanking amino acid residues. The diagram beneath the VEGF-C structure shows the scale in amino acid residues. The 8 conserved cysteine residues in the VEGF homology domain are indicated by open circles.

1.5.4. VEGF-C and VEGF-D signalling

In common with the other members of the VEGF family, ligand binding is responsible for receptor dimerization, intracellular tyrosine kinase activity and autophosphorylation, leading to the activation of various intracellular signalling pathways. Proteolytic processing of the ligands and heterodimerization of VEGF family members influence the affinity of binding and outcome of receptor activation (reviewed in Karkkainen & Petrova, 2000). VEGF-C and -D are ligands for VEGFR2 and VEGFR3, but cannot activate VEGFR1 (Joukov et al., 1996; Achen et al., 1998). Mature VEGF-C and -D bring about functions mediated through VEGFR2, while all forms of VEGF-C and -D mediate the effects of VEGFR3 (**Table 7, page 42**).

VEGFR3 signalling, through VEGF-C or -D ligand binding, is crucial for the development of the vascular system and for maintenance and function of the lymphatic system (see **section 1.3.3, page 46**).

1.5.5. VEGF-C and VEGF-D function: *in vitro* studies

As VEGF-C and VEGF-D activate both VEGFR2 and VEGFR3, they are consequently implicated in both angiogenic and lymphangiogenic pathways (**Table 7, page 42**). Both growth factors exhibit mitogenic effects for vascular and lymphatic endothelial cells and promote survival of lymphatic endothelial cells through VEGFR3 (Lee et al., 1996; Joukov et al., 1997b; Achen et al., 1998; Marconcini et al., 1999; Makinen et al., 2001b; Veikkola et al., 2001). Angiogenic pathways involving endothelial cell migration and activation, are stimulated by VEGF-C but with much less efficiency than by VEGF-A (Joukov et al., 1996; Joukov et al., 1997b; Cao et al., 1998). In three-dimensional culture systems VEGF-D stimulates angiogenesis (Marconcini et al., 1999).

1.5.6. VEGF-C and VEGF-D function: *in vivo* studies

The role of VEGF-C and -D in angiogenic and lymphangiogenic pathways has been investigated in a variety of animal models. Fully processed VEGF-C acts like VEGF-A, promoting blood vessel permeability in guinea pig skin in a dose-dependent manner (Joukov et al., 1997b) and inducing angiogenesis in ischaemic tissues (Witzenbichler et al., 1998). In the corneal assay, both VEGF-C and -D induce angiogenesis (Cao et al., 1998; Marconcini et al., 1999).

The lymphangiogenic potential of VEGF-C has been examined in the differentiated chorioallantoic membrane (CAM) (Oh et al., 1997). Lymphatic vessels were identified by various methods including ISH with probes for VEGFR2 and VEGFR3. The application of recombinant VEGF-C to the CAM induced lymphangiogenesis but not angiogenesis and caused proliferation of lymphatic endothelial cells as detected by bromodeoxyuridine labelling (Oh et al., 1997).

The roles of VEGF-C and VEGFR3 in dermal lymphatic development have been demonstrated using transgenic mouse models. These models use the keratin promoter K14 to control expression of the transgene and localise its expression to the basal layer of the epidermis. An increase in K14-driven transgene expression is seen from embryonic day 14.5 and transgenic mice producing VEGF-C in the skin manifest dermal lymphatic vessel enlargement and lymphatic endothelial cell proliferation without any alteration in blood vasculature (Jeltsch et al., 1997). Similar effects have been shown using transgenic VEGF-D and a mutant form of VEGF-C possessing VEGFR3 activating ability only (Veikkola et al., 2001).

Transgenic mice producing the soluble fusion protein VEGFR3-Ig in the skin under the control of the same promoter, demonstrate inhibition of lymphatic development (Makinen et al., 2001a). Dermal lymphatics developed in early fetal life but regressed after E14.5 and were absent by birth. The transgenic mice were characterised by

swollen feet at birth and older mice showed thickened dermal and subcutaneous layers of the skin and a complete lack of dermal lymphatics but again, no effect was seen on the development of the vascular system (Makinen et al., 2001a).

Combination of the models to produce a double transgenic mouse expressing both mutant VEGF-C or VEGF-D in the skin and soluble VEGFR3-Ig fusion protein showed inhibition of lymphatic hyperplasia although transgene expression remained high (Veikkola et al., 2001). Consequently, dermal lymphangiogenesis is stimulated by VEGF-C and VEGF-D and is mediated via VEGFR3 (Veikkola et al., 2001). Animal models of lymphoedema have been created by inactivating mutations of VEGFR3 in the germline (Karkkainen et al., 2000; Karkkainen et al., 2001) and will be useful in the study of therapeutic interventions for this condition.

Novel animal models of lymphoedema have been constructed in the rabbit ear (Szuba et al., 2002; Yoon et al., 2003), the mouse tail (Boardman & Swartz, 2003; Yoon et al., 2003) and the regenerating lizard tail (Daniels et al., 2003). These models will provide an exciting arena in which to investigate the actions of the lymphangiogenic cytokines in greater detail. Using the rabbit ear and mouse tail models, Yoon et al. (2003) have confirmed that VEGF-C naked plasmid DNA is capable of ameliorating lymphoedema and has a lymphangiogenic effect.

Tumour models in which overexpression of VEGF-C or VEGF-D is demonstrated have been constructed in melanomas (Skobe et al., 2001a), breast cancer (Karpanen et al., 2001; Skobe et al., 2001b; Stacker et al., 2001; Mattila et al., 2002), pancreatic islet cell tumours (Mandriota et al., 2001) and gastric tumours (Yanai et al., 2001). These studies show increased aggressiveness of the transfected cancer cell lines, intra-tumoural lymphangiogenesis, dilated and increased numbers of peri-tumoural lymphatics, lymph node metastases and tumour angiogenesis. In some studies, these effects have been

abrogated by antibodies directed against VEGFR3 (Karpanen et al., 2001) or VEGF-D (Stacker et al., 2001).

Although VEGF-C and VEGF-D cause developmental lymphangiogenesis acting through VEGFR3, whether lymphangiogenesis occurs within spontaneously arising tumours has been questioned. The presence of lymphatics within VEGF-C overexpressing animal tumour models has been demonstrated immunohistochemically in xenograft studies (Karpanen et al., 2001; Skobe et al., 2001b; Stacker et al., 2001). In a transgenic model, mice expressing VEGF-C driven by the rat insulin promoter (RIP) targeted to the β-cells of the endocrine pancreas were crossed with Rip1Tag2 mice who develop non-lymphangiogenic, non-metastatic pancreatic β-cell tumours. The consequent VEGF-C overexpressing tumours developed lymphatics that were located around and in the peripheries of the developing tumour (Mandriota et al., 2001). It is possible that in xenograft models, the injection of tumour cell suspensions to create tumours entraps pre-existing lymphatics within the developing tumour mass, whereas transgenic animal models may more accurately reflect the situation in spontaneously arising human tumours (Karpanen & Alitalo, 2001).

The question of whether immunohistochemically detected intra-tumoural lymphatics are functional has also been hotly debated (Jain & Fenton, 2002). Previously, functional lymphatics have only been found within 100μm of the edge of murine sarcoma xenografts, increased interstitial pressure within the tumour contributing to the lack of functioning lymphatics within the tumour mass (Leu et al., 2000). More recently, Padera et al. (2002) demonstrated a lack of viable intra-tumoural lymphatics and the presence of functional peri-tumoural lymphatics in mouse xenografts, which showed clearly that peripheral peri-tumoural lymphatics were sufficient to enable metastasis via the lymphatic route. This mirrors the situation in the transgenic mouse pancreatic tumour model (Mandriota et al., 2001).

1.5.7. VEGF-C and VEGF-D function: *ex vivo* studies

From the *in vitro* and animal studies, it is clear that VEGF-C induces the development of new lymphatic vessels (Oh et al., 1997) and hyperplasia of pre-existing lymphatics (Jeltsch et al., 1997), acts as a survival factor for lymphatic endothelial cells (Makinen et al., 2001b; Veikkola et al., 2001) and influences angiogenesis (Cao et al., 1998). However, the precise lymphangiogenetic involvement of VEGF-C, VEGF-D, and VEGFR3 in human malignancy is not fully understood (reviewed in Clarijs et al., 2001a; Pepper, 2001; Jain & Fenton, 2002; Van Trappen & Pepper, 2002).

In human cutaneous melanoma, lymphatics, but not lymphangiogenesis, were observed at the tumour margin; these peripheral lymphatics were pre-existing and trapped within the expanding tumour mass (de Waal et al., 1997), more recent studies however, support a case for lymphangiogenesis (Dadras et al., 2003; Straume et al., 2003). Dadras et al. (2003) studied a retrospective series of metastatic and non-metastatic melanomas and demonstrated proliferating intra- and peri-tumoural lymphatic vessels. Furthermore, the number of intra-tumoural, peri-tumoural lymphatics, average lymphatic vessel size and lymphatic vessel area was higher in metastatic melanomas than in matched non-metastastic tumours. Further evidence for tumour-derived lymphangiogenesis is gleaned from work by Beasley et al. (2002). These authors demonstrated the existence of proliferating lymphatics within head and neck cancer stroma and an association of intra-tumoural lymphatic vessel density (LVD) with neck node metastases (Beasley et al., 2002). These studies have raised the possibility of intra- or peri-tumoural LVD as a potential prognostic indicator of poor outcome. However, the presence of proliferating lymphatic endothelial cells within a tumour may not equate with functional lymphatic channels, and no assessment of function was possible in these studies. Different tumour types may vary with respect to the relative importance of lymphangiogenesis as a means

of lymphatic metastasis. Recent work has shown a lack of proliferating lymphatics in and around breast cancer, despite active angiogenesis, and a low LVD in comparison to MVD (Williams et al., 2003). Despite these findings, breast cancer was still able to spread to the regional lymph nodes, presumably through pre-existing peri-tumoural lymphatics (Williams et al., 2003).

In summary, these studies have demonstrated the existence and proliferation of intra-tumoural lymphatic vessels and endothelial cells within some tumour types, a possible differing importance of intra-tumoural/peri-tumoural lymphatics with varying tumour types and a potential role for LVD as a prognostic marker. These recent studies have all employed antibodies to lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 (see **section 1.6.1, page 82**). It is not clear whether similar findings will be shown with other lymphatic markers nor what the clinical relevance of these findings will be in the context of other human malignancies, particularly those which spread predominantly via the lymphatic route. No investigations have focussed on this area in colorectal cancer. Additional studies are required to examine the relationship of LVD and lymphangiogenesis to tumour expressed lymphangiogenic growth factors.

1.6. THE STUDY OF LYMPHATIC VASCULATURE

Study of the lymphatic vasculature has been difficult until recently and hampered by a lack of specific lymphatic markers and commercially available antibodies, the lack of lymphatic endothelial cells for culture and limited animal models. Discrimination of lymphatic from blood vessels on morphological appearance alone is difficult and

subjective. Lymphatics typically appear as thin-walled, single cell lined, irregular vessels that lack a continuous basement membrane and pericytes, and hence are characterised by wide inter-endothelial gaps (Ryan, 1989). To improve the accuracy of detection of lymphatic vessels from blood vessels, immunohistochemical techniques using various novel antibodies and antibody combinations have recently been used in an attempt to exploit differences between the two vessel types (Sleeman et al., 2001).

1.6.1. Lymphatic vessel markers

Traditional methods of identification of lymphatic vessels included the absence of Weibel-Palade bodies in the lymphatic endothelial cell cytoplasm, lack of immunostaining for vWF (Ryan, 1989) and an absence of basement membrane components such as laminin, collagen IV and fibronectin (Nerlich & Schleicher, 1991). Recent advances have included the production of new antibodies directed against antigens that are differentially expressed between lymphatic and vascular endothelial cells. However, all methods for lymphatic vessel staining described to date have limitations some of which will now be explored and summarised in **Table 11**.

Enzyme histochemistry is described to detect lymphatic endothelial cells using 5′-nucleotidase (5′-NA). 5′NA activity varies in different types of endothelial cells being very high in lymphatic endothelial cells and low or absent in blood vessel endothelia. However, whether activity is upregulated in new vessels undergoing angiogenesis is unclear. The method involves partial inhibition of 5′NA activity by paraformaldehyde fixation of cryostat sections, followed by an enzyme reaction induced by the addition of substrate materials, to identify optimally the excess activity in lymphatics compared to blood vessels (Ohta et al., 1999). Unfortunately, this technique is difficult, subjective and small changes to methodology may significantly alter the results.

Single antibody immunostaining methods are described using antibodies that bind to podoplanin, Prox-1, VEGFR3 or LYVE-1. Podoplanin is a glomerular podocyte membrane mucoprotein identified in rats, which is also expressed on endothelial cells of normal lymphatics (Breiteneder-Geleff et al., 1997; Birner et al., 2000; Birner et al., 2001a; Birner et al., 2001b). Prox-1 is a homeobox gene product essential for the regulation of early development of a variety of tissues including the lens, heart, liver, pancreas, central nervous system and the lymphatic system (Wigle & Oliver, 1999). Prox-1 null mice fail to develop a lymphatic system but vasculogenesis and angiogenesis remain unaffected (Wigle & Oliver, 1999). Immunohistochemical staining for Prox-1 demonstrates lymphatic but not blood vessels (Carreira et al., 2001).

Although VEGFR3 is restricted to lymphatics in the normal adult circulation (Kaipainen et al., 1995; Jussila et al., 1998; Lymboussaki et al., 1998), it is upregulated on tumour neovasculature. Consequently, VEGFR3 immunostaining lacks specificity for lymphatics in tumours (Partanen et al., 1999; Valtola et al., 1999; Niki et al., 2001). Monoclonal antibodies to VEGFR3 are reported to show specific endothelial cell staining, but problems associated with the use of polyclonal antibodies include high background staining and unreliable, irreproducible vascular staining (Birner et al., 2000; Birner et al., 2001b; Clarijs et al., 2002; Moller et al., 2002).

LYVE-1 is a lymphatic endothelial cell receptor for the extracellular matrix glycosaminoglycan hyaluronan. It is closely related to CD44, the major receptor for hyaluronan in epithelial, mesenchymal and lymphoid cells (Banerji et al., 1999). Staining for LYVE-1 highlights lymphatic vessels specifically (Jackson et al., 2001; Mattila et al., 2002) but LYVE-1 is also found on hepatic blood sinusoidal endothelial cells (Carreira et al., 2001) and may be expressed in up to 10% of tumour neovasculature (Padera et al., 2002).

The use of double staining techniques has been used to demonstrate lymphatic and blood vessels selectively and enhance the accuracy of detection. These methods have included combinations of PAL-E/CD31 (de Waal et al., 1997) and PAL-E/VEGFR3 (de Waal et al., 1997; Clarijs et al., 2001b). PAL-E is a monoclonal antibody that recognises an unknown human endothelial cell antigen that only stains blood vessel endothelia. In combination with CD31, PAL-E obscures the CD31 stain on blood vessels. Consequently PAL-E positive vessels are defined as blood vessels and CD31 positive vessels as lymphatics (de Waal et al., 1997; Clarijs et al., 2001a). In a similar fashion, PAL-E staining hides the stain derived from VEGFR3 on angiogenic blood vessels leaving PAL-E positive vessels defined as blood vessels and VEGFR3 positive vessels as lymphatic in origin (Valtola et al., 1999). Unfortunately, studies using PAL-E are limited to frozen tissue specimens. Anti-LYVE-1 antibodies have also been used in combination with anti-endothelial cell markers. Beasley et al. (2002) used a LYVE-1/CD34 antibody cocktail to illustrate the two different cell populations of blood vessel (CD34 positive/LYVE-1 negative) and lymphatic endothelial cells (CD34 negative/LYVE-1 positive) in human head and neck cancers. With this technique they could demonstrate 'hotspots' of intra-tumoural proliferating lymphatics and correlation between the density of intra-tumoural lymphatics and neck node metastases. Antibodies to LYVE-1 have also been used in combination with CD31 antibodies by Dadras et al. (2003) in cutaneous melanomas, identifying 'hotspots' of intra- and peri-tumoural lymphatic vessels. Finally, the combined use of Prox-1/CD31 has been reported by Wilting et al. (2002). Prox-1 is a specific lymphatic endothelial marker in the fetal lymphatic circulation and in adult lymphatics in both healthy and diseased tissue. Prox-1 co-localised with VEGFR3 and CD31 in lymphatics. In blood vessel endothelial cells, Prox-1 and PAL-E were mutually exclusive and Prox-1 was not found on the blood vessel endothelium. Specificity of Prox-1 for lymphatic endothelium was higher than

that of VEGFR3, which was upregulated in some blood vessel endothelia in haemangiomas (Wilting et al., 2002).

In summary, anti-LYVE-1 antibodies have been well studied and appear to offer the most specific staining for lymphatic endothelial cells. Studies comparing the specific lymphatic endothelial cell antibodies alone and in combination are required to confirm the best antibody or antibody combination for specific lymphatic endothelial cell discrimination.

Table 11: Immunohistochemical staining methods for the detection of lymphatic endothelial cells

Antibody(ies) used	Sites of antigen expression	Advantages	Disadvantages
VEGFR3	LEC, tumour neovasculature	LEC specific in normal tissue	Lack of specificity for LEC in tumours Non-specific stain with polyclonal forms, lack of commercial antibody availability for monoclonal forms
Podoplanin	LEC, glomerular podocytes	LEC specific	Lack of commercial antibody availability
Prox-1	LEC	LEC specific	Lack of commercial antibody availability
LYVE-1	LEC, hepatic sinusoidal cells, (?) neovasculature	LEC specific	Lack of commercial antibody availability Possible crossreaction with neovasculature
Enzyme histochemistry (5′NA activity)	All endothelial cells at different levels		Findings difficult to interpret Hampered by non-specific staining
CD31/PAL-E	Pan-EC marker/BEC	Enhanced discrimination accuracy	Frozen tissue required
VEGFR3/PAL-E	LEC, angiogenic BEC/BEC	Enhanced discrimination accuracy	Frozen tissue required, lack of commercial antibody availability for monoclonal VEGFR3
LYVE-1/CD34	LEC/Pan-EC marker	Enhanced discrimination accuracy	Lack of commercial antibody availability
Prox-1/CD31	LEC/Pan-EC marker	Enhanced discrimination accuracy	Lack of commercial antibody availability

Abbreviations: LEC, lymphatic endothelial cells; 5′NA, 5′-nucleotidase; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; BEC, blood vascular endothelial marker; Pan-EC marker, pan-endothelial cell marker

1.6.2. Lymphatic endothelial cell lines *in vitro*

The study of lymphatic endothelial cells in culture has been hampered by the lack of available cells from the lymphatic microvasculature. Most studies have used endothelial cells from large lymphatic vessels such as the thoracic duct but these cells tend to alter in culture, losing the expression of known lymphatic antigens (Pepper et al., 1998) and may not reflect the characteristics of the microvascular lymphatic endothelial cell *in vivo*. Recent studies have clarified the origin of subtypes of cells contained within cultured cell lines and this should provide a source of lymphatic endothelial cells for future work. For example, the human dermal microvascular endothelial cell (HDMEC) line has been shown to consist of a mixture of two separate, stable lineages of vascular and lymphatic endothelial cells that could be separated using antibodies to exploit their differential expression of the cell surface receptor VEGFR3 (Makinen et al., 2001b). Consistent expression of VEGFR3 was seen on the lymphatic endothelial cells *in vitro*, in contrast to other novel lymphatic cell markers, such as podoplanin and LYVE-1, which were heterogeneously expressed. The blood vascular endothelial cells in culture also showed heterogeneity of expression of the pan-endothelial markers, von Willebrand factor (vWF) and CD31, reflecting their likely origin from different sized blood vessels (Makinen et al., 2001b). Similar methodology exploiting the differential expression of podoplanin on lymphatic and blood vessel endothelial cells has been used to separate the two cell populations from dermal cell suspensions (Kriehuber et al., 2001). This study reported that podoplanin positive cells (ie. lymphatic endothelial cells) were also positive for VEGFR3 and LYVE-1 and the expression of podoplanin was stable after repeated passages of the lymphatic endothelial cells. Exploitation of the differential expression of VEGFR3, podoplanin and LYVE-1 on lymphatic endothelial cells in comparison to vascular endothelial cells *in vitro*, together with flow cytometry, may provide a reliable source of these cells to allow further investigation of lymphatic

endothelial cell biology. Hirakawa et al. (2003) have used a further modification of this immunomagnetic technique to separate human skin blood and lymphatic endothelial cells by their differential expression of CD34 and CD31, as lymphatic endothelial cells were consistently CD34 negative and CD31 positive. Lymphatic endothelial cells obtained in this way and maintained in culture, exhibited stable expression of the novel lymphatic specific cell markers over repeated passages, hence validating the use of these specific antigens for the detection of lymphatic vessels in tissues (Hirakawa et al., 2003). Furthermore, the identification of differential expression of lineage-specific genes in blood vascular and lymphatic endothelial cells confirmed the close relationship between the two cell types, in that only relatively small differences were found between their expression profiles, although these differences provided insights into the specialised functions of the cells as well as pinpointing further molecular targets for investigation of vascular development, regulation of the immune response and lymphangiogenesis (Hirakawa et al., 2003).

Culture of human microvascular endothelial cells, consisting of both blood and lymphatic endothelial cells, in three-dimensional systems shows the development of tubular structures that wrap around one another (Kriehuber et al., 2001) resembling the *in vivo* arrangement of blood and lymphatic vessels. The same study also demonstrated the secretion of the chemokine receptor 7 ligand, secondary lymphoid tissue chemokine (SLC)/CCL21 from the basolateral surface of lymphatic endothelial cells. This chemokine is implicated in the regulation of dendritic cell migration and may be involved in chemotaxis of chemokine receptor positive malignant cells towards the lymphatic vessel (Kriehuber et al., 2001). This observation may be of great relevance for the treatment of human malignancy, as chemokine receptor blockade could provide a new strategy to inhibit the invasion of lymphatic vessels and hence the metastatic potential of neoplastic cells.

1.6.3. Animal models for lymphatic vessel research

With the upsurge of interest in lymphangiogenesis research, animal models have become increasingly sought. Animal assays to study the process of lymphangiogenesis have been described and use techniques adapted from the field of angiogenesis research such as the CAM (Oh et al., 1997) and corneal angiogenesis assay (Kubo et al., 2002). Transgenic mouse models expressing lymphangiogenic growth factors or soluble receptor fusion proteins have demonstrated the developmental functions of VEGF-C and VEGF-D and provide tools for further investigation (Jeltsch et al., 1997; Makinen et al., 2001a) (see **section 1.5.6, page 77**). Genetic knock-out models have shown the importance of VEGFR3 in the development of the embryonic vascular system (Dumont et al., 1998) as well as in maintanence and function of the lymphatic system. Animal lymphoedema models created by inactivating mutations of VEGFR3 in the germline (Karkkainen et al., 2000; Karkkainen et al., 2001), the rabbit ear, mouse tail and regenerating lizard tail models discussed in **section 1.5.6 (page 77)** will be useful in the study of therapeutic interventions for this condition. The combination of VEGF-C and VEGF-D overexpressing animal tumour models in conjunction with specific lymphatic vessel markers allows more thorough investigation of the process of lymphatic metastasis.

1.7. VEGF-C AND VEGF-D IN HUMAN GASTROINTESTINAL MALIGNANCIES

The expression of VEGF-C mRNA is increased in a variety of human malignancies (Salven et al., 1998). Positive associations have been found between the expression of VEGF-C in multiple types of malignant tissue with negative clinicopathological features including lymphatic invasion and lymph node metastasis. These associations have been made in breast, cervical, colorectal, gallbladder, gastric, oesophageal, pancreatic, prostate, thyroid, NSCLC, lung adenocarcinoma, oral and laryngeal cancers. Clinically important areas of interest are the association between VEGF-C and -D expression, intra- and peri-tumoural LVD, lymphatic and venous invasion, lymph node metastasis and survival.

1.7.1. Methodological considerations

Many published reports conflict in their outcomes and conclusions. This may be partly explained by the use of different methodologies between studies.

Immunohistochemical techniques and microvessel counting examine the tissue as near its condition *in vivo* as possible. Even so, results obtained evaluating malignant tissue at the invasive edge of tumours may not concur with results from central and superficial parts of the tumour (Furodoi et al., 2002). As with MVD evaluation, scoring methods for transcript quantification and immunohistochemical staining vary between studies, with consequent difficulties in extrapolation of results.

As RNA extraction necessarily entails tissue disruption, studies examining mRNA levels provide an estimate of overall expression in the tissue fragment analysed, including tumour cells, stroma and normal mucosa. Consequently, analysis of global

cellular mRNA levels may miss subtleties of tissue expression that are key to understanding tumour behaviour.

Current knowledge of the role of VEGF-C and VEGF-D in gastrointestinal tumours has been summarised in **Tables 12, 13 and Appendix I** (Duff et al., 2003b).

1.7.2. Oesophageal cancer

Oesophageal cancer has an extremely poor prognosis defined by the existence of lymph node metastases at presentation. Limited and conflicting evidence exists for the role of VEGF-C in oesophageal cancer and no data are available for VEGF-D. Kitadai et al. (2001) analysed the relationship between the expression of VEGF-C and clinicopathological characteristics in oesophageal squamous cell carcinoma. *In vitro* RT-PCR and immunocytochemical analysis demonstrated that 4 of the 5 oesophageal carcinoma cell lines studied expressed VEGF-C mRNA, but only one cell line expressed VEGFR3 mRNA. *Ex vivo* analysis of 12 human oesophageal squamous carcinoma tissues showed the presence of VEGF-C mRNA in 67% of cases. In 48 archival specimens, 40% showed positive immunohistochemical staining for VEGF-C. Positive VEGF-C expression in the cancer cells correlated with stage of disease, lymphatic invasion, venous invasion and lymph node metastasis and with depth of tumour invasion. Interestingly, the number of blood vessels detected by immunohistochemical staining for CD34 was significantly higher in the VEGF-C positive tumours when compared with the VEGF-C negative tumours (Kitadai et al., 2001). This indicates that VEGF-C may be involved in both angiogenic and lymphangiogenic processes, via VEGFR2 and VEGFR3 in angiogenic vessels and VEGFR3 on lymphatic endothelial cells. However, angiogenic vessels in the oesophageal squamous cell carcinoma specimens could not be identified by positive staining for VEGFR3. Positive VEGFR3 immunoreactivity was detected in the

cytoplasm of lymphatic endothelial cells, defined as such by their morphological appearance only (Kitadai et al., 2001). However, a similar study examining larger numbers of oesophageal squamous cell carcinomas failed to detect significant associations between expression of VEGF-C protein and any clinicopathological variable other than histological grade (Noguchi et al., 2002) (**Table 12**).

The human model of neoplastic progression from Barrett's oesophageal epithelium to dysplasia and adenocarcinoma offers an insight into VEGF-C involvement in the development of adenocarcinoma as opposed to squamous cell carcinoma. Normal squamous oesophageal mucosa does not express VEGF-C whilst expression is increased in Barrett's epithelium, dysplasia and invasive adenocarcinoma. This is paralleled by a similar increase in VEGFR3 on lymphatic vessels (Auvinen et al., 2002).

1.7.3. Gastric cancer

Gastric cancer is an important cause of death from cancer worldwide, with lymph node status an important predictor of survival. The incidence in the UK is 10,000 new cases per year, with a 5-year survival rate in the order of 20%. The role of VEGF-C, lymphatic invasion and lymph node metastasis in gastric cancer has been investigated in several studies (**Table 12**). To date, only a single study has assessed the role of VEGF-D in gastric malignancy (Ishikawa et al., 2003) (**Table 12**).

Immunohistochemical analysis of tumour tissues has demonstrated that VEGF-C immunoreactivity is restricted to the cytoplasm of gastric cancer cells (Yonemura et al., 1999; Ichikura et al., 2001; Yonemura et al., 2001). By contrast, VEGFR3 immunoreactivity is restricted to endothelial cells of mucosal and submucosal lymphatic vessels and to a very few small blood vessels. Consequently, the majority of VEGFR3 positive vessels in gastric cancer are thought to be lymphatics (Yonemura et al., 1999; Yonemura et al., 2001). The percentage of gastric tumours that are positive for VEGF-C

protein expression varies in different studies from 26% to 51% (**Table 12**) (Yonemura et al., 1999; Ichikura et al., 2001; Kabashima et al., 2001; Takahashi et al., 2002). This may be a function of variable scoring methods that are semi-quantitative based on the number (Takahashi et al., 2002), or percentage (Yonemura et al., 1999; Ichikura et al., 2001; Kabashima et al., 2001; Amioka et al., 2002), of positive cells in varying numbers of high-power fields.

Lymphatic invasion and lymph node status in gastric cancer are correlated positively with tissue expression of VEGF-C (Yonemura et al., 1999; Ichikura et al., 2001; Kabashima et al., 2001; Amioka et al., 2002; Takahashi et al., 2002; Ishikawa et al., 2003) (**Table 12**). In early gastric cancer (confined to the mucosa), positive VEGF-C tissue expression was significantly associated with lymphatic invasion, potentially predicting a subset of patients where minimal surgical resection might be inappropriate (Kabashima et al., 2001). Similar associations are demonstrated with the expression of VEGF-C and VEGFR3 mRNA in gastric cancer tissue. Malignant tissue expressed increased VEGF-C mRNA compared with adjacent normal mucosa [47% vs. 13% (Yonemura et al., 1999); 55% vs. 13% (Yonemura et al., 2001)] and this correlated with expression of VEGFR3 mRNA (Yonemura et al., 1999; Yonemura et al., 2001). VEGF-C mRNA expression was significantly associated with positive lymph node status and lymphatic and venous invasion (Yonemura et al., 1999).

Microvessel counts for VEGFR3 positive vessels showed a non-significant increase in vessel count in tumour stroma compared with normal gastric mucosa (4.62 +/-5.85 vs. 2.48 +/- 1.64, $p=0.067$), but a significant increase in VEGF-C mRNA positive tumours compared to VEGF-C mRNA negative tumours (6.96 +/-6.05 vs. 2.16 +/- 2.00, $p<0.001$) (Yonemura et al., 2001). Similar increases in VEGFR3 positive vessel counts are seen in gastric cancers that are lymph node positive, show lymphatic invasion or are

poorly differentiated (Yonemura et al., 2001). These results suggest that VEGF-C and VEGFR3 act together in a paracrine fashion in the gastric tumour microenvironment.

The association between VEGF-C expression and prognosis however is not clear cut (**Table 12**). Non-significant trends towards reduced survival in VEGF-C expressing gastric cancers have been found (Ichikura et al., 2001). Yonemura et al. (1999) have shown a significant difference in survival in 117 patients with gastric cancer where high levels of VEGF-C expression were associated with poorer prognosis. Further significant differences in survival with VEGF-C status have been reported by Takahashi and colleagues (2002) in a group of 65 cancer patients. An additional finding highlighted by this study was that the density of dendritic cells in the tumour correlated inversely with VEGF-C expression. The effect of VEGF-C on survival may be due, in part, to its regulatory function on dendritic cells and potential enhancement of evasion of immunosurveillance by the tumour (Kabashima et al., 2001). This mirrors the situation for VEGF-A expression in gastric cancer and NSCLC, where VEGF-A influences the number of infiltrating dendritic cells and has a negative impact on prognosis (Saito et al., 1998; Inoshima et al., 2002) (see **section 1.4.3, page 54**).

Ishikawa et al. (2003) investigated the role of VEGF-D and VEGF-C in 105 early gastric carcinomas (**Table 12**) and found that the overall rate of tumour positivity for VEGF-D was lower than that of VEGF-C (22/105 vs. 83/105, $p<0.001$). Lymph node metastasis was significantly related to expression of VEGF-C and VEGF-D in adenocarcinomas of undifferentiated type, but not in differentiated tumours.

To summarise, in gastric cancer, expression of VEGF-C mRNA is seen in the majority of tumour cell lines, and at higher levels in gastric cancer than normal mucosa. VEGF-C mRNA and protein expression in gastric cancer correlates with lymphatic invasion and lymph node metastasis, and in some studies correlates with venous invasion and reduced five-year survival. There is limited evidence available for VEGF-D aside from

the observation that expression of this growth factor may predict lymph node metastases in early undifferentiated gastric carcinomas.

1.7.4. Colorectal cancer

The role of VEGF-C and VEGF-D in colorectal carcinoma is less well understood than in that of gastric carcinoma. Recent publications illustrate conflicting results regarding protein and gene expression in relation to clinicopathological features (Duff & Jayson, 2003) (**Table 13**).

Several authors have demonstrated associations between VEGF-C expression and less favourable clinicopathological outcomes (Akagi et al., 2000; Furodoi et al., 2002). Furodoi et al. (2002) showed that the microanatomical location of VEGF-C within a tumour is relevant. This group studied 152 cases of advanced colorectal cancer demonstrating immunohistochemical detection of VEGF-C in 47% at the deepest invasive site of colorectal carcinoma. Expression correlated with lymphatic and venous invasion, lymph node status, Duke's stage, liver metastasis, depth of invasion, poorer histological grade and MVD. In contrast, positive correlations were not seen on examination of the central and superficial parts of the tumour. VEGF-C expression and lymph node metastasis were prognostic factors for five-year survival on multivariate analysis (odds ratio (OR) 9.10, p=0.0272 and OR 8.52, p=0.0322 respectively). The study emphasised the paracrine nature of the interaction between VEGF-C and the tumour microenvironment and the positive relationship between VEGF-C and tumour angiogenesis (Furodoi et al., 2002). Similar associations between tissue VEGF-C expression and clinicopathological factors have been described in colorectal cancer by Akagi et al. (2000), but with a non-significant trend towards decreased survival in VEGF-C positive groups (p=0.3227) and the additional finding of concordant patterns of VEGF-C expression in involved lymph nodes and primary tumours. Unfortunately,

no attention has been paid to whether the tumours assessed in these studies exhibited ulcerative or polypoidal phenotypes. These tumour types differ in their microenvironments and hence, the factors driving growth factor expression will possess varying levels of importance in the different tumours.

Contradictory evidence exists concerning the role of VEGF-C in lymphatic metastasis in colorectal cancer. Studies examining the mRNA levels of various VEGF family members tend to show variable associations between such levels and clinicopathological factors. George et al. (2001b) showed an increase in VEGF-A and VEGF-C mRNA in carcinomas (p=0.006 and p=0.004, respectively) but not in colonic polyps (p=0.22 and p=0.5, respectively). However, no association was found between the increased level of VEGF-C mRNA and lymph node status, although a relationship existed between positive lymph nodes and VEGF-A mRNA expression. Patterns of VEGF-C mRNA expression were similar in the primary tumour and lymphatic metastases, reflecting the findings for protein expression described by Akagi et al. (Akagi et al., 2000). The mRNA findings for VEGF-A and VEGF-C were confirmed by immunohistochemistry, demonstrating no correlation between positive staining for VEGF-A, VEGF-C or VEGF-D and lymphatic spread (George et al., 2001b). Further analyses of VEGF family mRNA levels in the adenoma-carcinoma sequence showed that only VEGF-A mRNA levels were consistently raised in malignant tissues and this became apparent early on in disease progression (Andre et al., 2000).

There are few studies on the role of VEGF-D in colorectal malignancy, but the published literature mirrors the areas of disagreement that are seen for VEGF-C expression (**Table 13**). The tumour expression of VEGF-D mRNA and protein has been found to be both lower (George et al., 2001b) and higher (White et al., 2002; Funaki et al., 2003) than normal mucosa by different authors.

Funaki et al. (2003) showed that 4 of 8 colorectal cancer cell lines expressed increased levels of VEGF-D mRNA on real-time quantitative RT-PCR analysis, which translated into increased protein levels in 5 of 8 cell lines. The authors proceeded to examine 83 colorectal cancer cases immunohistochemically, showing a significant association between VEGF-D expression and lymph node metastasis ($p<0.01$), although no statistically significant relationship with survival (5 year survival rates: 66% VEGF-D positive cases vs. 79% VEGF-D negative cases, $p=0.15$). A further study of 84 colorectal cancer specimens confirmed an increased level of VEGF-D protein expression in colorectal cancer (colorectal cancer vs. adenomatous polyps and normal mucosa, $p<0.001$), which was associated with lymph node involvement ($p=0.02$) and reduced overall- and disease-free survival (White et al., 2002).

On the other hand, lower VEGF-D mRNA expression in colorectal cancer specimens was demonstrated by George et al. ($p=0.002$, colorectal cancer vs. normal mucosa; $p=0.0002$, colonic polyps vs. normal mucosa). However, in this study, despite the lower level of VEGF-D mRNA in the tumours, the number of tumours demonstrating immunohistochemically detected VEGF-D protein expression was paradoxically higher than that seen in normal mucosa or polyps (31% cancers vs. 0% normal mucosa and 18% adenomas) (George et al., 2001b). The authors postulated that the reduction in VEGF-D levels in the progression of malignancy may act permissively, allowing the more potent angiogenic cytokines VEGF-A and VEGF-C, to bind to the signalling receptors. However, this seems unlikely, because despite a reduction of tumour VEGF-D mRNA, there was an actual increase in VEGF-D protein detected immunohistochemically. Consequently, reduced VEGF-D mRNA levels in tumours may not reflect a real decrease in expressed protein, and is likely to be a fundamental problem of the methodology used. For example, because VEGF-D is expressed by

vascular smooth muscle cells, the tissue homogenates required for RNA extraction may not appropriately quantify VEGF-D levels in carcinoma and normal mucosa.

Recently, Hanrahan et al. (2003) have examined the mRNA levels of all the VEGF and VEGFR family members by ribonuclease protection assay, RT-PCR and immunohistochemistry in normal colorectal tissue, adenomas and carcinomas. This is the only study to examine the differential expression patterns of all the growth factors and receptors at different stages of the adenoma-carcinoma sequence and has illustrated that the growth factors are switched on and off at different stages of progression. An increase in VEGF-A and VEGF-B mRNA was found in adenomas over normal mucosa, an increase in VEGF-A and VEGF-C mRNA in carcinomas over normal mucosa and a decrease in VEGF-D mRNA in adenomas and carcinomas in comparison to normal tissue. These mRNA findings were confirmed by immunohistochemistry. The authors postulated that VEGF-A and VEGF-B act as initiators, important in early tumour development at the stage of adenoma formation, VEGF-A and VEGF-C sustain tumour progression and play a role in advanced disease and that loss of VEGF-D may be important in some tumour subsets (Hanrahan et al., 2003).

In conclusion, in colorectal malignancy, conflicting reports exist for the precise roles of VEGF-C and VEGF-D in tumour behaviour and there are no studies that have examined the expression of the growth factors in the liver metastases of colorectal cancer. The areas of controversy noted may be partly explained by methodological issues and differences in tumour biology. Methodological issues include the importance of appropriate sampling from the invasive edge of the tumour, the choice of appropriate examination techniques, consistent methods for assessment for immunohistochemical staining and scoring, all of which are fundamental to interpretation and comparison across studies. Differences in individual tumour biology and behaviour relate to the balance between various members of the VEGF family, their relative levels within a

tumour, the extent of proteolytic processing and receptor availability. Individual levels alter with different stages of tumour progression and interaction between VEGF family members and their receptors may hold some clues to the microenvironmental balance of lymphangiogenic and angiogenic influences in the progression of colorectal cancer.

Table 12: Immunohistochemical detection of VEGF-C and VEGF-D expression in upper gastrointestinal malignancy

Tumour type	Number of cases	VEGF-C IHC positive (%)	Clinicopathological associations of increased growth factor expression with regard to :				Reference
			Lymphatic invasion	Venous invasion	Lymph node metastasis	Prognosis	
VEGF-C expression							
Oesophageal SCC	48	39.6%	***p*<0.01**	***p*<0.01**	***p*<0.01**	NA	Kitadai et al., 2001
Oesophageal SCC	71	53.5%	*p*=0.51	*p*=0.092	*p*=0.085	*p*=0.80	Noguchi et al., 2002
Gastric adenocarcinoma	117	26%	***p*<0.05**	***p*<0.01**	***p*<0.05**	***p*<0.001**	Yonemura et al., 1999
Gastric adenocarcinoma	76	45%	***p*=0.04**	*p*=0.07	NS	NS trend	Ichikura et al., 2001
Early gastric adenocarcinoma	105	29%	***p*=0.02**	NS increase	NS increase	NA	Kabashima et al., 2001
Gastric adenocarcinoma	65	51%	***p*<0.05**	NS	***p*<0.05**	***p*<0.01**	Takahashi et al., 2002
Gastric adenocarcinoma	139	32% strongly positive	***p*<0.05**	NS	***p*<0.05**	NA	Amioka et al., 2002
Early gastric adenocarcinoma	105	79%	***p*=0.01**	*p*=0.27	*p*=0.09	NA	Ishikawa et al., 2003
VEGF-D expression							
Early gastric adenocarcinoma	105	21% overall: differentiated cancer (18/41) vs. undifferentiated (4/64), ***p*<0.0001**	***p*=0.01**	NS	***p*=0.03**	NA	Ishikawa et al., 2003

Abbreviations: SCC, squamous cell carcinoma; IHC, immunohistochemistry; NS, non-significant; NA, not assessed. Figures in bold type indicates significant results.

Table 13: Immunohistochemical detection of VEGF-C and VEGF-D expression in colorectal cancer

Tumour type	Number of cases	IHC positive (%)	Clinicopathological associations of increased growth factor expression with regard to:				Reference
			Lymphatic invasion	Venous invasion	Lymph node metastasis	Prognosis	
VEGF-C expression							
Colorectal adenocarcinoma	99	56%	***p*<0.01**	NS	***p*<0.01**	NS trend	Akagi et al., 2000
Colorectal adenocarcinoma	59	35%	NS	NA	NS	NA	George et al., 2001b
Advanced colorectal adenocarcinoma	152	46.7% at deepest invasive site	***p*<0.01**	***p*<0.01**	***p*<0.01**	***p*<0.05**	Furodoi et al., 2002
VEGF-D expression							
Colorectal adenocarcinoma	59	31%	NS	NA	NS	NA	George et al., 2001b
Colorectal carcinoma/adenomas	84	100%	NA	NA	***p*=0.02**	***p*=0.037**	White et al., 2002
Colorectal carcinoma	83	31%	NS	NS	***p*<0.01**	p=0.15	Funaki et al., 2003

Abbreviations: IHC, immunohistochemistry; NS, non-significant; NA, not assessed. Figures in bold type indicates significant results.

1.8. SUMMARY AND PROPOSED STUDIES

The relative balance of VEGF family member expression and receptor availability could explain the conflicting associations that are reported for clinicopathological variables, MVD and growth factor expression in colorectal malignancy and offer valuable insights into the potential role for anti-VEGF/anti-VEGFR agents in adjuvant therapy. The hypothesis arises that the VEGF family may influence the natural history of colorectal cancer by affecting angiogenic, lymphangiogenic and autocrine/paracrine pathways, altering the growth and metastatic potential of a tumour.

Areas that are unresolved in the literature include:

- the topographical location and pattern of distribution of VEGF family member/receptors throughout the primary tumour
- the relationship between VEGF family member/receptor expression in the primary site and metastases of colorectal cancer
- evaluation of lymphangiogenesis and lymphatic vessels within colorectal cancer and their quantification
- relationships between primary tumour LVD/MVD, expression of VEGFs and patterns of metastasis

Additional information in these areas may provide insight into the functional roles of the VEGF family in colorectal cancer and tumour biology.

To investigate and clarify the roles played by the VEGF family and lymphangiogenesis in colorectal cancer, studies were designed to determine whether the topographical distribution of the angiogenic and lymphangiogenic VEGF proteins and their receptors

influenced metastatic potential in colorectal cancer. The following investigations were performed:

I – Expression of VEGF family members and receptors in the primary and metastatic sites of colorectal cancer

- To extend and define observations regarding the topographical distribution of VEGF-A, VEGF-C, VEGF-D, VEGFR2, VEGFR3 in primary colorectal cancer specimens and determine patterns of tissue expression.
- To examine the expression of the above growth factors/receptors in metastatic sites (hepatic and lymph node) and explore the relationship between primary and secondary site expression and the potential for prediction of metastatic behaviour from expression at the primary site.
- To determine whether lymphatic vessels are present within and around colorectal cancer and to assess LVD of the primary tumour, using a specific lymphatic vessel marker, LYVE-1.
- To analyse the relationship between primary tumour LVD/MVD and VEGF family member expression and patterns of metastasis.

In addition, it can be hypothesised that circulating VEGF-C and VEGF-D are tumour markers that can identify patients with cancer and potentially identify those with or at risk of lymph node involvement or lymphatic invasion. Studies were designed to investigate whether circulating VEGF-C could be measured and whether circulating VEGF-C and VEGF-D levels held any prognostic significance in colorectal cancer. In order to answer these questions, the following studies were performed:

II – The role of circulating VEGF-C and -D in the prediction of tumour behaviour

- To develop and validate an enzyme-linked immunosorbent assay (ELISA) for the measurement of VEGF-C in body fluids.
- To perform a pilot study to investigate VEGF-C levels in cancer patient and control serum and plasma.
- To determine the clinical potential of the VEGF-C ELISA by performing prospective studies utilising the ELISA, in conjunction with commercially available assays for VEGF-D and VEGF-A, in colorectal cancer patient and control groups.

2. MATERIALS AND METHODS

2.1. IMMUNOHISTOCHEMISTRY

The aims of this section of the study were to:

a) develop immunostaining protocols for formalin fixed, paraffin embedded colorectal tumour specimens, to stain for blood vessel endothelial cells (CD31, CD34, CD105), lymphatic endothelial cells (LYVE-1), tumour-expressed growth factors (VEGF-C, VEGF-D, VEGF-A) and their receptors (VEGFR2/flk-1/KDR and VEGFR3/flt-4).

b) define the tissue distribution pattern and investigate relationships between and differences in patterns of growth factor/receptor expression in primary colorectal adenocarcinomas and their metastases and quantify tumour LVD and its relationship with MVD, growth factor and receptor expression and mode of metastasis.

2.1.1. Development of immunohistochemical methods

Slide preparation

Plain glass slides were prepared by coating with 3-aminopropyl triethoxysilane (APES; Sigma, St Louis, MO). Plain glass slides were degreased in 95% industrial methylated spirit (IMS; Genta Medical, York) for 2 minutes, rinsed three times in tap water, once each in distilled water and 99% IMS. After drying in an incubator at 37°C for one hour, the slides were immersed in a solution of 3% APES in acetone (BDH, Poole) for 2 minutes in a fume cupboard, rinsed in acetone, washed in distilled water and dried in a

50°C incubator overnight. The APES coated slides were stored in slide boxes until required.

Tissue selection and preparation

Formalin-fixed paraffin embedded blocks from 21 cases of colorectal cancer were obtained from the histology archives of Withington Hospital. These cases had previously undergone detailed histopathological staging with intensive pathological examination of lymph nodes as part of a xylene clearance study of pathological staging. Paraffin blocks containing the invading edge of the tumour and when present, involved lymph nodes, were selected following review of haematoxylin and eosin (H & E) stained slides.

The blocks were cooled in a freezer at -20°C for a minimum of one hour prior to sectioning and kept cool in a freezing waterbath during each sectioning session. Five μm sections were cut using a Leitz microtome (Leitz Wetzlar Microtome, Model 1512), floated in a warm water bath and mounted either onto plain glass slides for H & E staining or onto APES coated slides for immunohistochemistry.

The sections for immunohistochemistry were dried in a 37°C incubator for a minimum of 24 hours.

H & E staining

A single slide from each tissue block was stained using haematoxylin (Harris's haematoxylin, RA Lamb Ltd, Eastbourne) and eosin (BDH). The mounted tissue section was dried on a hot plate at 50°C for 30 minutes and stained as follows:

- Dewaxed by immersion in a series of 4 xylene solutions, 2 minutes in each pot, rehydrated through a series of 4 IMS solutions, with a few seconds in each pot and washed in running tap water

- Immersed in haematoxylin for 5 minutes and washed in running tap water until the water ran clear
- Differentiated by dipping 3-4 times into 1% acid/alcohol (1% hydrochloric acid (BDH), 75% IMS) solution and then washed in cold running water then hot running water to 'blue' the slides
- Immersed in eosin for 2 minutes
- Dehydrated back up through the series of IMS solutions and cleared through the series of xylene solutions
- The slides were wiped dry and mounted in a drop of XAM mountant (BDH) which was applied to a glass coverslip and the stained slide placed face down on the coverslip. Air bubbles were expelled with direct pressure and the slides were left to dry in a fume cupboard for a minimum of 30 minutes.

Buffers

Buffer solutions for washing and antigenic retrieval were employed throughout the immunostaining procedures described and can be summarised here:

- Tris buffered saline (TBS) – 0.05M Tris (BDH)/sodium chloride (BDH), pH 7.6
- TBS-Tween 0.01% (Tween 20, Sigma)
- Citrate buffer (trisodium citrate, BDH), 0.01M, adjusted to pH 6.0
- Tris/EDTA buffer, 10mM Tris and 1mM EDTA (BDH), pH 9.0

Antigen retrieval

Tissue sections were obtained from formalin fixed, paraffin embedded specimens. Tissue fixation inactivates endogenous lysosomal activity and bacterial attack in tissue but results in cross-linking of proteins that can mask tissue antigens. Antigen unmasking and retrieval may be necessary prior to antigen immunostaining. Methods of antigen

retrieval that may be utilised include proteolytic enzyme digestion (eg. trypsin or pepsin), microwave antigen recovery (Shi et al., 1991), pressure cooker antigen retrieval and combination methods (eg. microwave and proteolytic digestion).

Where particular methods for the primary antibody were recommended by the manufacturer, these were employed. If no particular treatment method was endorsed, different retrieval methods were evaluated for each specific antibody.

Immunostaining procedures

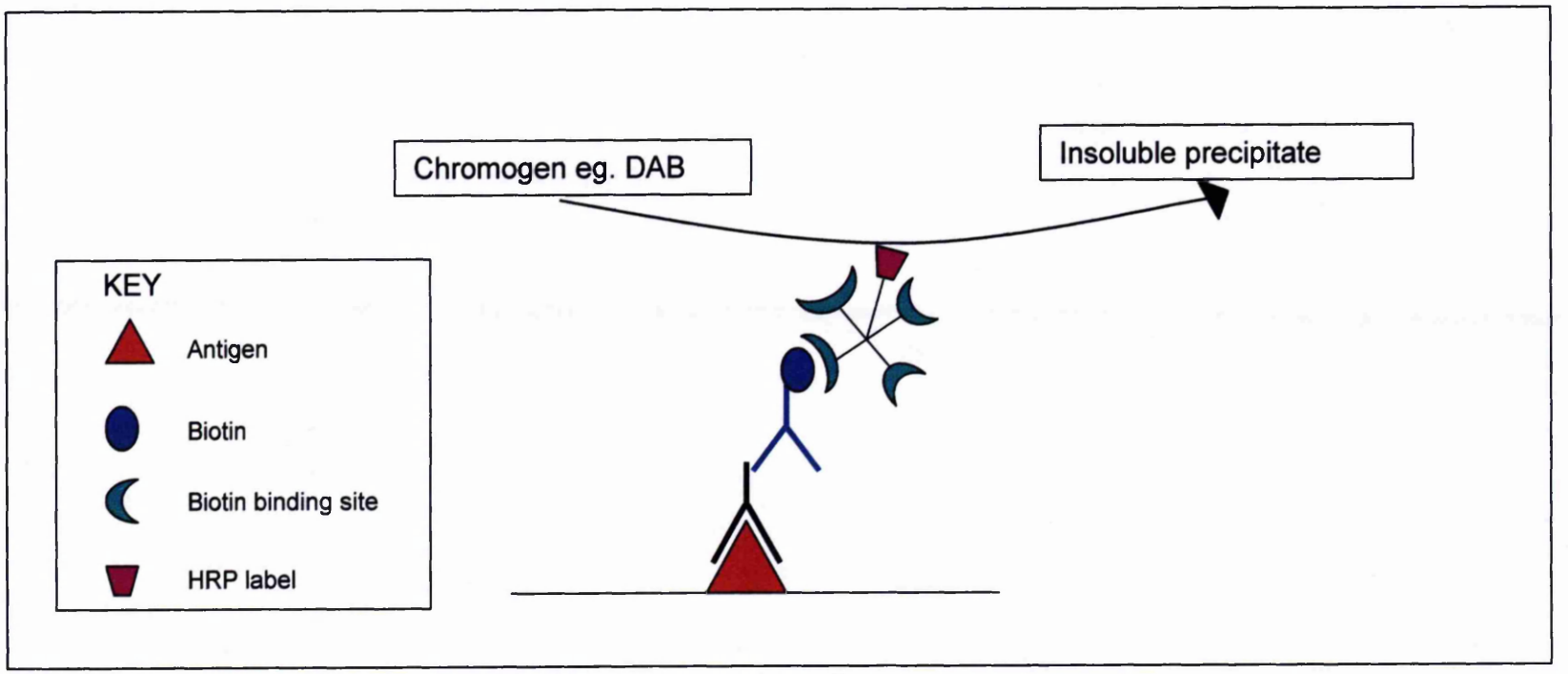
The immunostaining procedures employed in these studies were based on the indirect immunoperoxidase technique, with the sequential application of primary antibody, incubation overnight at 4°C, application of a biotinylated secondary antibody directed against the species of the primary antibody, followed by horseradish peroxidase (HRP) conjugated streptavidin then chromogenic substrate (**Figure 3**). The basic procedure followed was:

- Dewaxed through 4 changes of xylene, for 10 minutes/pot, rehydrated through 4 changes of alcohol, for 2 minutes/pot, then washed briefly three times in tap water and once in distilled water
- Pretreatments for antigen retrieval (if required, see **page 107**), followed by washing three times for 5 minutes in distilled water (if antigen retrieval was performed)
- Blockade of endogenous peroxidase by quenching in 3% hydrogen peroxide (Sigma) solution for 15-30 minutes at room temperature, shaking, followed by washing three times for 5 minutes in distilled water. Endogenous peroxidase has a physiological role in numerous cells and tissues and has the ability to oxidise the chromogen producing unwanted signal. Prior incubation of the tissue section with hydrogen peroxide exhausts or

'quenches' endogenous enzyme activity and prevents such non-specific signal production.

- Serum block with 1:10 normal serum, diluted in TBS, for 15-30 minutes. The serum used was that of the species in whom the secondary antibody for that run was raised. This step was employed to reduce non-specific staining which can result from hydrophobic interaction between proteins, such as tissue protein and primary antibody. Proteins within the blocking serum competitively bind to and block the hydrophobic binding sites on tissue sections.
- Primary antibody application, using primary antibodies diluted in 1:20 normal serum. Slides were incubated in a refrigerator at 4°C overnight then washed in TBS-Tween, three times for 5 minutes.
- Biotinylated secondary antibody application (DAKO, Denmark) at 1:500 dilution in TBS. Incubated at room temperature for 45 minutes, shaking, then washed in TBS-Tween, three times for 5 minutes.
- Application of HRP-streptavidin (DAKO) at 1:500 dilution in TBS. Incubated at room temperature for 45 minutes, shaking then washed in TBS-Tween for 3 times for 5 minutes, then distilled water once for 5 minutes.
- Addition of chromogen, 3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma)
- Finally, washed in distilled water, counterstained lightly with Meyer's haematoxylin (BDH), dehydrated through the alcohol series, cleared through the xylene series and mounted.

Figure 3: The indirect immunoperoxidase technique



Primary antibody directed against the antigen of interest was applied. This was followed by biotinylated secondary antibody, directed against the species in which the primary antibody was raised. HRP-streptavidin was added. The biotin-binding sites of the HRP-streptavidin molecule bind to the biotinylated secondary antibody. Finally, the chromogen was added in its soluble form. The enzymatic action of HRP converted the colourless soluble chromogen to a coloured insoluble precipitate, which was deposited over the antigen of interest, hence highlighting its location in the tissue examined.

Immunostaining for vascular endothelial cells

Three primary antibodies were investigated for immunostaining vascular endothelial cells. The antibodies were monoclonal and directed against the antigens CD31, CD34 and CD105. The aim was to determine the best antibody to achieve maximum endothelial cell detection with clean, specific immunostaining in paraffin embedded colorectal cancer tissues. CD31 is a 130 kD glycoprotein located on the surface of endothelial cells, platelets, monocytes and granulocytes that acts as a surface adhesion molecule. CD34 is a 110kD glycosylated protein present in endothelial and lymphopoietic cells. Different studies have recommended both of these endothelial markers for use in determining MVD in colorectal cancer (see **section 1.2.2, page 32**). CD105 is a 180 kD protein preferentially expressed on angiogenic endothelial cells (reviewed in Duff et al., 2003a). It has been suggested that determination of MVD using CD105 immunostaining predicts prognosis better than that obtained with conventional antibodies (Wang et al., 1994a; Brewer et al., 2000; Tanaka et al., 2001; Akagi et al., 2002), although this may rely on the specific anti-CD105 antibody used for immunostaining (Balza et al., 2001).

a) Immunostaining for CD31

The primary antibody used was a mouse anti-human monoclonal antibody (clone JC/70; DAKO) (Parums et al., 1990).

An indirect immunostaining procedure was followed as outlined above (**page 108 & Figure 3**). Pretreatment of slides for antigen retrieval was performed using heating in a microwave (Matsui M196T) in Tris/EDTA buffer, pH 9.0 for 5 minutes (as recommended by the manufacturer) at high power. Endogenous peroxidase was blocked for 30 minutes, 1:10 goat serum was used as a blocking agent for 30 minutes and biotinylated goat antimouse IgG (DAKO) employed as the secondary antibody.

Negative control slides were incubated with equivalent concentration of irrelevant mouse IgG in place of primary antibody and with omission of primary antibody. Antibody dilution runs determined the optimum dilution of the primary antibody to be 1:100 (4.5μg/ml).

b) Immunostaining for CD34

The primary antibody used was a mouse monoclonal antibody, clone QBEnd/10 (DAKO). Pretreatment of tissue sections for antigen retrieval was recommended by the manufacturer but no specific method endorsed.

Indirect immunostaining procedures were followed as outlined above. The optimal antigen retrieval method was determined by testing various methods at a primary antibody concentration of 1:25 (1.6μg/ml) (**Table 14**).

Table 14: Pre-treatment methods evaluated for CD34 immunostaining

	Pre-treatment method	**H202 block (minutes)**	**Blocking agent**	**Primary antibody (overnight at 4°C)**
1	None	30	1:10 goat serum	Omission of primary antibody
2	None	30	1:10 goat serum	Diluted in 1:20 goat serum
3	10mmol citrate buffer, 1 x 5 min, high power	30	1:10 goat serum	Diluted in 1:20 goat serum
4	10mmol citrate buffer, 2 x 5min, high power	30	1:10 goat serum	Diluted in 1:20 goat serum
5	10mmol Tris/1mmol EDTA buffer, 1 x 5 min, pH 9, high power	30	1:10 goat serum	Diluted in 1:20 goat serum
6	Trypsinisation, 37°C, 15min (1mg/ml, Sigma)	30	1:10 goat serum	Diluted in 1:20 goat serum

Optimisation of immunostaining was achieved using Tris/EDTA buffer, pH 9.0 and microwave pre-treatment for 5 minutes (5^{th} method in **Table 14**). Negative control

slides were incubated with equivalent concentration of irrelevant mouse IgG in place of primary antibody and with omission of primary antibody. The optimal antibody titration was achieved with a dilution of 1:50 (0.8μg/ml).

c) Immunostaining for CD105

The primary antibody used was a mouse monoclonal antibody, SN6h (DAKO).

Initial staining runs employed an indirect immunohistochemical technique and four different pre-treatment methods at a primary antibody concentration of 1:10 as recommended by the manufacturer (**Table 15**).

Table 15: Pre-treatment methods evaluated for CD105 immunostaining

Pretreatment	**H2O2 block (minutes)**	**Serum block**
No pre-treatment	30	1:10 goat serum
Trypsinisation - 15 minutes at 37°C	30	1:10 goat serum
Citrate buffer, pH 6.0 – 2 x 5 minutes, high power	30	1:10 goat serum
Tris/EDTA buffer, pH 9.0 – 1 x 5 minutes, high power	30	1:10 goat serum

Lack of endothelial cell staining for CD105 using any of the above pre-treatment protocols led to the use of two signal amplification techniques to enhance specific staining for CD105; the Envision System and the Catalysed Signal Amplification System (CSA) (DAKO).

The Envision System is an indirect method of immunostaining, utilising a secondary antibody conjugated to a polymer backbone to which many enzyme (HRP) moieties are attached. Slides to be stained with the Envision System were pretreated with citrate buffer, pH 6.0 in a microwave for 2 x 5 minutes at high power. The SN6h antibody dilutions used were 1:25 and 1:50.

The CSA system is an indirect method of immunostaining using biotinyl tyramide to enhance the signal. No pre-treatment of sections is necessary and much higher antibody dilutions can be used. Varying antibody dilutions from 1:100 –1:2000 were employed and a dilution of 1:500 was selected as optimum.

In both cases negative controls used nonimmune mouse IgG at the equivalent concentration to the lower antibody dilution. In both cases using signal enhancement, commercial protein blocking and antibody diluent solutions were utilised (DAKO).

Immunostaining for lymphatic endothelial cells

The primary antibody used for lymphatic endothelial cell staining was rabbit anti-human LYVE-1 IgG (a gift from Dr D Jackson, Oxford). This was a polyclonal affinity purified antibody raised against an IgG_1 fusion protein of the human lymph vessel endothelial hyaluronan receptor, LYVE-1. This antibody has been demonstrated to be lymphatic endothelial cell specific (see **section 1.6.1, page 82** and Jackson et al., 2001). An indirect immunostaining procedure was followed using the Envision detection system (DAKO). Antigen retrieval was performed by heating in a microwave in 0.01M citrate buffer, pH 6.0, at medium power for 2 x 5 minutes. Endogenous peroxidase was blocked for 30 minutes, 1:10 swine serum used as a blocking agent (for 30 minutes) and the secondary antibody of the Envision kit employed. Negative control slides were incubated with equivalent concentrations of irrelevant rabbit IgG in place of primary antibody. Antibody dilution runs determined the optimum dilution of the primary antibody to be 1:60 (7μg/ml).

Immunostaining for growth factors

Immunohistochemical protocols were developed to stain for VEGF-A, VEGF-C and VEGF-D.

a) Immunostaining for VEGF-A

The primary antibody used was an affinity-purified polyclonal rabbit anti-human VEGF-A IgG (Santa Cruz), raised against a peptide mapping at the amino terminus of VEGF-A of human origin. It was reactive with the 165, 189 and 121 amino acid splice variants of VEGF-A.

Indirect immunostaining was performed using 1:10 swine serum as blocking agent, 1:20 swine serum as antibody diluent and biotinylated swine antirabbit IgG (DAKO) as the secondary antibody. Positive tissue control was skin and internal positive control was tissue macrophages. Negative controls used irrelevant rabbit IgG at equivalent concentration to the primary antibody. The primary antibody was used at a dilution of 1:400 (0.5μg/ml).

b) Immunostaining for VEGF-C

The following three different primary polyclonal antibodies were assessed for VEGF-C staining:

- goat anti-human VEGF-C IgG, (SC-1881, Santa Cruz), an affinity-purified antibody raised against a peptide mapping at the carboxy terminus of VEGF-C of human origin (amino acid residues 135-155).
- goat anti-human VEGF-C IgG, (SC-7133, Santa Cruz), an affinity-purified antibody raised against a peptide mapping at the amino terminus of the precursor form of VEGF-C of human origin

- rabbit anti-human VEGF-C IgG (18-2255, Zymed, South San Francisco, CA), an affinity-purified antibody raised against a peptide mapping to the carboxy terminus of human VEGF-C.

Problems with immunostaining were encountered with both goat antibodies (see **section 3.1.3, page 142**), hence, the Zymed rabbit anti-human VEGF-C IgG was used for definitive immunostaining. Positive tissue controls were normal colonic epithelium, negative tissue controls included muscle and skin. Procedural controls included the omission of primary antibody and use of irrelevant rabbit IgG at equivalent antibody concentration. Indirect immunostaining was performed as outlined above. Pre-treatment of the slides for antigen retrieval was performed by microwave heating in citrate buffer pH 6.0 for 2 x 5 minutes at medium power, according to the manufacturers' recommendation. Endogenous peroxidase was blocked for 30 minutes. Serum blocking used 1:10 normal swine serum and biotinylated swine antirabbit IgG (DAKO) was used as the secondary antibody.

Antibody titration studies determined the optimal dilution of primary antibody as 1:50 (1μg/ml).

c) Immunostaining for VEGF-D

The primary antibody employed was mouse monoclonal anti-human VEGF-D IgG (R & D Systems), produced from a murine hybridoma elicited from a mouse immunized with purified, NS0-derived, recombinant human VEGF-D.

Indirect immunostaining was performed using 1:10 goat serum as blocking agent, 1:20 goat serum as antibody diluent and biotinylated goat antimouse IgG (DAKO) as the secondary antibody. Positive tissue control was skin and internal positive control was the normal colonic mucosa. Negative controls used irrelevant mouse IgG as the primary antibody. The primary antibody was used at a dilution of 1:500 (2μg/ml).

Immunostaining for VEGF receptors

a) VEGFR3 (flt-4)

Two polyclonal antibodies directed against VEGFR3 were assessed for immunostaining:

- goat anti-human VEGFR3 IgG (R & D Systems, Abingdon), an affinity-purified antibody, raised against purified, NS0-derived, recombinant human VEGFR3 extracellular domain
- rabbit anti-human VEGFR3 IgG (sc-321: Santa Cruz), an affinity-purified rabbit antibody raised against a peptide mapping at the carboxy terminus of VEGFR3 of human origin

Initial immunostaining used the goat anti-human VEGFR3 antibody (R & D Systems) with antigen retrieval by microwave heating for 2 x 5 minutes in citrate buffer, pH 6.0 at medium power. Rabbit serum at a dilution of 1:10 was used for serum blocking and commercial antibody diluent (DAKO) used to dilute the primary antibody to 1:6.66 as recommended by the manufacturer (15μg/ml). Negative control slides used irrelevant goat IgG at equivalent concentration to the primary antibody and omission of primary antibody.

Due to problems with background staining and negative control section staining (see **section 3.1.4, page 147**), an alternative anti-human VEGFR3, rabbit anti-human VEGFR3 IgG (Santa Cruz) was evaluated. Indirect immunostaining was performed using 1:10 swine serum as a blocking agent, 1:20 swine serum as the primary antibody diluent and biotinylated swine antirabbit IgG (DAKO) as the secondary antibody. Slides were stained with and without pre-treatment using either trypsin for 15 minutes at 37°C or microwave antigen retrieval using citrate buffer, pH 6.0 at medium power for 2 x 5

minutes. The primary antibody concentration used was 5μg/ml. Negative controls used nonimmune rabbit IgG at equivalent concentration.

b) VEGFR2

The primary antibody used was mouse monoclonal anti-VEGFR2 (Santa Cruz), an IgG_1 antibody raised against a recombinant protein corresponding to amino acids 1158-1345 mapping at the carboxy terminus of Flk-1 of mouse origin. It was reactive with Flk-1 of mouse, rat and human origin.

Indirect immunostaining was performed employing 1:10 goat serum as blocking agent, 1:20 goat serum as the antibody diluent and biotinylated goat antimouse IgG (DAKO) as the secondary antibody. Antigen retrieval was recommended by the manufacturer but no specific method endorsed. Various pre-treatment methods were assessed, including microwave treatment in citrate buffer, pH 6.0 and trypsinisation. Best results were achieved by pre-treatment with microwave heating in citrate buffer, pH 6.0 at medium power for 2 x 5 minutes. The primary antibody was used at a dilution of 1:50 (4μg/ml). Negative control slides used irrelevant mouse IgG at equivalent concentration to the primary antibody.

In order to confirm the results obtained with the above antibody, another antibody [a polyclonal rabbit anti-VEGFR2 antibody (Abcam, Cambridge, UK)] was used. This antibody is raised against a synthetic peptide derived from the C-terminus of the precursor form of mouse VEGFR2 and cross-reacts with human VEGFR2. Indirect immunostaining was performed using 1:10 swine serum as blocking agent, 1:20 swine serum as antibody diluent and biotinylated swine antirabbit IgG as the secondary antibody. Antigen retrieval by microwave heating in a citrate buffer, pH 6.0 at medium power for 2 x 5 minutes was performed as recommended by the manufacturer. The primary antibody was used at a dilution of 1:50 and negative control slides used

irrelevant rabbit IgG at a similar dilution. A section of human placenta was used as a positive tissue control.

Double staining for VEGF-C and VEGFR2

To demonstrate the relative locations of two different antigens, a double immunoperoxidase technique was utilised. This technique relied on the sequential application of a primary/secondary antibody combination, followed by HRP-streptavidin and chromogen, followed by a second primary and secondary antibody, then HRP-streptavidin and an alternative chromogen of different colour. Here, the methods used for immunostaining VEGF-C using rabbit polyclonal IgG were followed with DAB as the chromogen which was followed by the addition of monoclonal anti-VEGFR2 IgG as the second primary antibody as described above. The second chromogen used was Vector® Very Intense Purple (VIP; Vector Laboratories, Burlingcame, CA) which produces a purple colour. Light counterstaining was performed with methyl green (Vector) at 37°C for a few seconds.

Four consecutive sections of colorectal tumours were stained: as a negative control, for VEGF-C alone (DAB as chromogen), for VEGFR2 alone (VIP as chromogen) and for VEGF-C/VEGFR2 (both DAB and VIP).

2.1.2. Primary colorectal cancer and metastases immunostaining study

Tissues from patients

Specimens from two cohorts of patients were studied:

a) 21 colorectal carcinoma cases (see **section 2.1.1, page 106**): primary tumours (n=21) and their lymphatic metastases (n=9)

b) 9 matched pairs of primary colorectal cancer specimens, involved lymph nodes (n=3) and their subsequent resected hepatic metastases (n=9). (These patients were the

subjects of a study approved previously by the North Manchester Ethics Committee). For this study, informed consent was sought from those subjects still alive at the time of the study as requested by the Ethics Committee. Representative formalin fixed, paraffin embedded tissue blocks of the primary colorectal cancer specimen, any involved lymph nodes and the subsequently resected hepatic metastases were obtained from the departments of pathology at North Manchester General Hospital and the original referring hospitals. The reported pathological findings were confirmed by a specialist consultant colorectal pathologist.

Clinicopathological details were available for both sets of patients.

Immunostaining

Sections of the tissue blocks were cut and mounted for H & E staining or immunohistochemistry (see **section 2.1.1, page 105**). Immunostaining was performed for CD34, CD31, CD105, VEGF-A, VEGF-C, VEGF-D, VEGFR3 and VEGFR2. Each individual antibody run was performed under optimised conditions, at a single sitting, for all the tumour samples in each set (primary tumours/lymph node metastases or primary tumours/liver metastases/lymph node metastases). Adjacent sections were used for different runs and all staining runs included previously defined positive and negative controls.

Immunostaining runs for liver metastases included the addition of biotin blockade in the staining protocols in order to block endogenous biotin and diminish non-specific background staining. This was performed using a Biotin Blocking Kit (DAKO), between the endogenous peroxidase block and serum blocking steps.

The LYVE-1 staining was performed for all the primary tumours in both sets in a single staining run. No biotin blockade was required as the Envision system used for detection eliminated the need for a biotinylated secondary antibody.

Scoring

Slides were reviewed independently by two reviewers blinded to clinical details.

Intensity of tissue staining was scored on a semi-quantitative scale from 0-5 (0: no stain; 5: strongest stain). Assessment was made at different areas of the tissue, including the normal colonic mucosa (N), mucosa at the junction between normal and malignant tissue (J), superficial tumour (TS), central tumour (TC) and tumour at the invading edge (TI).

The percentage of cells staining at particular intensities was determined for the liver metastases and the majority percentage determined the score given for the intensity of liver metastasis staining.

The presence or absence of LYVE-1 positive vessels in the superficial, central and deep areas of the tumour was assessed on an all or none basis.

In the event of discrepancies between the scorers, the slides were reviewed and scoring agreed by consensus.

Counting vessels

Both blood and lymphatic vessels were counted in the same way. Sets of slides were counted independently using a Chalkley point grid on two separate occasions with the assessor blinded to clinical details (Chalkley, 1943; Fox et al., 1995). The slide was scanned at low power x40 (x10 ocular, x4 objective) to identify four separate vascular hotspots within the tumour itself or in its immediate vicinity. Identification of vessels was performed using the method specified by Weidner (1991), in which 'any brown staining endothelial cell or cell cluster that was clearly separate from adjacent microvessels, tumour cells and other connective tissue elements was considered a single, countable microvessel'. All positively stained endothelial cells in contact with

the points on the Chalkley grid were counted. Vessels were counted at x200 (x10 ocular, x20 objective). Four x200 fields were scored and the results averaged to give a mean score. Scoring was repeated for the whole set of slides at a different sitting to quantify the reproducibility of the method and the mean score of the two counts used for analysis.

Statistical analysis

Advice on statistical analysis was taken from Dr D. Ryder, Medical Statistics Department, Christie Hospital. All statistical analyses were performed using the Statistics Package for the Social Sciences (SPSS) Version 10.1 (SPSS Inc, Chicago, Illinois). Non-parametric statistical tests were employed. Differences in median location were assessed with Mann-Whitney U tests for two independent groups and Kruskall-Wallis tests for more than two groups. Repeated measures of related variables within individuals were compared with the Wilcoxon signed rank test (for 2 variables) and the Friedman test (for greater than 2 variables). Correlations between variables were examined with Spearman's rank correlation coefficients. A p value of less than 0.05 was taken as significant and all statistical tests were two-sided.

2.2. IMMUNOASSAYS

2.2.1. Development of indirect ELISA for VEGF-C

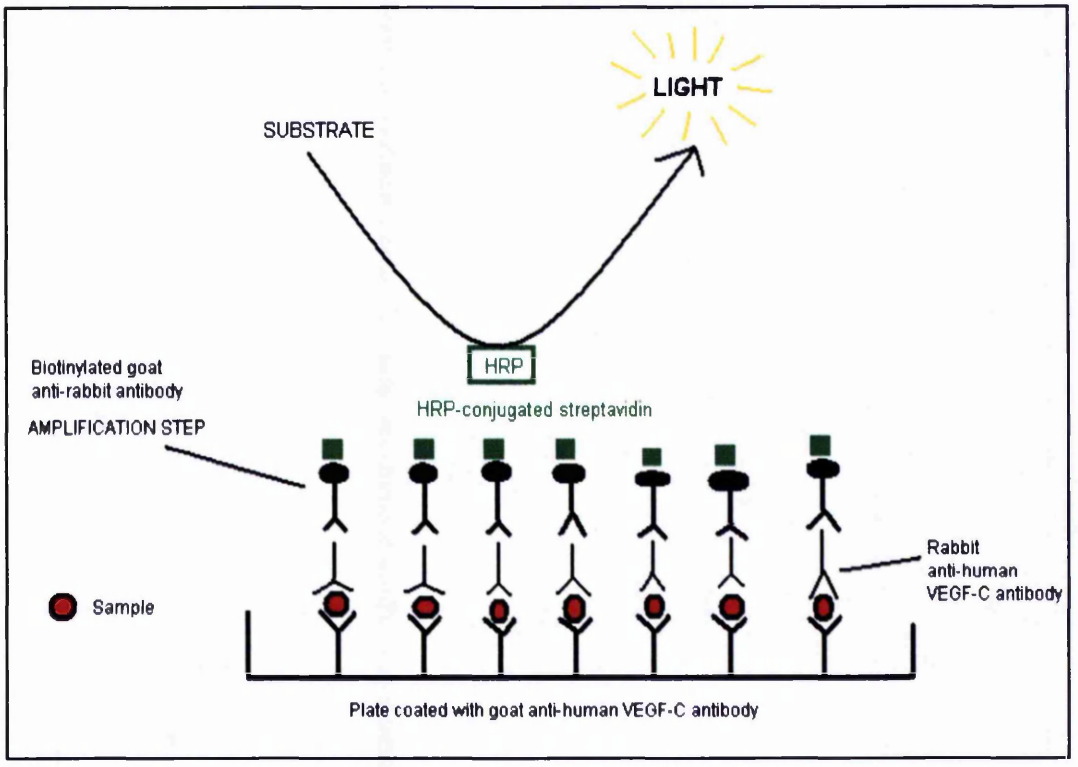
Since VEGF-A and VEGF-D in the circulation can be detected using immunoassays, it seemed reasonable to postulate that VEGF-C could also be quantified by an ELISA system.

VEGF-C present in plasma and serum samples was captured using goat polyclonal anti-human VEGF-C antibody (R & D Systems, Abingdon, UK). This antibody is produced in goats immunized with purified, *E.coli*-derived, recombinant human VEGF-C (rhVEGF-C) peptide, corresponding to amino acid residues 104 to 330. The detection antibody used was rabbit polyclonal anti-human VEGF-C antibody (Zymed Laboratories Inc, CA), raised against a synthetic peptide corresponding to the carboxyl terminal of human VEGF-C. A standard curve could not be created on the ELISA plate using the aforementioned rhVEGF-C (R & D Systems) as this peptide was not recognised by the detection antibody (see **section 4.1.4, page 187**), consequently a known human plasma with high levels of VEGF-C was used to form a standard curve.

Ninety-six well white microtiter plates (Dynex Technologies, Worthing, UK) were coated with 100µl/well goat anti-human VEGF-C antibody (1µg/ml in 0.1M carbonate buffer, pH 9.6). The coated plates were incubated overnight in a humidified chamber at 4°C then blocked with 100µl/well of 1% BSA (w/v) in 0.1M PBS and 0.1% Tween 20 (Sigma-Aldrich Ltd; PBS-Tween) for three hours at room temperature. Test samples diluted 1 in 2 in PBS-Tween were added to the plates in duplicate. Plasma taken from a patient with a multisystem autoimmune disorder known to have a high level of VEGF-C by immunoprecipitation (see **sections 2.2.3, page 125 & 4.1.4, page 187**) was serially diluted to generate a standard curve on each plate. The concentration of VEGF-C was defined as 100 units/ml in the standard plasma. After overnight incubation in a humidified chamber at 4°C, 100µl/well of rabbit anti-human VEGF-C antibody was added at a final concentration of 0.5µg/ml diluted in PBS-Tween and incubated at 4°C for three hours. Amplification of the signal was achieved by the addition of 100µl/well of biotinylated goat-antirabbit IgG (DAKO Ltd, Cambridgeshire, UK) at 1/1000 dilution in 1% BSA (w/v) and PBS-Tween, and incubated with shaking at room temperature for 1 hour. Horseradish peroxidase (HRP)-streptavidin (DAKO) was

diluted at 1/2000 in 1% BSA (w/v) and PBS-Tween and 100µl/well added. The plates were incubated with shaking at room temperature for 30 minutes. Three washes with PBS-Tween were carried out between each of the procedures. Finally, 100µl/well signal reagent (Orthoclinical Diagnostics, Bucks, UK) was added and the light emission immediately measured at 420nm in a plate reader (Kodak Clinical Diagnostics, Aylesbury, UK) (**Figure 4**).

Figure 4: ELISA for the detection of circulating human VEGF-C



2.2.2. Validation of indirect ELISA for VEGF-C

Intra- and inter-assay variation

To evaluate the reproducibility of the assay, the intra-assay coefficient of variation (CV) was measured using the same quantity of standard plasma in 22 wells on a single plate and the inter-assay CV measured using the same quantity of standard plasma across 8

separate experiments. The CV values were calculated by dividing the standard deviation by the mean and multiplying by 100. All other steps in the assay were the same as described above.

Specificity

The specificity of the system was determined by the substitution of the standard plasma for known concentrations of recombinant VEGF-A, VEGF-D, TGF-β1, TGF-β3 and IL-6 (all from R & D Systems) to assess for any evidence of cross-reactivity.

Specificity was further verified by substitution of the capture antibody with alternative antibodies (all monoclonal antibodies directed against human TGF-β1, TGF-β3, CD105, CD31 and CD34), and addition of standard plasma and detection antibody as detailed above.

2.2.3. Immunoprecipitation and Western blotting of VEGF-C

To confirm the presence and reveal the molecular forms of VEGF-C present in the standard plasma and validate its use to create a standard curve, immunoprecipitation and immunoblotting were performed. To eliminate non-specific binding of plasma proteins, the plasma sample was pre-cleared with Protein L-agarose (Santa Cruz Biotechnology Inc, Santa Cruz, CA) by mixing 1/10 diluted standard plasma (2μl plasma plus 18μl PBS) with 10μl Protein L-agarose at 4°C for 4 hours. The beads were pelleted by centrifugation for 5 minutes at 1000*g* and the supernatant collected. VEGF-C was specifically precipitated from the pre-cleared supernatant with goat anti-human VEGF-C antibody coupled to Protein L-agarose. Goat anti-human immunoglobulins (Sigma-Aldrich Ltd) coupled to Protein L-agarose were used as a negative control. The pre-cleared plasma samples were made up to 1ml with PBS, 10μl of protease inhibitor cocktail (Calbiochem Biosciences UK, Nottingham, UK) was added followed by 10μg

goat anti-human VEGF-C antibody or negative control antibody. Following one hour of rotation at 4°C, 10µl Protein L-agarose was added and incubation continued overnight. Immunoprecipitates were isolated by centrifugation at 1000*g* for 5 minutes, 500µl NET buffer (50mM Tris-HCl, 150mM sodium chloride, 0.5M EDTA (Sigma-Aldrich), 1% Igepal CA-630 (Sigma-Aldrich)) and 10µl of protease inhibitor cocktail were added and the mixture centrifuged again. This process was repeated twice and finally, the bead pellet was washed with PBS, centrifuged, the supernatant discarded and the beads dried. Reducing sample buffer [0.5M Tris-HCl, pH 6.8, 10% SDS, 0.05% bromophenol blue in 20% glycerol solution (Sigma) in distilled water] was added to the tube, which was boiled for 10 minutes followed by cooling on ice, briefly centrifuged and loaded into a 4-10% SDS-PAGE gel and electrophoresed at 100V for 90 minutes at room temperature. Electrophoretic transfer onto a PVDF membrane (Hybond-C Super, Amersham) at 100V followed for one hour. Molecular weight markers were run on the gel to aid detection of the molecular weights of the species observed (BioRad, Hercules, CA). Filters were washed with PBS-Tween and blocked with 4% Marvel-PBS-Tween for 2 hours at room temperature. To detect VEGF-C, the filters were divided and incubated overnight at 4°C with goat anti-human VEGF-C antibody at 1/1000 dilution or rabbit anti-human VEGF-C antibody at 1/500 dilution in blocking solution. Finally, the blots were incubated with rabbit anti-goat antibody (1/1000) or mouse anti-rabbit antibody (1/1000) conjugated with HRP (both from DAKO), for 2 hours at 4°C. The precipitated VEGF-C was detected using an enhanced chemiluminescence (ECL) system (Orthoclinical Diagnostics, Amersham, UK).

2.2.4. Evaluation of ELISA in human blood samples

Plasma and serum of healthy controls

VEGF-C was measured in serum (n=40) and plasma (n=31) of healthy volunteer staff members of the University of Manchester (19 paired serum and plasma samples). Blood samples were collected in EDTA or serum separator Vacutainer® (Becton Dickinson, Oxford, UK) tubes, plasma was harvested following centrifugation for 5 minutes at 1000*g* at 4°C, serum tubes were allowed to clot for 20-30 minutes at room temperature, then centrifuged for 5 minutes at 1000*g* at 4°C and the serum separated. Plasma and serum samples were aliquoted and stored at -80°C until required.

Plasma of colorectal cancer patients and controls

VEGF-C was measured in the plasma from 31 normal controls (described above) and 41 patients with colorectal cancer. Patients with colorectal cancer were diagnosed histologically, blood samples, taken prior to resectional surgery were collected into an EDTA Vacutainer® bottle and plasma was harvested following centrifugation for 5 minutes at 1000*g* at 4°C. Plasma samples were aliquoted and stored at -80°C until required. The samples from colorectal cancer patients had been stored frozen for a period of at least 2 years from the time of sampling. Blood samples were taken with the permission of the South Manchester Research Ethics Committee (Reference No:SOU/00/001).

Statistical analysis

All statistical calculations were carried out using SPSS 10.1 statistical software. Unless specified, the data were expressed as median and range. The distribution of the data was examined and parametric and non-parametric tests applied as appropriate. The control group data were normally distributed, so parametric tests were applied for initial

evaluation. Paired and independent sample two tailed t-tests were used to examine differences within and between groups of controls. The combined data for VEGF-C levels in the cancer patients and controls was not normally distributed, which necessitated the use of the non-parametric Mann-Whitney U and Kruskall-Wallis tests to examine the differences between groups of cancer patients and controls. A level of $p<0.05$ was accepted as statistically significant.

2.3. IMMUNOASSAYS IN COLORECTAL CANCER

To investigate whether circulating levels of VEGF-C and VEGF-D could provide clinical information and act as tumour markers predicting advanced disease and risk of developing lymphatic involvement, based on initial promising results from the pilot study described above (**section 2.2.4, page 127 & section 4.1.6, page 190**), a prospective study to measure VEGF-C, VEGF-D and VEGF-A levels in colorectal cancer patients and controls was designed.

The ELISA for the detection of VEGF-C described above and measurement of VEGF-D and VEGF-A was evaluated in two cohorts of colorectal cancer patients with respective controls to assess their clinical utility. The patient cohorts comprised:

a) a large cohort of pre-operative colorectal cancer patients and controls, in whom assessment was made of the use of the immunoassays in the prediction of clinicopathological factors and

b) a cohort of patients undergoing liver resection for colorectal cancer metastases and controls undergoing laparotomy for non-malignant conditions. This phase of the study examined the pre-operative, peri-operative and post-operative levels of plasma growth factor profiles in the malignant and non-malignant disease groups and changes of

growth factor levels with time following surgery. This phase of the study also investigated whether plasma levels of growth factors were reflected in the expression of the same proteins in the resected tumour metastases (**section 2.1.2, page 119**).

2.3.1. Pre-operative colorectal cancer patients and controls

Based on the preliminary data obtained from the initial evaluation study detailed above (**section 2.2.4, page 127 & section 4.1.6, page 190**), a sample size calculation was made to allow an 80% power of detecting a difference of 0.1 in the $\log_{10}$ mean VEGF-C level, between early and advanced cancer patients (see **section 4.1.7, page 195**). This required the recruitment of 120 colorectal cancer patients and 50 controls.

Recruitment

Colorectal cancer patients were recruited at the Christie Hospital and South Manchester University Hospitals NHS Trusts. Patients at the Christie Hospital were those with histologically diagnosed rectal cancer undergoing pre-operative radiotherapy. Recruitment was made at the time of attendance for a planning CT scan. Patients at South Manchester Hospital were those with histologically confirmed colorectal cancer admitted for cancer resection surgery. Patients were given an information sheet and written informed consent obtained. Inclusion and exclusion criteria were as detailed in **Table 16**.

Table 16: Inclusion and exclusion criteria for cytokine study

Inclusion criteria	Exclusion criteria
Histologically confirmed colorectal cancer	Previous cancer within last 5 years
No previous radiotherapy	Age less than 18 years
No previous chemotherapy	Unable to give written informed consent
Performance status 0-2	
Able to give written informed consent	

Control patients were recruited from those undergoing flexible sigmoidoscopic or colonoscopic evaluation of the lower gastrointestinal tract for investigation of symptoms, family history of bowel cancer or surveillance for and polyp follow-up. All patients had an endoscopic examination clear of cancer, polyps or inflammatory bowel disease.

Written informed consent was obtained for the study which had been approved by South Manchester Ethics Committee (LREC Ref: 02/SM/128).

Data collection

The age and gender of the control patients were recorded. For the cancer patients, additional details were collected including haematological variables (pre-operative platelet counts and CEA level) and pathological information, in particular:

- Dukes' stage
- tumour size
- tumour differentiation
- presence of lymphatic invasion
- presence of venous invasion
- presence of lymph node metastases
- number of lymph nodes identified and number of positive lymph nodes

- presence of marginal involvement
- TNM stage

All patient details were recorded on a case record form (CRF) and into an anonymised computer database (Excel, Microsoft).

Sample collection and processing

Venous blood was taken from control patients prior to their lower gastrointestinal endoscopic examination and from cancer patients prior to their surgery or planning CT scan. Blood was collected, processed and stored as previously described.

Plasma samples were assayed in the ELISA for VEGF-C according to the method described (**section 2.2.1, page 122**). Plasma samples were assayed by ELISA for VEGF-D and VEGF-A using Quantikine® ELISA kits (R & D Systems, Inc., Minneapolis, USA) according to the manufacturer's instructions. All samples were assayed in duplicate. These ELISA kits use the quantitative sandwich enzyme immunoassay technique and are colorimetric in nature.

The VEGF-D Quantikine ® ELISA kit (catalogue number DVED00) uses a monoclonal antibody specific for VEGF-D pre-coated onto a microplate. Standards and samples are added to the wells and any VEGF-D present is bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked monoclonal antibody specific for VEGF-D is added to the wells. After further washing, a substrate solution is added to the wells and colour develops in proportion to the amount of bound VEGF-D. The colour development is stopped and the intensity of the colour measured in a microplate reader at 450nm, with a correction wavelength of 540 or 570nm. The standard contained in the kit is *Sf*21-expressed, recombinant human VEGF-D against which the monoclonal antibodies are raised. The intra-assay variation is reported by the

manufacturer as a maximum of 6.2% and the inter-assay variation as a maximum of 8.0%. The mean minimum detectable amount of VEGF-D varies from 4.7-31.3 pg/ml.

The VEGF-A Quantikine ® ELISA kit (catalogue number DVE00) is similar to that for VEGF-D. It utilises *Sf*21-expressed, recombinant human $VEGF_{165}$ as a standard and antibodies raised against it. The capture antibody pre-coated onto the microplate is a specific anti-$VEGF_{165}$ monoclonal antibody and detection is by a polyclonal anti-$VEGF_{165}$ antibody. The intra-assay variation is reported by the manufacturer as a maximum of 6.7% and the inter-assay variation as a maximum of 8.8%. The minimum detectable amount of VEGF-A in plasma and serum is typically less than 9.0 pg/ml. No significant cross-reactivity or interference is seen with either ELISA kit for multiple related and unrelated recombinant cytokines.

Statistical analysis

Derivation of the values of VEGF-C, VEGF-D and VEGF-A in the samples was made using the standard curve created on each ELISA plate (for VEGF-C, **section 4.1.3, page 185 & Figures 37-38**). For VEGF-D and VEGF-A, the duplicate values for each standard sample were averaged and the value of the well containing no cytokine subtracted to correct for background. Linear logistic regression analysis was performed using SPSS 10.1 computer software on the logarithmically transformed standard concentrations and corrected optical density values obtained, to calculate the equation of a regression line (see **section 4.2.1, page 200 & Figure 43**). This equation was then used by interpolation to calculate the concentrations of the test samples corrected for background (by subtraction of the value of the zero pg/ml well as above).

Statistical analyses were performed using the χ^2 test for categorical variables. Assessment of normality of distribution of data was performed with the Kolmogorov-Smirnov test. The levels of growth factors were not normally distributed, so further data

analysis was performed using the Mann-Whitney U test to compare medians between two independent groups and the Kruskal-Wallis test to compare the medians of greater than two groups. A p value of less than 0.05 was considered significant and all tests were two-tailed.

2.3.2. Hepatic resection and control laparotomy patients

Recruitment

Patients were those who had participated in a previous study at North Manchester General Hospital, entitled 'Impact of laparotomy and liver resection on serum and peritoneal fluid concentrations of basic fibroblast growth factor, vascular endothelial growth factor and hepatocyte growth factor' (see also **section 2.1.2, page 119**). The continuation/extension of the study was approved by the North Manchester Ethics Committee. Ten patients undergoing resectional surgery for colorectal cancer hepatic metastasis were recruited. All patients had confirmation of their diagnosis by pre-operative imaging. 10 patients undergoing laparotomy for non-malignant conditions were recruited. All patients were given a study information sheet and signed a consent form.

Data collection

Patient demographics, operative details, histological details and follow-up were recorded on a case record form.

Sample collection and processing

Venous blood was taken from subjects into an EDTA Vacutainer bottle pre-operatively, intra-operatively and 1, 6 12, 24, 48, 96 hours and 7 days post-operatively. The blood

was transported on ice, centrifuged at 4°C at 1000*g* for 5 minutes and the plasma separated and stored at -80°C until analysis.

Plasma samples from the seven time points were assayed for VEGF-C levels as described above (**section 2.2.1, page 122**).

Analysis of plasma samples from the pre-operative and seven day post-operative time points were assayed for VEGF-D and VEGF-A concentrations using Quantikine® ELISA Kits as detailed above.

Statistical analysis

Calculation of VEGF-C, VEGF-D and VEGF-A values were made from the standard curve on each ELISA plate as described.

Statistical analyses were performed using χ^2 and Fisher's exact test for categorical variables where appropriate. Time series data for VEGF-C were normalised to the pre-operative value to examine changes in levels with time free of interference from different individual baseline levels. Data were analysed with two-tailed non-parametric tests and a p value of <0.05 was considered significant.

3. RESULTS: IMMUNOHISTOCHEMISTRY

The results in this chapter are presented in two sections. The first discusses the methodology, the second details the application of the optimised protocols in patients with primary colorectal cancer and metastases.

3.1. METHODOLOGY

Protocols were developed to immunostain for vascular and lymphatic endothelial cells, VEGF family members and VEGFRs. The development of protocols in each of these areas will be discussed in turn.

3.1.1. Vascular endothelial cells

Three anti-endothelial cell antibodies, directed against CD31, CD34 and CD105, were evaluated to determine which was optimal for staining vascular endothelial cells in paraffin embedded colorectal cancer specimens.

Immunostaining for CD31

Initial immunostaining runs used 15 minute steps for blocking endogenous peroxidase and serum blocking. Slides stained in this way contained some weak non-specific background staining. An increase in the length of both steps to 30 minutes eliminated this background staining and thus were employed for all subsequent immunohistochemical staining runs.

Serial primary antibody dilutions (1:20 – 1:300) demonstrated optimal blood vessel staining at 1:100 (4.5μg/ml) and hence this dilution was selected for staining the tissue sections.

No staining was identified on the negative control slides using equivalent concentration of irrelevant mouse IgG or omission of primary antibody. The immunostained sections showed specific endothelial cell staining, indicated by the brown staining in **Figure 5a**.

Immunostaining for CD34

Optimisation of pre-treatment methods for antigen retrieval showed that the clearest most intense staining was in the slides pre-treated by microwave heating for 5 minutes at high power in Tris/EDTA buffer, pH 9.0. Little background staining and intense positive staining of endothelial cells was seen as illustrated in **Figure 5b**.

Serial antibody titration runs used dilutions of 1:25 - 1:100. Optimal staining was achieved with 1:50 (0.8μg/ml) (**Figure 5b**). Higher dilutions (1:100) gave heterogeneous vessel staining and lower dilutions (1:25) resulted in excessive positive staining of stromal constituents such as fibroblasts and collagen.

No staining was identified on the negative control slides using equivalent concentration of irrelevant mouse IgG or omission of primary antibody.

Immunostaining for CD105

Use of the routine pre-treatment methods for antigen retrieval showed no endothelial cell staining at a primary antibody concentration of 1:10. Consequently, signal amplification techniques were employed. Slides stained with the Envision System showed no positive endothelial cell staining. However, sections stained with the CSA System did show specific vascular endothelial cell staining without background staining with positive endothelial cells seen predominantly at the invading edge of the tumour.

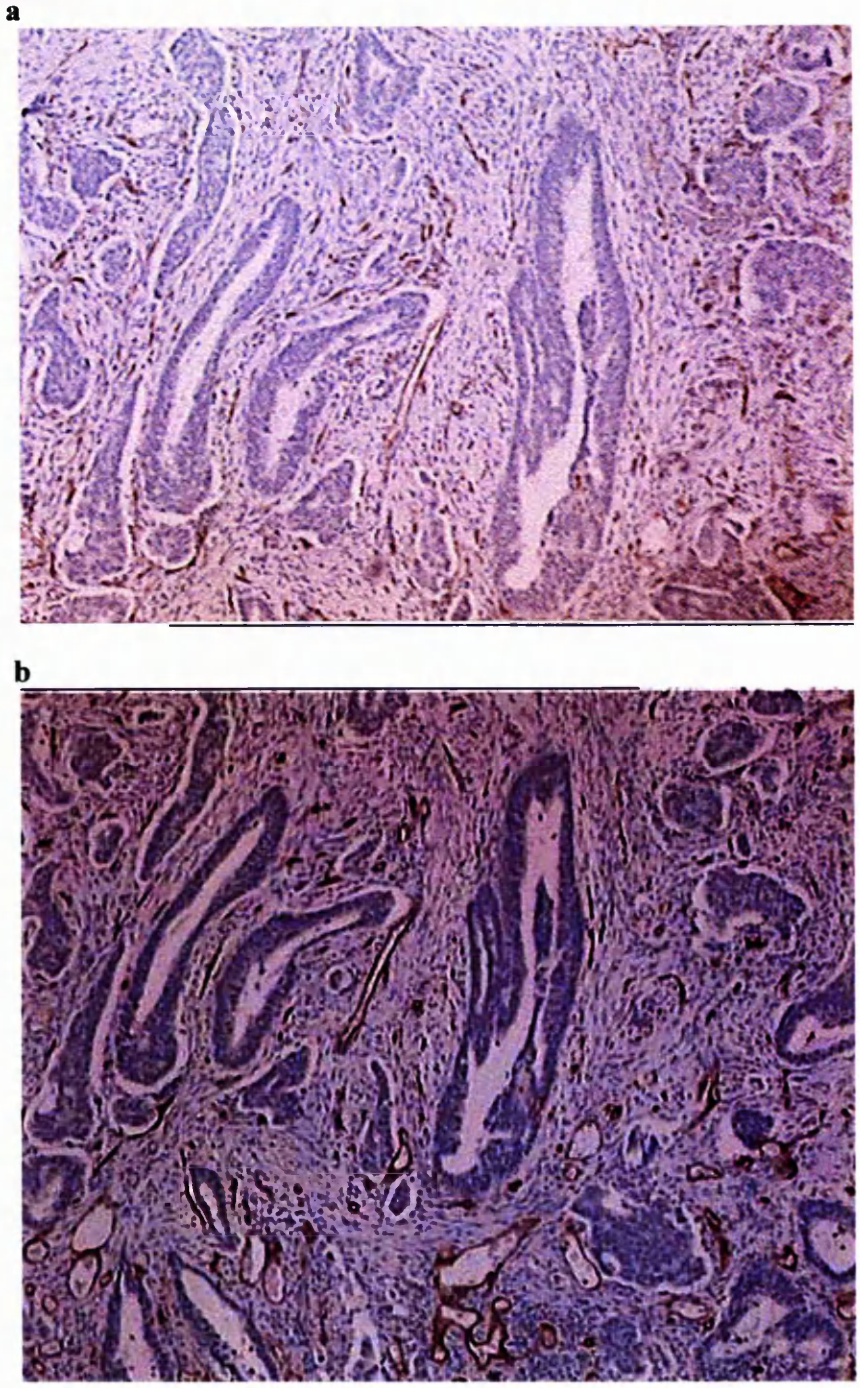
Optimal staining signal was achieved using the CSA system at a primary antibody dilution of 1:500 (0.584μg/ml). Lower dilutions gave strong staining of vascular endothelial cells with peri-cellular blurring of the signal. No staining was seen in the negative control sections.

Assessment of MVD

The optimal staining protocol for highlighting colonic tumour blood vessels in paraffin embedded tissue was obtained with anti-CD34, which identified all sizes of blood vessel within the section with the greatest intensity (**Figure 5b**). Anti-CD31 staining produced a much weaker stain, which was harder to identify, in comparison with the staining associated with anti-CD34 (**Figure 5a**). Anti-CD105 staining only produced endothelial cell staining in half the cases, mainly at the invading margin of the tumour. While anti-CD105 immunostaining may be useful for the determination of prognosis as discussed above (see **section 2.1.1, page 111**), the limited number of specimens available for study necessitated the use of a more reliable endothelial cell marker. Thus, MVD was assessed in anti-CD34 stained sections.

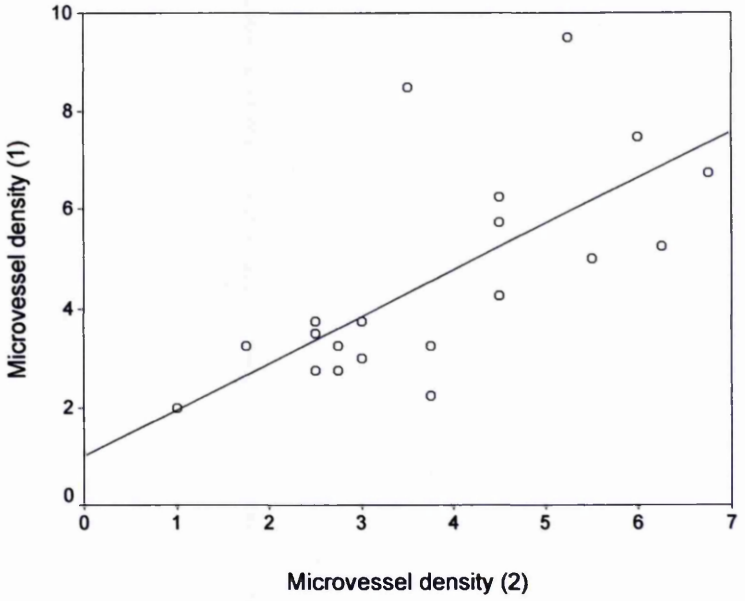
MVD of CD34 stained sections was assessed using a Chalkley point grid as described in **section 2.1.2, page 121**. The level of correlation between the two counts was high, with a Spearman's rank correlation coefficient of 0.72, $p<0.001$ (**Figure 6**).

Figure 5: Immunostaining of vascular endothelial cells in colorectal cancer



Sections of a moderately differentiated colonic adenocarcinoma immunostained for CD31 (a) and CD34 (b). Endothelial cells are immunostained with DAB (brown colour) and the tissue has been counterstained with haematoxylin. Original magnification x40.

Figure 6: Correlation between microvessel density counts



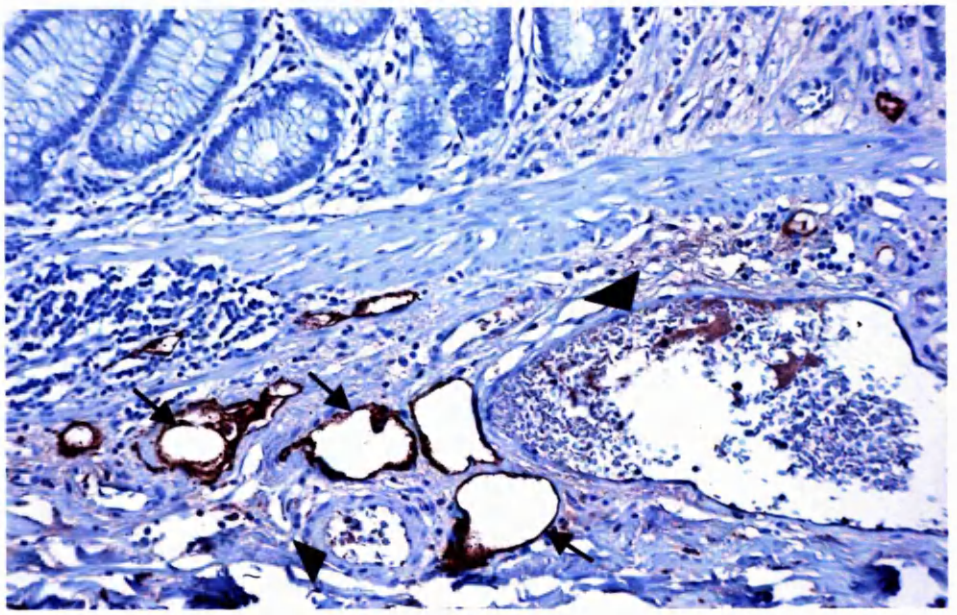
A high level of correlation was seen between the two sets of MVD counts in primary colorectal tumours, Spearman's rank correlation coefficient, 0.72, $p<0.001$

3.1.2. Lymphatic endothelial cells

Lymphatic endothelial cells were highlighted with rabbit anti-LYVE-1 antibody, using microwave antigen retrieval in 0.01M citrate buffer, pH 6.0 and the Envision detection system. Serial antibody dilution runs demonstrated lymphatic endothelial cell staining with primary antibody diluted to 1-10μg/ml. The concentration of 7μg/ml was selected for the definitive staining run of tissue sections. No staining was identified on the negative control sections.

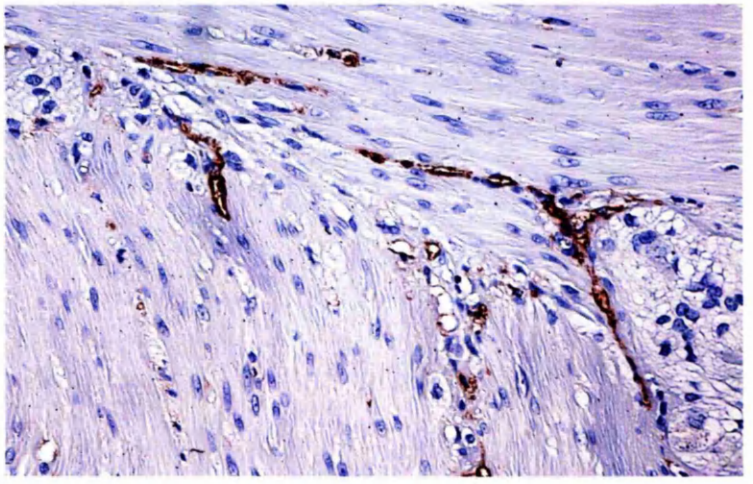
Figure 7: Immunostaining of lymphatic endothelial cells

a



Normal tissue adjacent to a moderately differentiated colonic adenocarcinoma immunostained for LYVE-1. Positive LYVE-1 staining highlights lymphatic vessels (arrows), with adjacent LYVE-1 negative blood vessels (arrowheads). The tissue was counterstained with haematoxylin. Original magnification x100.

b



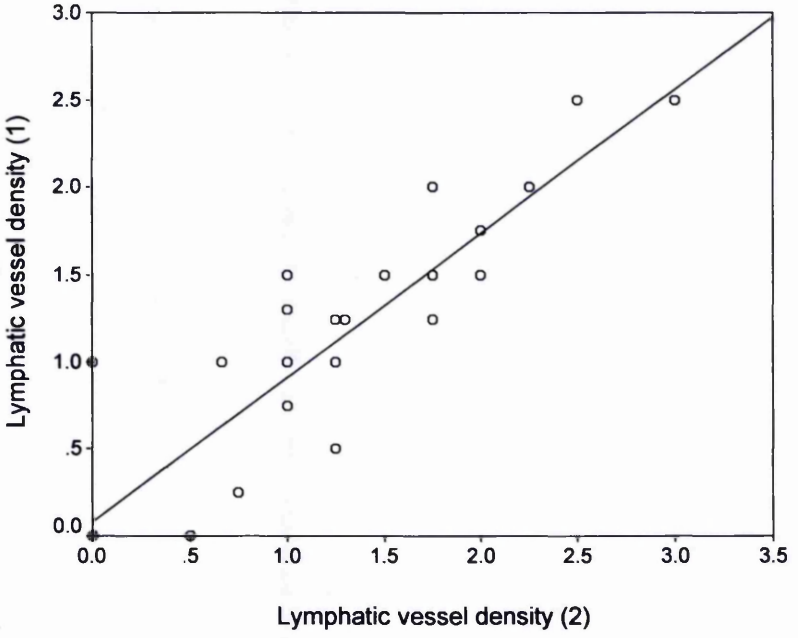
Normal musculature adjacent to a moderately differentiated colonic adenocarcinoma immunostained for LYVE-1. Positive lymphatic vessels can be seen between the layers of the muscularis propria. The tissue was counterstained with haematoxylin. Original magnification x100.

LYVE-1 positive vessels were easily identified in the normal colonic tissue, in particular in the submucosa. The morphology of these vessels were typical of lymphatics (thin-walled, irregular, large lumen, lacking red blood cells and pericytes, accompanying blood vessels) and were frequently seen in close proximity to arteries and veins, both of which were LYVE-1 negative (**Figure 7a**). Thus, in paraffin embedded colorectal cancer specimens, anti-LYVE-1 antibody produced specific staining of lymphatic vessels.

Assessment of LVD

LVD was measured with a Chalkley point grid. The level of correlation between the two sets of LVD counts was high, with a Spearman's rank correlation coefficient of 0.86, $p<0.001$ (**Figure 8**).

Figure 8: Correlation of lymphatic vessel density counts



A high level of correlation was seen between the two sets of LVD counts, Spearman's rank correlation coefficient, 0.86, $p<0.001$.

3.1.3. Growth factors

Immunostaining for VEGF-A

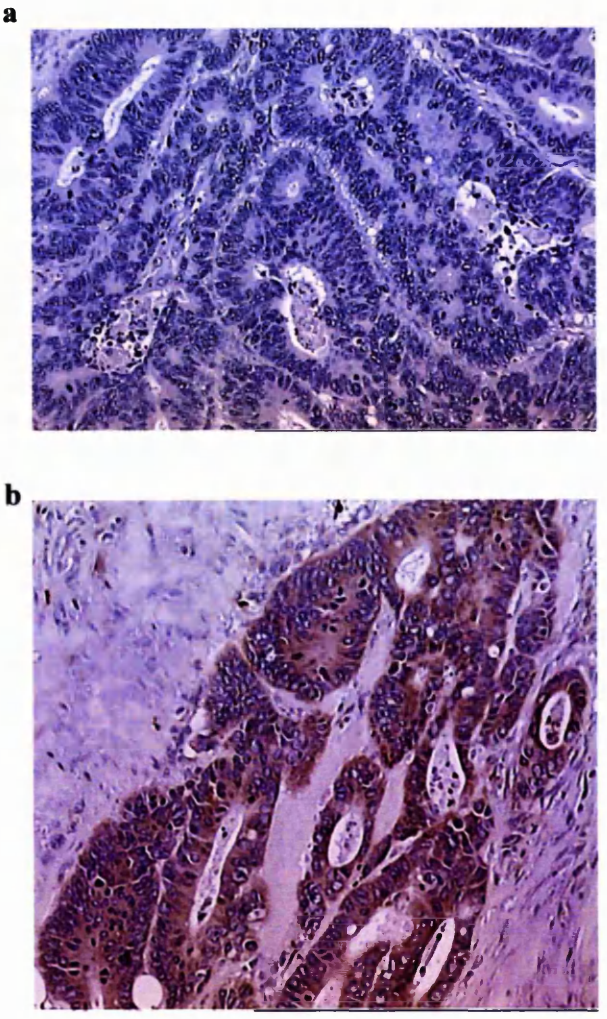
Initial staining methods that did not use antigen retrieval pre-treatment demonstrated strong immunostaining of tissue macrophages, with staining also observed on inflammatory cells, colonic and tumour epithelial cells (**Figure 9**) and some vascular endothelial cells. The optimal concentration of primary antibody was found to be 1:400 (0.5μg/ml) which gave strong positive staining of tissue macrophages and a high signal:background ratio. No immunostaining was seen on the negative control slides.

Immunostaining for VEGF-C

Initial attempts to stain colorectal tumours for VEGF-C utilised the two goat antibodies (SC-1881, SC-7133, Santa Cruz). However, problems were encountered with both the antibodies. The antibodies were assessed at the manufacturers' recommended dilutions and at lower dilutions, with and without methods for antigen retrieval, with longer peroxidase quenching times, with and without commercial protein blocking solutions and antibody diluents (in place of 1:10 and 1:20 rabbit serum). Staining was either intense and non-specific over the entire tissue section including the negative control or negligible, with some focal weak endothelial cell and scattered malignant cell staining. Known positive tissue controls (placenta) exhibited weak and inconsistent staining.

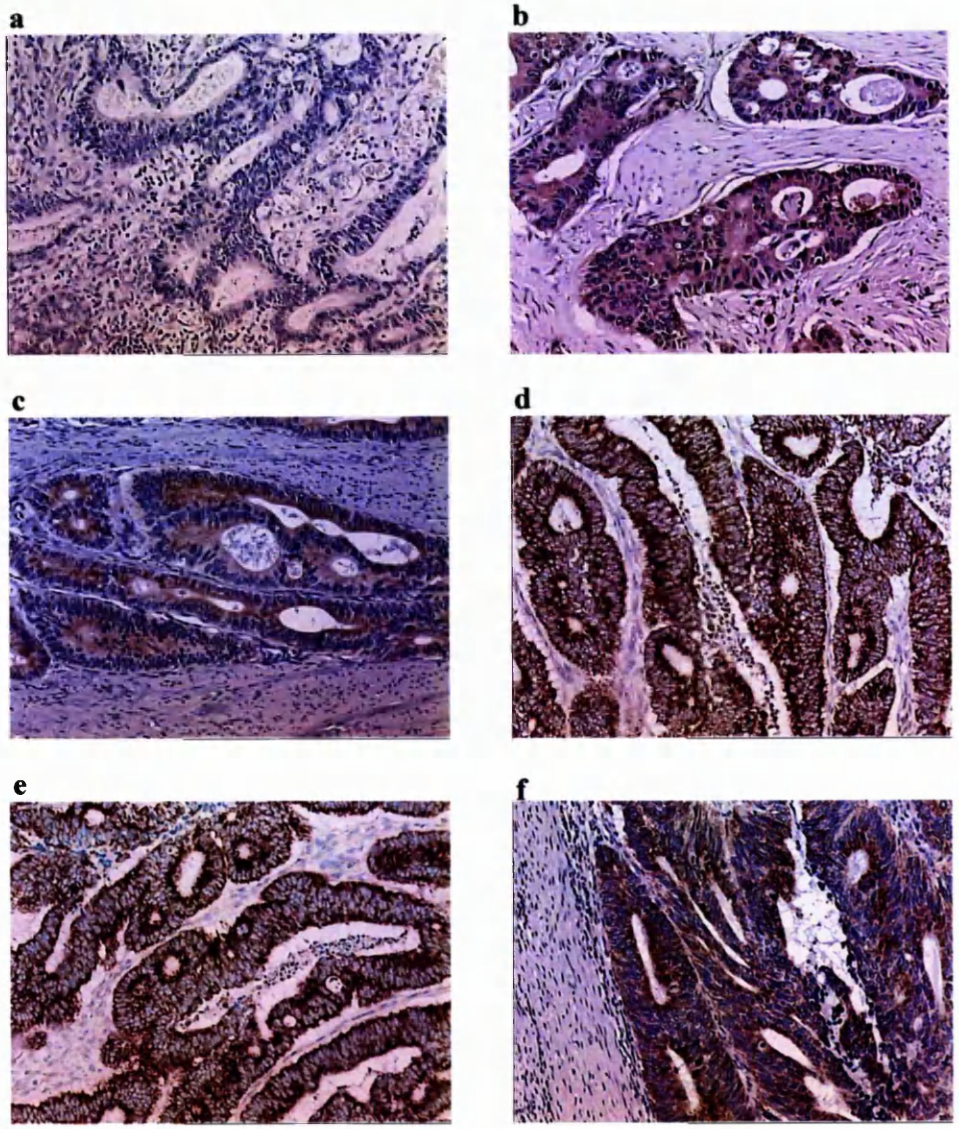
Attempts to use the goat antibodies were abandoned and an alternative rabbit primary antibody was substituted, rabbit anti-human VEGF-C IgG (18-2255, Zymed). The cytoplasm of malignant epithelial cells stained positively for VEGF-C, with weaker staining seen in the normal colonic epithelium while no staining was seen in the negative control sections (**Figure 10**).

Figure 9: Immunostaining for VEGF-A in colorectal cancer



Moderately differentiated sigmoid adenocarcinoma. a, negative control; b, immunostained for VEGF-A, both counterstained with haematoxylin. Original magnification x40.

Figure 10: Immunostaining for VEGF-C in colorectal cancer



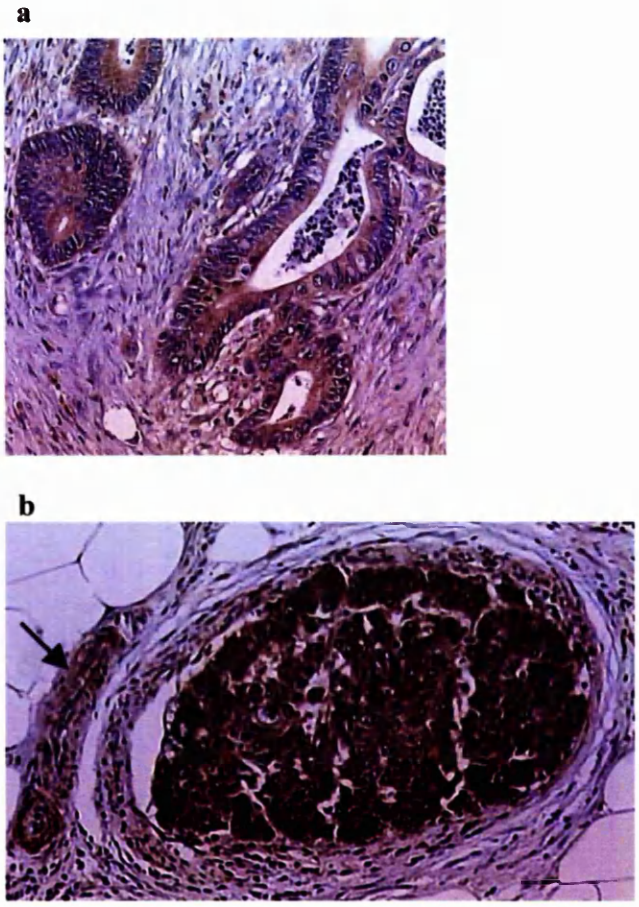
Primary colorectal cancers immunostained for VEGF-C. a, moderately differentiated rectal adenocarcinoma, negative control; b, moderately differentiated rectal adenocarcinoma; c, moderately differentiated rectal adenocarcinoma; d, moderately differentiated colonic carcinoma; e, moderately differentiated colonic carcinoma; f, moderately differentiated colonic carcinoma. All sections were counterstained with haematoxylin with the exception of e) which was counterstained with methyl green. Original magnifications x40.

Antibody titration studies were performed using the primary antibody at dilutions of 1:10 - 1:200. The optimal concentration giving the highest signal:background ratio was 1:50 (1μg/ml).

Immunostaining for VEGF-D

Initial staining procedures without specific antigen retrieval pre-treatment demonstrated strong tumour epithelial, normal colonic mucosa, muscle and stromal staining at the manufacturers' recommended concentration of 10μg/ml (1:100). Antibody dilution runs demonstrated an optimal concentration for immunostaining when used at 2μg/ml (1:500). Positive VEGF-D staining was found in normal colonic mucosa, particularly in the upper third of the colonic crypts, tumour epithelial cells (**Figure 11a**), inflammatory cells and fibroblasts. Intense positive staining was seen in the smooth muscle cells of medium and large-sized arterial vessels (**Figure 11b**). No immunostaining was seen on the negative control slides.

Figure 11: Immunostaining for VEGF-D in colorectal cancer



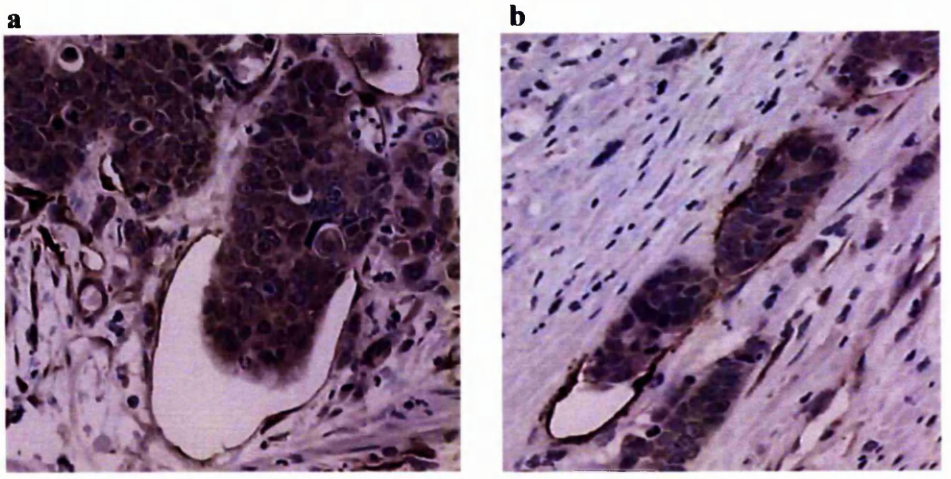
Tissue sections immunostained for VEGF-D. a, moderately differentiated colonic adenocarcinoma; b, poorly differentiated rectal carcinoma with vascular permeation, note positively stained tumour cells within a vessel and positive staining of the smooth muscle of an arteriolar wall (arrow). Slides counterstained with haematoxylin. Original magnifications: a, x40; b, x100.

3.1.4. VEGF receptors

Immunostaining for VEGFR3 (flt-4)

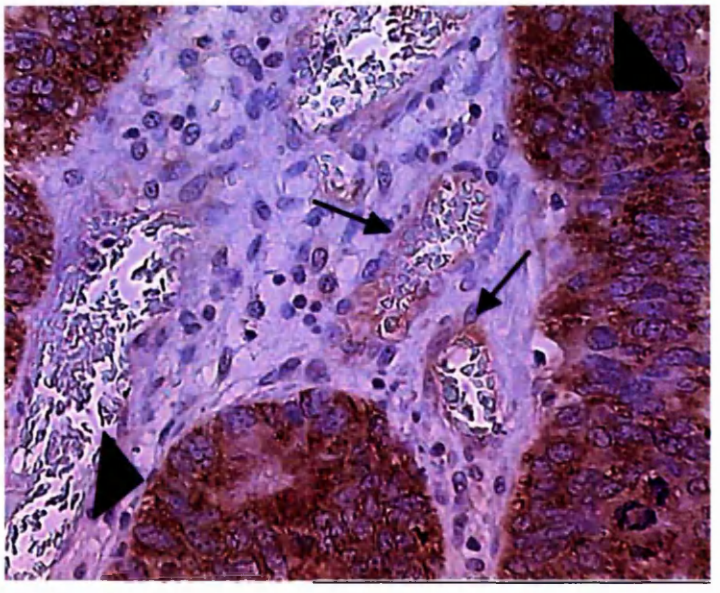
Immunostaining for VEGFR3 was performed in an attempt to define whether VEGFR3/VEGF-C/VEGF-D autocrine circuits exist in colorectal cancer (Witte et al., 2002; Kawakami et al., 2003) and if LVD could be quantified by counting VEGFR3 positive vessels. Two anti-VEGFR3 antibodies were evaluated. The first antibody assessed was goat anti-human VEGFR3 IgG. Pre-treatment by microwave heating in citrate buffer, pH 6.0 gave better immunostaining results than pre-treatment with trypsinisation, which produced widespread non-specific staining. Results obtained with citrate pre-treatment demonstrated staining of the endothelial cells of vessels that were typical of lymphatics from their morphological appearance. These positively stained vessels were seen in and around the tumour, in some cases with tumour cells within the vessel (**Figure 12**). Positive staining was also observed on smooth muscle cells, fibroblasts and tumour epithelial cells. Non-specific positive staining was also encountered throughout the control sections where irrelevant goat IgG at equivalent concentration to the primary antibody was used (although not on the control sections where primary antibody was omitted). The non-specific staining of the negative control was considered to be due to the high concentration of antibody used (15μg/ml). This staining did not diminish even when the time of endogenous peroxidase blocking or serum blocking was increased, or by using proprietary agents such as protein blocking solutions, antibody diluents and biotin blocking kits.

Figure 12: Immunostaining for VEGFR3



Immunostaining for VEGFR3 with goat anti-human VEGFR3 IgG, in a poorly differentiated rectosigmoid adenocarcinoma. Note positively stained malignant epithelial cells within positively stained vessels (a & b), note also however, stromal staining (a), limiting the usefulness of this antibody for discrimination of specific VEGFR3 positive staining. Slides were counterstained with haematoxylin. Original magnifications x100.

Figure 13: Immunostaining for VEGFR2



Immunostaining for VEGFR2 with monoclonal anti-VEGFR2 IgG, in a moderately differentiated colonic adenocarcinoma. Note weak positive staining of endothelial cells (arrows) and stronger positive staining of malignant epithelial cells (arrowheads).

Comparisons of paired tumour and control sections illustrated that more intense staining was present in the tumour in the test section compared to the non-specific staining of the negative control and this was particularly intense at the invading edge of the tumour. Endothelial cell staining was only observed on the test sections. Thus, tumour epithelial and endothelial cell immunostaining for VEGFR3 in the test sections was specific, in that it did not occur in the negative controls. This specificity was confirmed by the use of shorter antibody incubation times in order to reduce the overall staining. Slides were incubated with primary antibody for just 1.5 hours at room temperature when endothelial cells just began to stain in the test sections with little background staining. The implication may be that the endothelial cell staining observed was specific as these cells were the first structures to acquire a positive stain.

Use of the alternative polyclonal rabbit anti-human VEGFR3 antibody showed strong staining throughout the whole of the test sections with and without pre-treatments for antigen retrieval. Tumour epithelium, muscle, inflammatory cells, fibroblasts and all vascular structures (blood vessels and lymphatic vessels) stained positively. No staining was seen in the negative control sections. Serial antibody dilutions were performed at low concentrations of primary antibody (0.1–2 μg/ml), however, a similar positive staining pattern of multiple structures was also seen at these concentrations. Consequently, this rabbit anti-human VEGFR3 IgG was less specific than the goat anti-human VEGFR3 IgG previously tested. Neither of these two polyclonal antibodies provided immunostaining results that allowed the clear distinction of positively stained endothelial cells and hence the possible discrimination of lymphatic vessels, nor clarification of whether malignant colorectal epithelial cells express VEGFR3. Similar problems have been encountered by others with the use of currently available polyclonal anti-human VEGFR3 antibodies (Birner et al., 2000; Birner et al., 2001b; Clarijs et al., 2002; Moller et al., 2002).

Immunostaining for VEGFR2

Using the monoclonal anti-VEGFR2 antibody, antigen retrieval method assessment showed that microwave pre-treatment in 0.01M citrate buffer gave excellent results, in contrast to poor staining without pre-treatment or with trypsinisation. Positive staining was found on endothelial cells, tumour epithelial cells and some stromal constituents (**Figure 13**). Serial antibody dilution runs used dilutions of 1:20 – 1:100 and optimal results were encountered with an antibody dilution of 1:50 (4μg/ml). No immunostaining was seen on the negative control slides.

The immunostaining results obtained and reported below using the monoclonal anti-VEGFR2 antibody were unexpected, consequently, an additional rabbit polyclonal anti-VEGFR2 antibody was used to confirm and verify the findings. Microwave pre-treatment in citrate buffer with a primary antibody dilution of 1:50 was used as recommended by the manufacturer. Positive staining was identified in a similar distribution to the first antibody (see **Figure 19**) and no staining was seen on the negative control slides.

3.1.5. Summary

In summary, using paraffin embedded colorectal cancer tissue:

i) protocols were developed to immunostain for vascular and lymphatic endothelial cells, using CD34 and LYVE-1 antibodies.

ii) MVD and LVD counts obtained were reproducible.

iii) immunostaining techniques for the growth factors VEGF-A, VEGF-C, VEGF-D and the receptor VEGFR2 were developed.

iv) immunostaining for VEGFR3 with available polyclonal antibodies was non-specific and could not be used to evaluate LVD nor to clarify whether malignant epithelial cells express this receptor specifically.

3.2. VEGF MEASUREMENTS IN COLORECTAL CANCER AND METASTASES

The optimised immunostaining protocols were used to examine the patterns of VEGF family member and receptor expression in primary and metastatic colorectal cancer specimens.

3.2.1. Clinical details of patient groups

Two groups of cases were studied whose demographic and clinicopathological details are summarised in **Table 17**. Group 1 patients (n=21) were those that participated in a previous study examining the use of xylene clearance methods to increase the lymph node yield in routine pathological specimens, whereas all Group 2 patients (n=9) had undergone hepatic resections for colorectal cancer liver metastases (see **sections 2.1.1, page 106 & 2.1.2, page 119**). In group 1, 12 patients had lymph node metastases (11 Dukes' C, 1 Dukes' D) and 1 patient had synchronous liver metastases at the time of primary resection. In group 2, 4 patients had lymph node metastases at the time of primary resection and all went on to have liver metastases resected. Consequently, 16 of the 30 cases assessed had lymph node metastases associated with their primary tumour and 10 had synchronous or metachronous liver metastases.

Table 17: Clinicopathological details of patients studied

Parameter	**Group 1 (n=21)**	**Group 2 (n=9)**
Age (years)[§]	71 (49-86)	53 (39-76)
Gender (male:female)	10 : 11	7 : 2
T stage		
T1	1	0
T2	4	0
T3	13	8
T4	3	1
N stage		
N0	9	5
N1	7	3
N2	5	1
Differentiation		
Well differentiated	0	0
Moderately differentiated	13	9
Poorly differentiated	8	0
Dukes' stage		
Dukes' A	1	0
Dukes' B	7	4
Dukes' C	11	4
Dukes' D	2	1

[§] Median (range)

Representative formalin fixed paraffin embedded tissue blocks were available from the following:

- primary colorectal cancers: n=30
- lymph node metastases: 9 from Group 1 and 3 from Group 2
- liver metastases: all 9 from Group 2

Blocks of the primary tumour specimens were selected to include, where possible, adjacent normal tissue, junctional mucosa at the area between normal mucosa and tumour epithelium, and a full section through the tumour encompassing the invading edge.

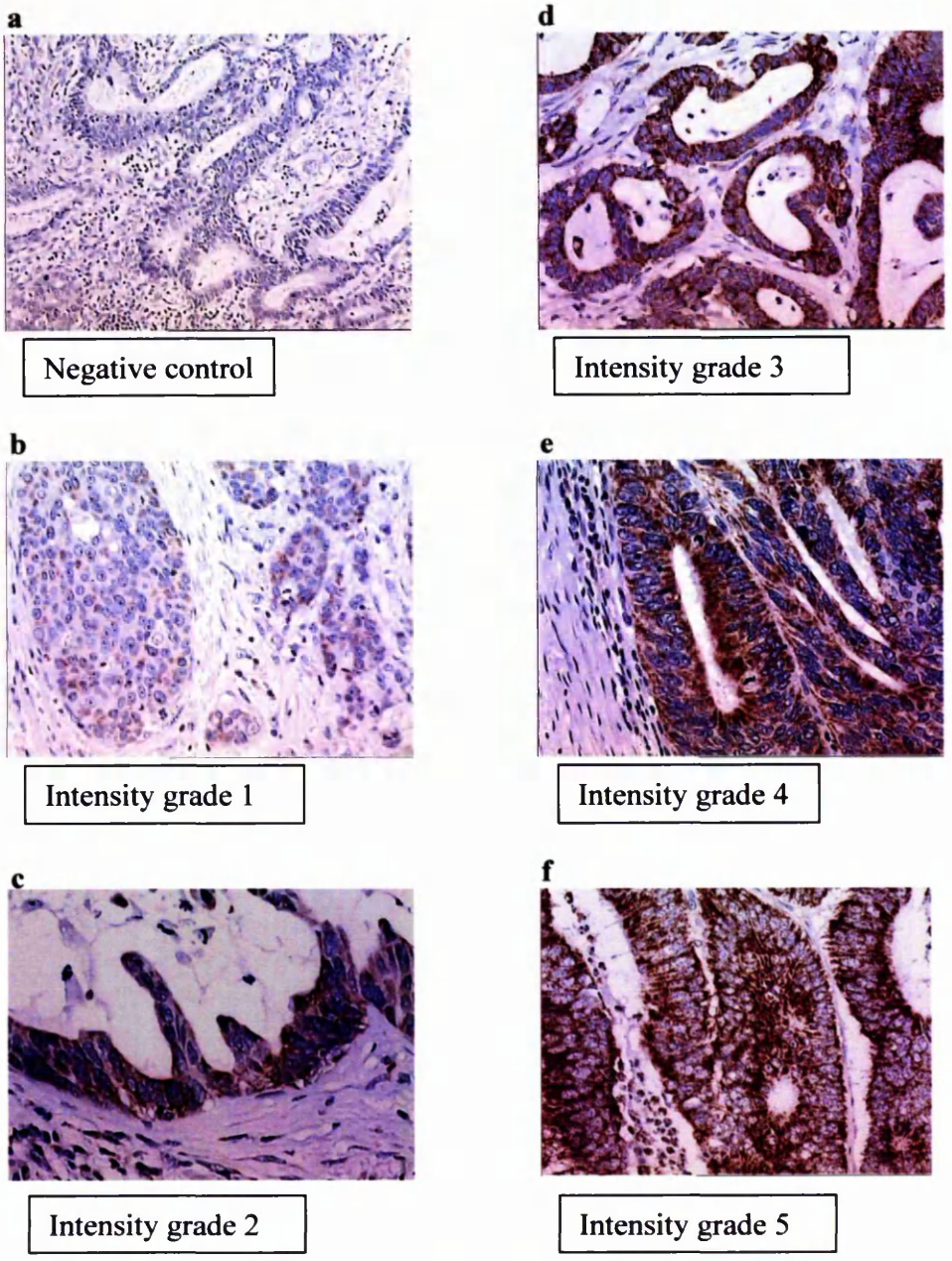
3.2.2. Distribution of growth factors in primary tumours

Assessment of staining intensity for VEGF-A, VEGF-C and VEGF-D was made at 5 different sites: normal mucosa (N), junctional mucosa (J), superficial tumour (TS), central tumour (TC) and invasive edge of tumour (TI). Assessment was made in a semi-quantitative manner, scoring the intensity of immunostaining from 0-5 at each site, so that 0 represented no staining and 5 represented maximum staining. Representative photomicrographs illustrating the different staining intensities are shown in **Figure 14.** Assessment of staining intensity was made by two observers and discrepancies agreed by consensus.

Agreement between the two observers was examined over 100 separate assessments. Complete agreement was seen on 61 occasions, the two scores were within 0.5 intensity units in 15 assessments and within 1.0 unit in 19 assessments. The two scores correlated closely with a Spearman's rank correlation coefficient of 0.92 ($p<0.001$).

The results of growth factor intensity staining are summarised in **Table 18** and **Figures 15-16.**

Figure 14: Immunostaining intensity scores



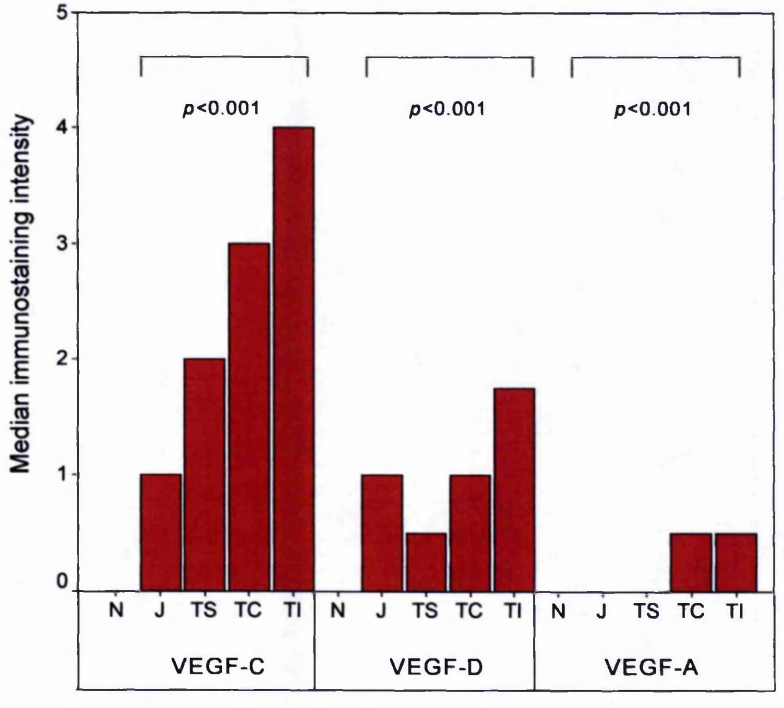
Examples of different scoring intensities for VEGF-C. a, moderately differentiated rectal adenocarcinoma, negative control, x40; b, poorly differentiated colonic adenocarcinoma, x100; c, moderately differentiated mucus secreting colonic adenocarcinoma, x100; d, moderately differentiated mucus secreting colonic adenocarcinoma, x100; e, moderately differentiated colonic adenocarcinoma, x100; f, moderately differentiated rectal adenocarcinoma, x100. All sections counterstained with haematoxylin.

Table 18: Growth factor staining intensity in epithelial cells at primary tumour sites

	Sites stained in primary tumours				
Growth factor	**N**	**J**	**TS**	**TC**	**TI**
VEGF-C					
Number	25	23	29	30	30
Median (range)	0 (0-2)	1 (0-4)	2 (0-4)	3 (0-5)	4 (0.5-5)**
VEGF-D					
Number	23	23	29	30	30
Median (range)	0 (0-2)	1 (0-3)	0.5 (0-2.5)	1 (0-3)	1.75 (0-4)**
VEGF-A					
Number	25	22	30	30	30
Median (range)	0 (0-0.5)	0 (0-1.5)	0 (0-1)	0.5 (0-3)	0.5 (0-3)**

N, normal mucosa; J, junctional mucosa; TS, superficial tumour; TC, central tumour; TI, invasive tumour edge. The full set of 30 primary tumours could not be assessed at all sites for all cases, due to the limitations of tissue availability on the slides assessed.
** $p<0.001$ (Friedman test) for difference in median staining intensity across the sites examined (see **Figure 15**).

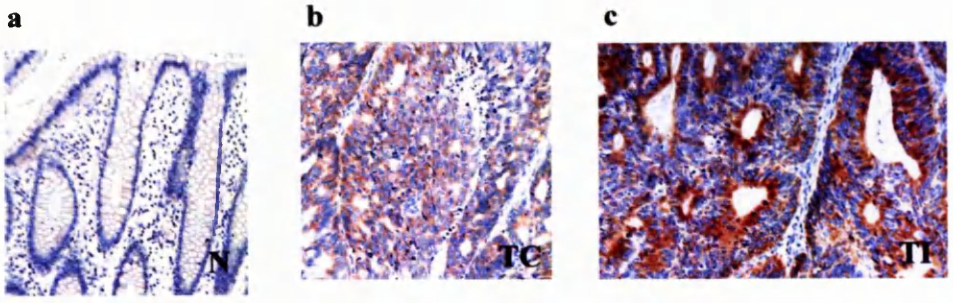
Figure 15: VEGF-C, VEGF-D and VEGF-A staining intensity in primary tumours



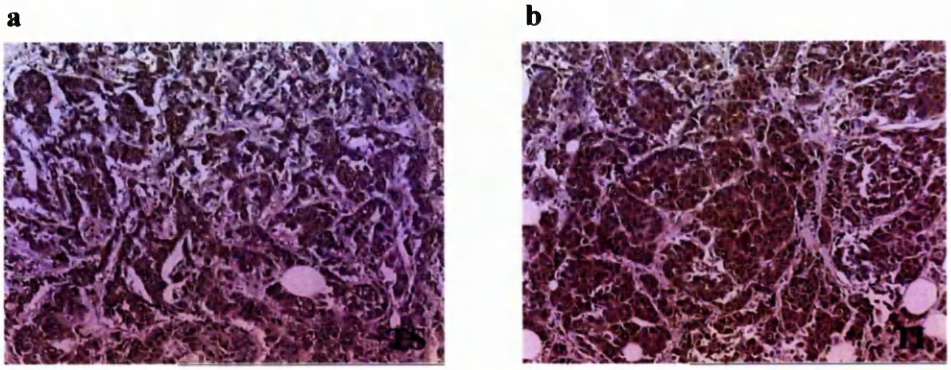
N, normal mucosa; J, junctional mucosa; TS, superficial tumour; TC, central tumour; TI, invasive tumour edge. The p values refer to the Friedman test for related variables, which examines differences in median score across the 5 tumour sites assessed within individual cases.

The pattern of growth factor expression was broadly similar for the three members of the VEGF family assessed in that the median intensity of expression was maximal at TI (**Figure 15** above and **Figure 16**). The differences in expression intensity across the 5 tumour sites examined were statistically highly significant for all three growth factors [Friedman test for related variables, $p<0.001$ for VEGF-C, VEGF-D and VEGF-A (**Figure 15**)]. In order to identify the significant differences between different tumour sites assessed, the Wilcoxon signed rank test was used to examine pairs of related data. The use of multiple comparisons in this way (10 for each growth factor) increased the risk of a Type I error, to avoid this pitfall, compensation was made by adjusting the level of statistical significance to $p<0.001$. Significant differences ($p<0.001$) were identified for VEGF-C between N vs TS, TC and TI, J vs TC and TI, TS vs TC and TI, TC vs TI, for VEGF-D between N vs TI and TS vs TI and for VEGF-A between N vs TC and TI, TS vs TC and TI.

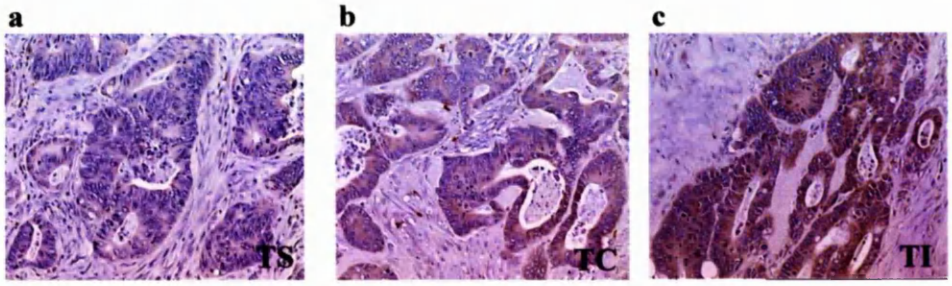
Figure 16: Expression of growth factors throughout primary tumours



VEGF-C expression in a moderately differentiated colonic adenocarcinoma: a, normal mucosa, N; b, central tumour, TC; c, invasive edge of tumour, TI. All sections counterstained with haematoxylin and original magnifications x40.



VEGF-D expression in a poorly differentiated rectal adenocarcinoma: a, superficial tumour, TS; b, invasive edge of tumour, TI. Sections were counterstained with haematoxylin, original magnification x40



VEGF-A expression in a moderately differentiated sigmoid adenocarcinoma: a, superficial tumour, TS; b, central tumour, TC; c, invasive edge of tumour, TI. Sections were counterstained with haematoxylin, original magnification x40.

3.2.3. VEGFR2 in primary tumours

Primary tumours were stained for VEGFR2 using a monoclonal anti-VEGFR2 antibody. Weak staining was identified on vascular endothelial cells (**Figure 17**) and strong immunostaining was seen on malignant colorectal epithelium (**Figure 18**). The pattern of immunostaining was similar to that of the VEGF family members, increasing in intensity throughout the tumour to TI (**Table 19 & Figure 18**). There was a significant difference in the intensity of expression of VEGFR2 across the different tumour sites assessed (Friedman test for related variables, $p<0.001$; using Wilcoxon signed rank test for pairs of related data, $p<0.001$ for N and TS vs TC and TI). Such intense expression of VEGFR2 on colorectal epithelial cells was unexpected, so in order to confirm this finding immunostaining was repeated using a different antibody, a rabbit polyclonal anti-VEGFR2 antibody, and results agreed with the initial findings (**Figure 19**).

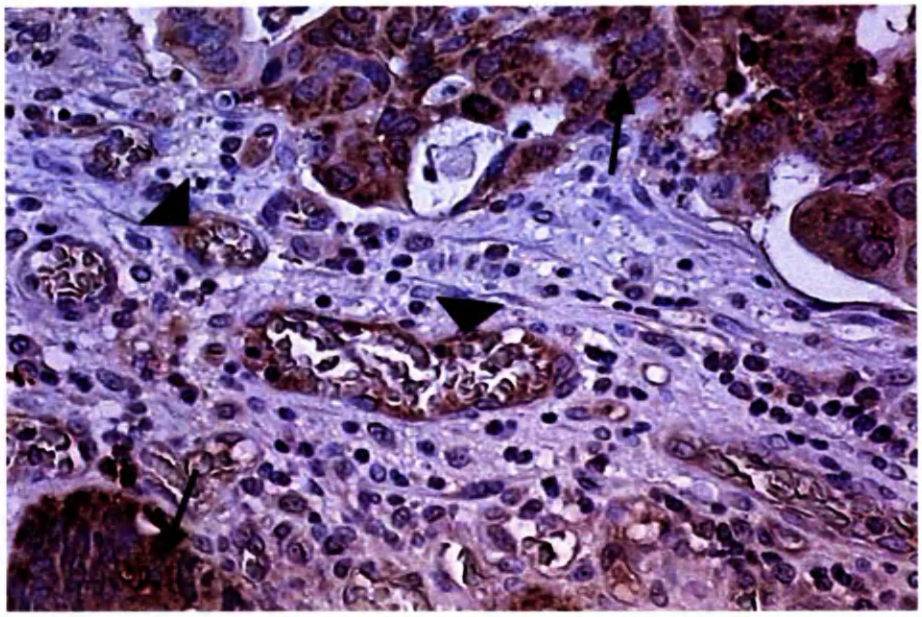
Table 19: VEGFR2 staining in primary tumours

	Sites stained in primary tumours				
Growth factor	**N**	**J**	**TS**	**TC**	**TI**
VEGFR2					
Number	26	20	30	30	30
Median (range)	0 (0-3)	0.75 (0-4)	0.5 (0-4)	3 (0-5)	4 (1-5)**

N, normal mucosa; J, junctional mucosa; TS, superficial tumour; TC, central tumour; TI, invasive tumour edge. The full set of 30 primary tumours could not be assessed at all sites for all cases, due to the limitation of tissue availability.

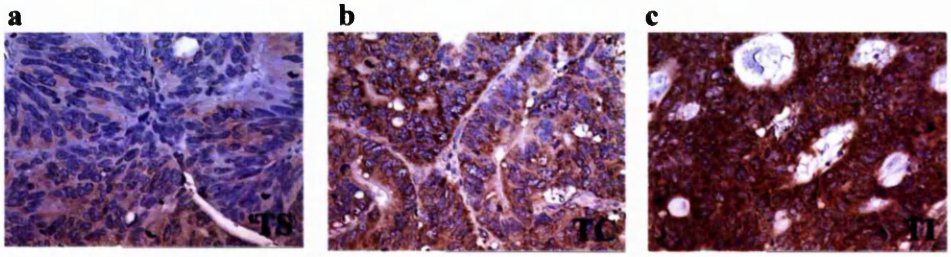
** $p<0.001$ (Friedman test) for difference in median staining intensity across the sites examined (see **Figure 18**).

Figure 17: VEGFR2 immunostaining in vascular endothelial cells



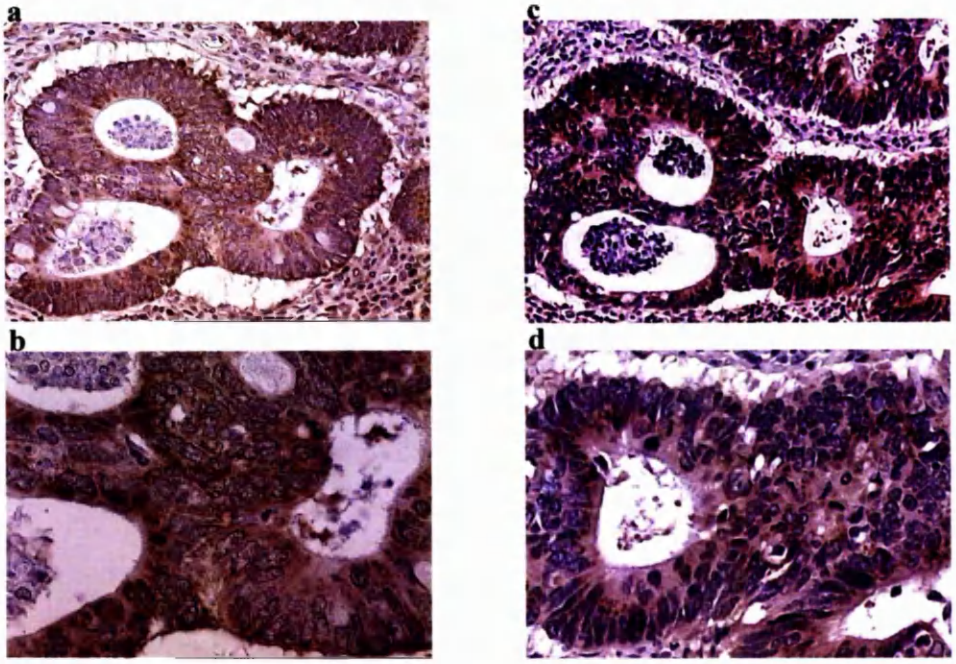
A moderately differentiated colonic adenocarcinoma immunostained for VEGFR2 with mouse monoclonal anti-human VEGFR2 antibody, counterstained with haematoxylin at magnification x200. Positive immunostaining is seen on endothelial cells (arrowheads) and on tumour epithelial cells (arrows).

Figure 18: VEGFR2 immunostaining in colorectal cancer cells throughout primary tumours

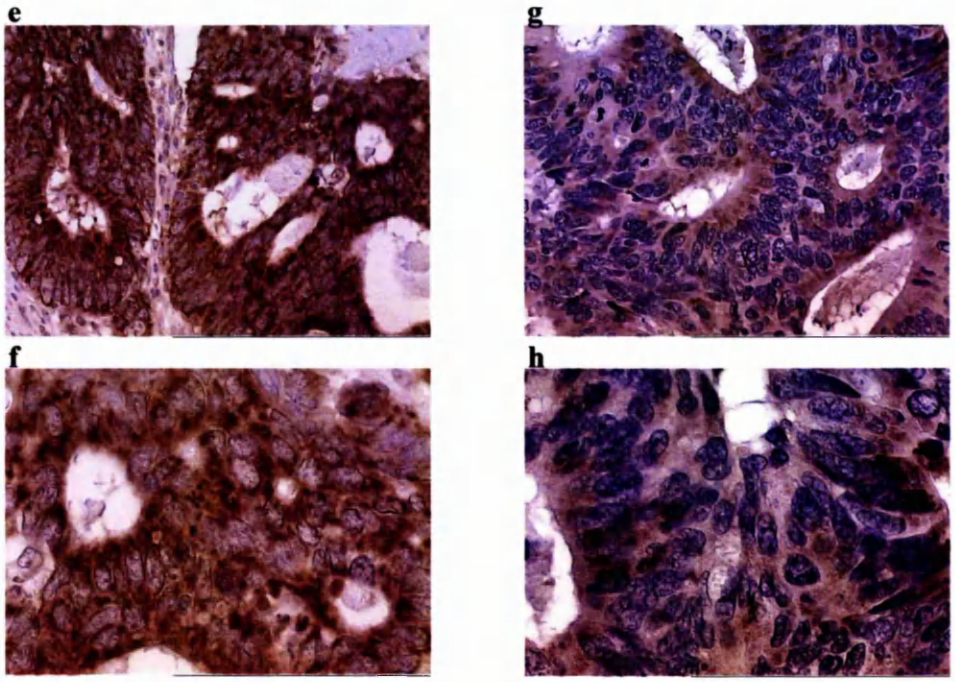


VEGFR2 expression in a moderately differentiated colonic adenocarcinoma immunostained with mouse monoclonal anti-human VEGFR2 antibody: a, superficial tumour, TS; b, central tumour, TC; c, invasive edge of tumour, TI. Sections counterstained with haematoxylin and original magnification x100.

Figure 19: Immunostaining for VEGFR2 in colorectal cancer with two different antibodies



Two moderately differentiated colonic adenocarcinomas (a-d and e-h) stained for VEGFR2. Serial sections were stained with mouse monoclonal anti-human VEGFR2 antibody (a-b, e-f) and rabbit polyclonal anti-human VEGFR2 antibody (c-d, g-h) and counterstained with haematoxylin. Magnifications: a, c, e, g, x100; b, d, f, h, x200.

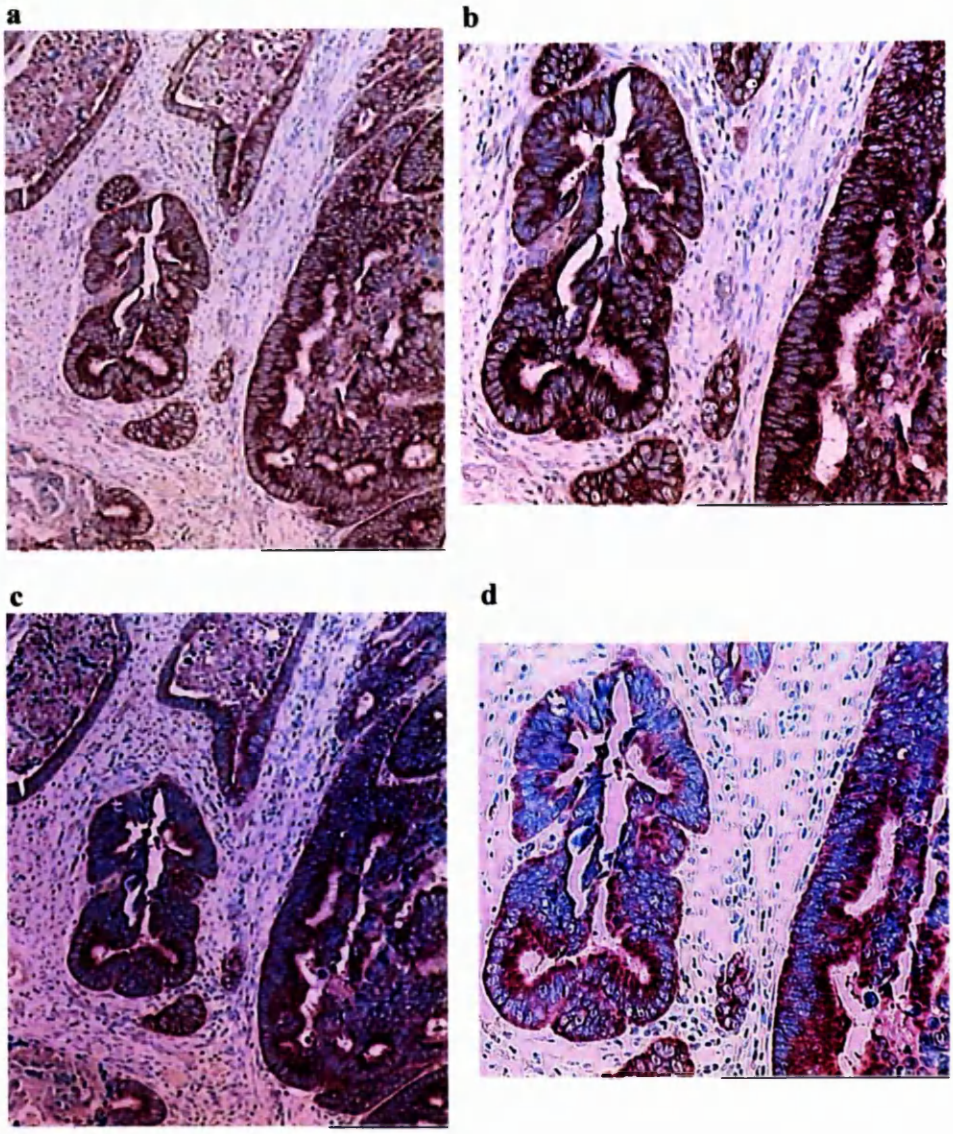


3.2.4. Growth factor/receptor expression in primary tumours

Correlations between the expression of VEGF-C, VEGF-D, VEGF-A and VEGFR2 at the different tumour sites were examined using Spearman's rank correlation coefficients. The expression intensity of VEGF-C and VEGFR2 in epithelial cells correlated consistently at four of the five assessed locations. Spearman's rank correlation coefficients for J, TS, TC and TI were 0.50, 0.41, 0.58, and 0.42, with corresponding *p*-values of 0.03, 0.028, 0.001 and 0.019, respectively. The intensity of epithelial cell expression of VEGF-C, -D and -A did not correlate with one another, nor did epithelial cell expression intensity of VEGF-D or VEGF-A correlate with that of VEGFR2 within the tumour sites examined.

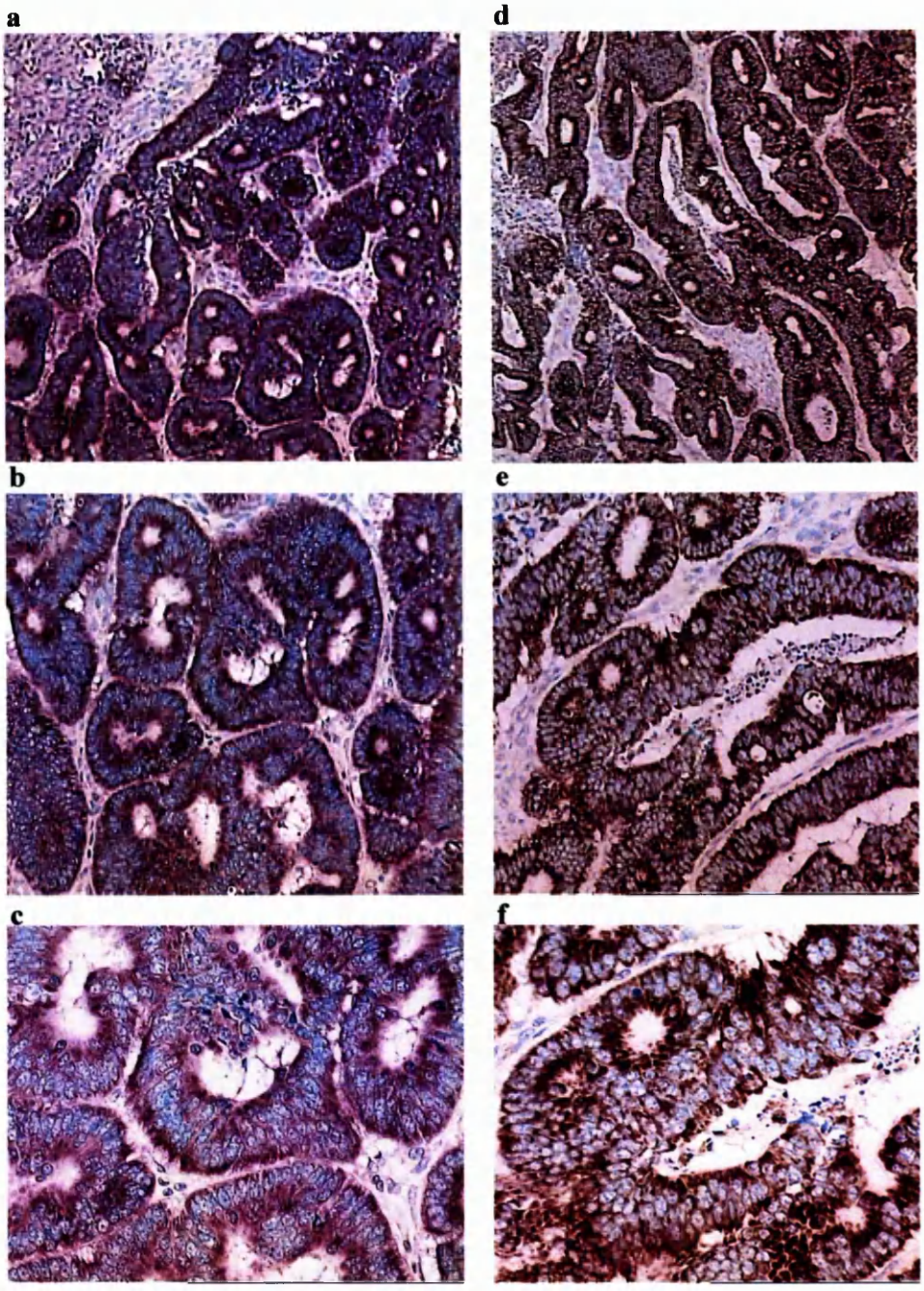
The similarity of the expression pattern (increasing throughout the tumour to TI) and the correlation between the expression intensity of VEGF-C and VEGFR2 may be important in colorectal cancer biology. Proteolytically processed VEGF-C can activate VEGFR2, consequently the expression of both molecules by the same cell populations highlights a potential autocrine circuit within the colorectal tumour. To further investigate the relationship between VEGF-C and VEGFR2, a double-staining technique was employed. Four consecutive sections were stained: a negative control, VEGF-C alone (DAB as chromogen), VEGFR2 alone (VIP as chromogen) and VEGF-C/VEGFR2 (both DAB and VIP). Serial sections stained for either antigen alone, clearly demonstrated co-localisation of VEGF-C and VEGFR2 within the same population of colorectal cancer cells (**Figures 20-21**). In colorectal cancer cells stained with both chromogens, the intensity of the DAB stain overwhelmed the pale purple colour of the VIP.

Figure 20: Co-localisation of VEGF-C and VEGFR2 in colorectal cancer



Serial sections of a moderately differentiated rectal adenocarcinoma immunostained for VEGF-C (a & b) and VEGFR2 (c & d) and counterstained with methyl green. The chromogen was DAB for sections a & b and VIP for sections c & d. Immunolocalisation of both antigens is to the cytoplasm of the malignant colonic epithelial cells. Original magnifications: a, c, x40; b, d, x100.

Figure 21: Co-localisation of VEGF-C and VEGFR2 in colorectal cancer



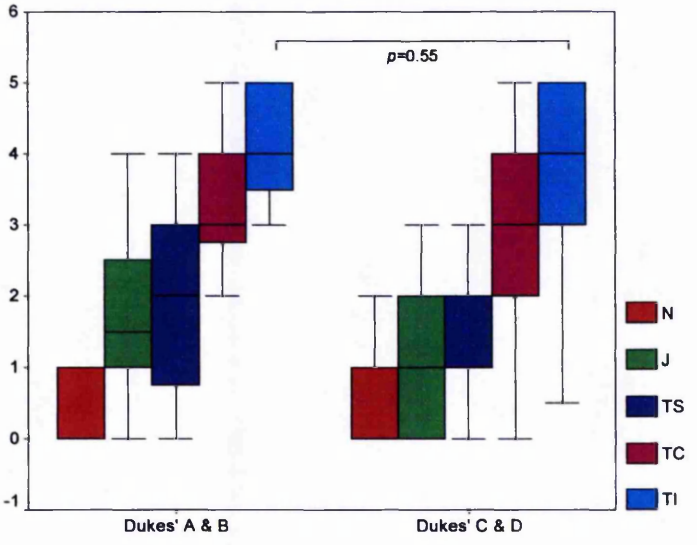
Serial sections of a moderately differentiated rectal adenocarcinoma immunostained for VEGFR2 (a-c) and VEGF-C (d-f), counterstained with methyl green. The chromogen was VIP for sections a-c and DAB for sections d-f. Original magnifications: a, d, x40; b, e, x100; c, f, x200.

3.2.5. Growth factor expression and disease stage

To investigate whether the patterns of growth factor and receptor expression differed in primary tumours at different stages, the group of 30 cases was divided into those with early disease (Dukes' A and B, n=12) and late disease (Dukes' C and D, n=18).

No obvious differences in expression of VEGF-C, VEGF-D, VEGF-A or VEGFR2 between cases with early and late stage disease were observed (illustrated in **Figure 22** for VEGF-C). This lack of difference in expression between early and late stage disease was confirmed by between group comparisons of expression at the TI site using the Mann-Whitney U test (early vs. late disease: VEGF-C, p=0.55; VEGF-D, p=0.67; VEGF-A, p=0.33; VEGFR2, p=1.0).

Figure 22: VEGF-C expression in early and late stage primary tumours



The boxplot illustrates the intensity of VEGF-C expression at the differing sites within and around colorectal cancers. There were no differences in median intensities at each of the sites assessed between early and late stage disease. Comparison of medians at TI by the Mann-Whitney U test, p=0.55.

3.2.6. Primary tumour growth factor expression and pattern of metastasis

To investigate whether the patterns of growth factor and receptor expression differed in primary tumours that spread by different routes, the group was examined with respect to the mode of metastasis. The group was divided into those with (n=16) or without (n=14) lymph node metastases, with (n=10) or without (n=20) liver metastasis and with either no metastases (n=9), lymph node metastases only (n=11), liver metastases only (n=5) or both lymph node and liver metastases (n=5).

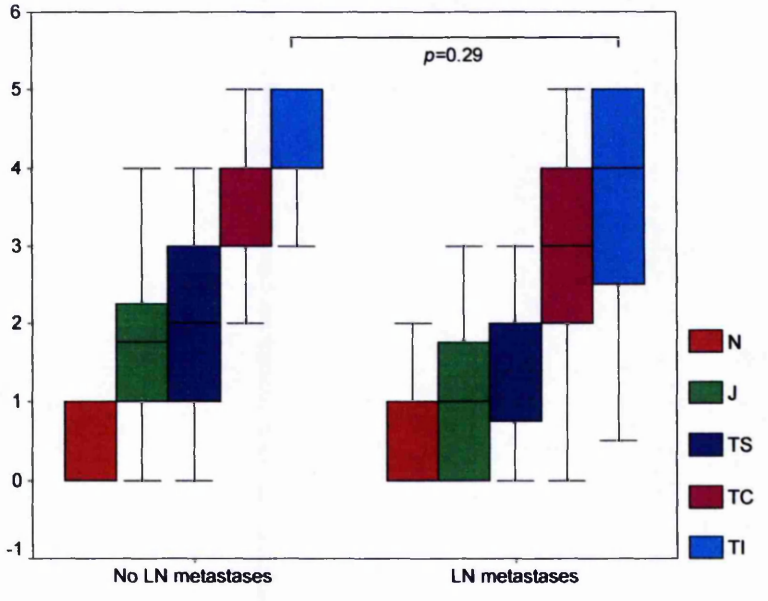
For the lymphatic route of tumour spread, no obvious differences in expression of VEGF-C, VEGF-D, VEGF-A or VEGFR2 at different sites were identified (illustrated in **Figure 23** for VEGF-C). This lack of difference was confirmed by between group comparisons of expression at the TI site using the Mann-Whitney U test (lymph node metastasis vs. no lymph node metastasis: VEGF-C, p=0.29; VEGF-D, p=0.67; VEGF-A, p=0.95; VEGFR2, p=0.79). Similarly, for the haematogenous route of tumour spread, no differences in expression for VEGF-D, VEGF-A or VEGFR2 at different sites were identified. However, the expression of VEGF-C decreased in tumours that went on to develop liver metastases (**Figure 24**). The observed patterns were confirmed by between-group comparisons of expression at the TI with the Mann-Whitney U test (liver metastasis vs. no liver metastasis: VEGF-C, p=0.005; VEGF-D, p=0.29; VEGF-A, p=0.09; VEGFR2, p=0.06).

When groups were compared by their combined route of metastatic spread – no metastases, lymph node metastases only, liver metastases only or both lymph node and liver metastases, similar findings of reduced TI expression intensity of VEGF-C emerged. Comparison of growth factor and receptor expression at the TI site between the four groups was made using the Kruskall-Wallis test. No differences were seen in expression by different modes of metastasis for VEGF-D (p=0.55), VEGF-A (p=0.31)

or VEGFR2 (p=0.16) but a significant difference was seen in VEGF-C expression (p=0.020) (**Figure 25**).

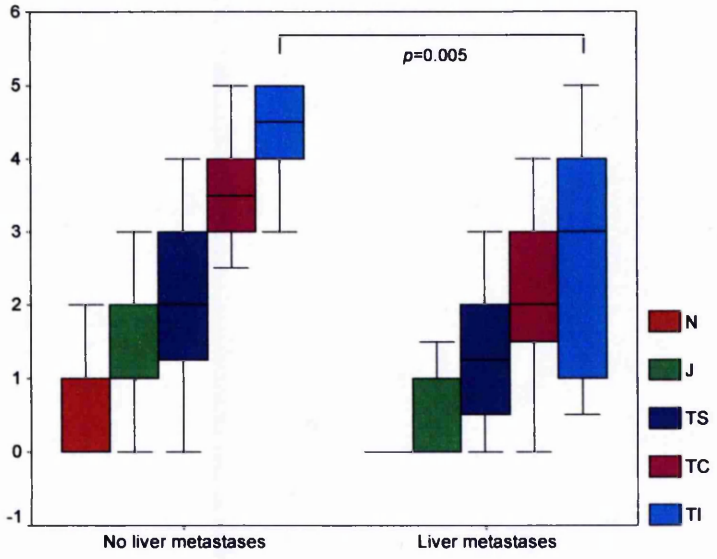
In summary, primary colorectal cancer expression intensity of VEGF-C, VEGF-D, VEGF-A and VEGFR2 at the TI did not differ between tumours that did and did not spread to lymph nodes. In primary colorectal cancers that spread to the liver, VEGF-C expression intensity at the TI was reduced, whereas no differences were seen for TI expression of VEGF-D, VEGF-A and VEGFR2.

Figure 23: VEGF-C expression in primary tumours with lymphatic metastasis



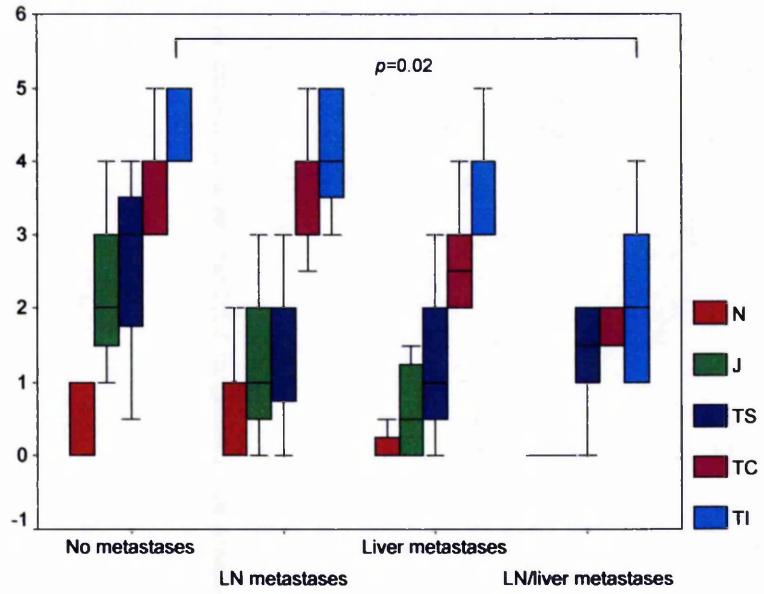
The boxplot illustrates the intensity of VEGF-C expression at the differing tumour sites. There were no differences in median intensities at each of the sites assessed between tumours with and without lymph node metastases. Comparison of medians at the TI by the Mann-Whitney U test, p=0.29.

Figure 24: VEGF-C expression in primary tumours with haematogenous metastasis



The boxplot illustrates the intensity of VEGF-C expression at 5 tumour sites. A significant difference was seen in median VEGF-C expression at the TI in tumours that did and did not develop liver metastases, Mann-Whitney U test, p=0.005. No such differences were seen for expression of VEGF-D, VEGF-A or VEGFR2.

Figure 25: VEGF-C expression in primary tumours with different metastatic routes



The boxplot illustrates the intensity of VEGF-C expression at 5 tumour sites. A significant difference was seen in median VEGF-C expression at the TI in tumours that spread by different metastatic routes, Kruskall-Wallis test, p=0.02 No such differences were seen for expression of VEGF-D, VEGF-A or VEGFR2.

3.2.7. Growth factors and receptors in metastatic sites

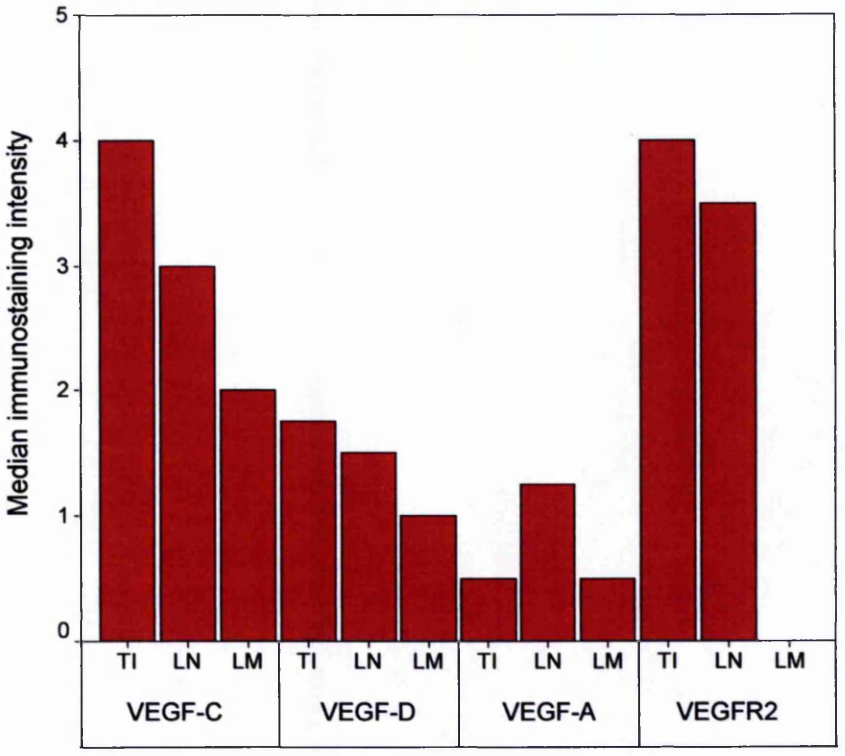
The expression of VEGF-C, VEGF-D, VEGF-A and VEGFR2 was examined in lymph node and liver metastases. The intensity of expression was assessed using the same scoring system that was used to study the primary tumours. The results are summarised in **Table 20** and **Figure 26**.

Table 20: Growth factor and receptor expression in metastatic sites and invasive tumour edge

Growth factor	TI (n=30)	Lymph node metastases (n=12)	Liver metastases (n=9)
VEGF-C			
Median (range)	4 (0.5-5)	3 (0.75-5)	2 (0.25-4)
VEGF-D			
Median (range)	1.75 (0-4)	1.5 (0.25-4)	1 (0-3)
VEGF-A			
Median (range)	0.5 (0-3)	1.25 (0.25-3.5)	0.5 (0-1.5)
VEGFR2			
Median (range)	4 (1-5)	3.5 (0-5)	0 (0-1.5)**

TI, invasive tumour edge.
** p=0.007, TI vs. liver met (Wilcoxon signed rank test)

Figure 26: Expression of growth factors/receptor in metastatic sites and at TI



TI, invasive tumour edge; LN, metastatic lymph node; LM, liver metastases

There were no statistically significant differences between median expression intensity of VEGF-C, VEGF-D, VEGF-A and VEGFR2 at the TI and in lymph node metastases. Nor were there any statistically significant differences between median expression intensity of VEGF-C, VEGF-D or VEGF-A at the TI and in liver metastases. However, VEGFR2 expression intensity was reduced in liver metastases in comparison to TI expression (Wilcoxon signed rank test, p=0.007).

3.2.8. Primary and metastatic site growth factor expression

The relationship between the intensity of expression of growth factors and receptors in the TI of primary tumours and metastatic sites were examined with Spearman rank correlation coefficients.

VEGF-C and VEGFR2 TI expression correlated with their corresponding expression intensity in metastatic lymph nodes (Spearman's rho, 0.72, p=0.009 and 0.66, p=0.02 respectively) (**Figures 27-28**) but not with expression in liver metastases. Neither VEGF-D nor VEGF-A expression in the primary TI site correlated with expression in metastatic lymph node or liver.

Just as VEGF-C and VEGFR2 expression correlated significantly at different sites within the primary tumour (**section 3.2.4, page 161**), expression between VEGF-C and VEGFR2 in metastatic lymph nodes also correlated (Spearman's rho, 0.72, p=0.009) (**Figure 29**). No correlation was seen between the expression of the two variables in liver metastases.

Figure 27: VEGF-C expression in TI and metastatic lymph nodes

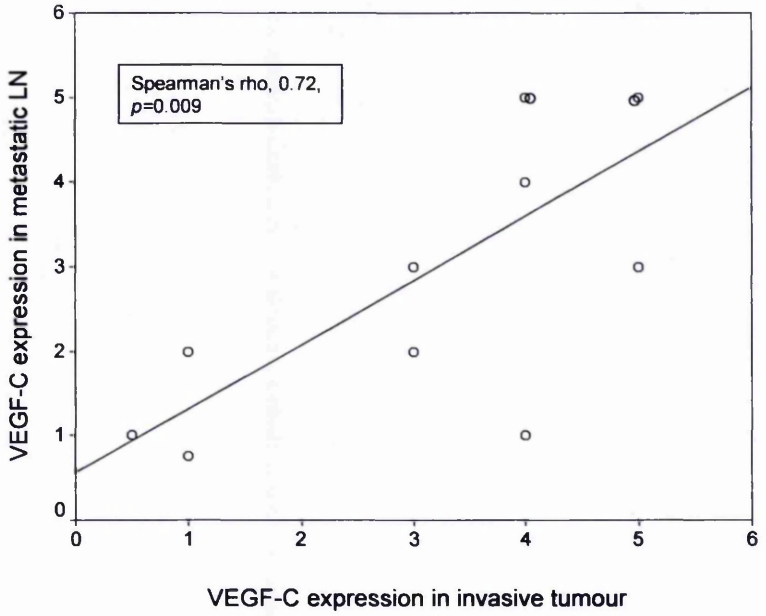


Figure 28: VEGFR2 expression in TI and metastatic lymph nodes

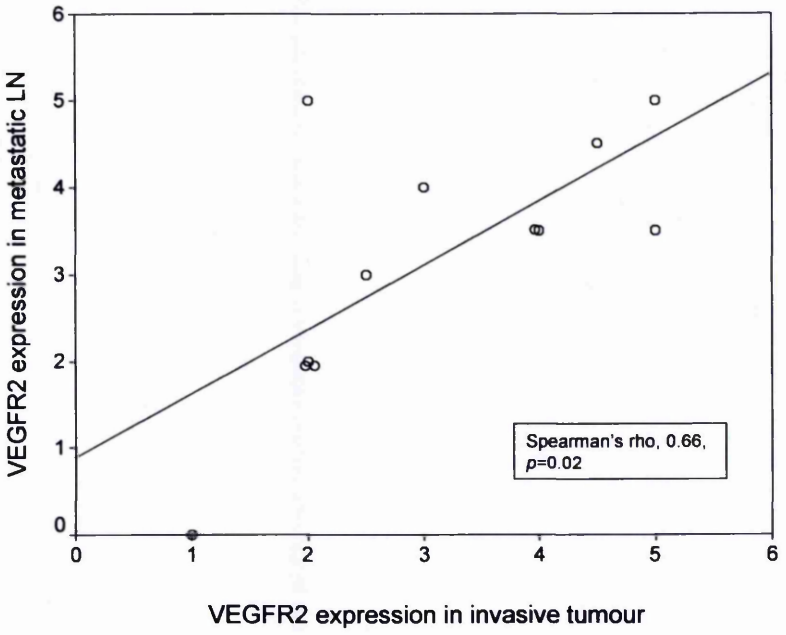
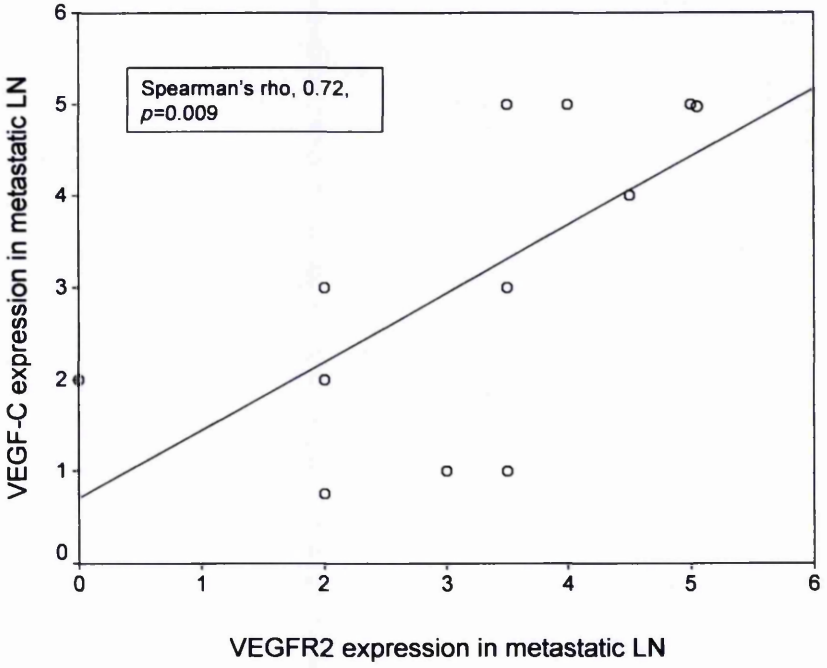


Figure 29: Relationship between VEGF-C and VEGFR2 in metastatic lymph nodes

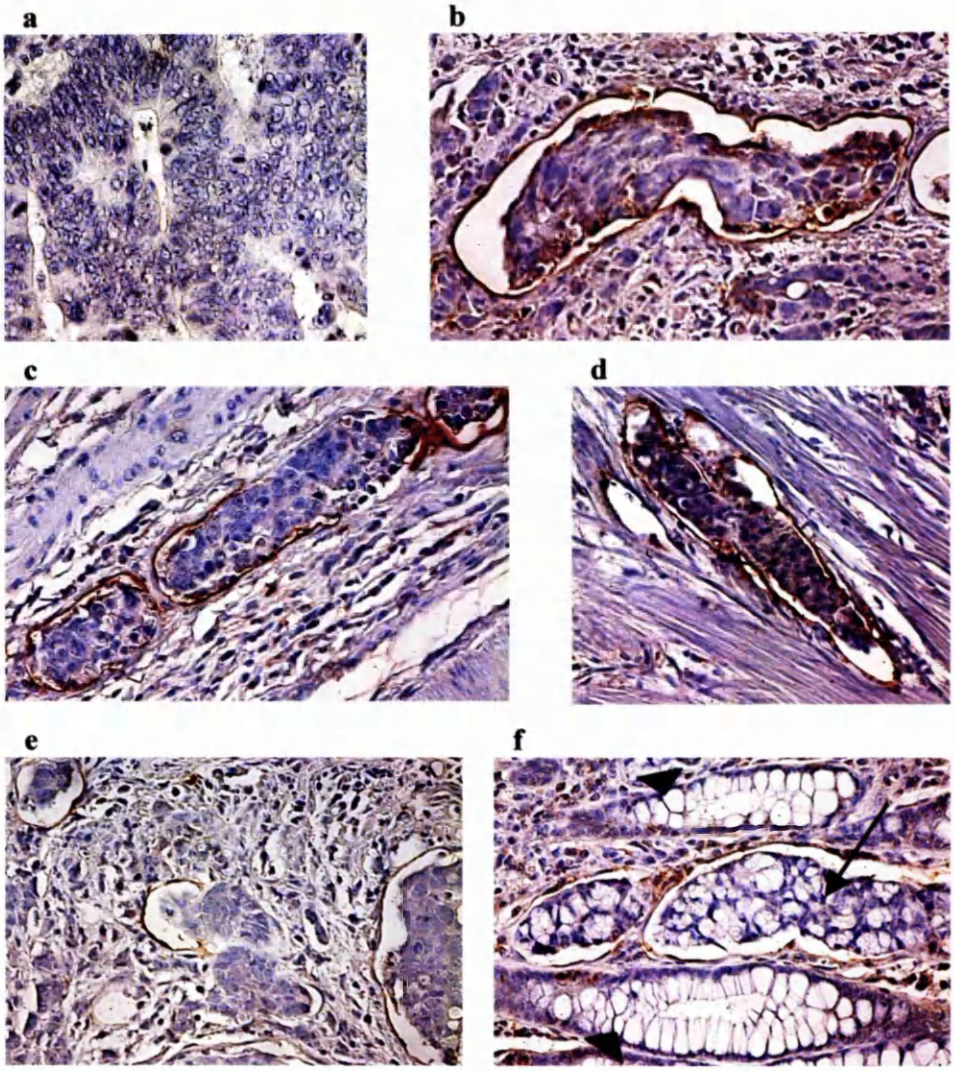


3.2.9. Lymphatic distribution in primary colorectal cancer and LVD

LYVE-1 immunostaining was used to highlight lymphatic vessels in the histological sections, in order to obtain a LVD score, to examine the relationship between LVD and primary tumour growth factor/receptor expression and to detail any differences in LVD scores in tumours with different metastatic behaviour.

LYVE-1 positive lymphatic vessels in the normal colonic mucsoa were easily identified on morphological grounds and were frequently seen in close proximity to arteries and veins, which were both LYVE-1 negative (**Figure 7a, page 140**). Thus, in paraffin embedded colorectal cancer specimens, anti-LYVE-1 antibody gave specific lymphatic vessel staining. In normal colonic mucosa, lymphatic vessels were prominent in the submucosa just beneath the muscularis mucosa. Lymphatic vessels were also frequently seen throughout the muscularis propria, clustering in particular between the muscle layers (**Figure 7b, page 140**). In a few colorectal cancers, tumour foci were seen within intra-tumoural or peri-tumoural lymphatic vessels (**Figure 30 b-f**). In the majority of cases, lymphatic vessels were located in the peri-tumoural area rather than within the tumour itself.

Figure 30: Colorectal cancer within LYVE-1 positive lymphatic vessels



Sections were immunostained for LYVE-1 and counterstained with haematoxylin. a, moderately differentiated colonic adenocarcinoma, negative control, x100; b-f, malignant cells within LYVE-1 positive lymphatic vessels - b-e, poorly differentiated rectal carcinomas, x100; f, a poorly differentiated mucinous adenocarcinoma, mucinous adenocarcinoma cells can be seen within LYVE-1 positive lymphatics (arrowed), sandwiched between normal colonic mucosal crypts (arrowheads), x100.

LVD was determined by Chalkley grid counting in lymphatic vessel hotspots within the tumour (if present) or in the immediate tumour vicinity. In addition, tumours were examined closely for the presence or absence of LYVE-1 positive lymphatic vessels within the three tumour areas – superficial (TS), central (TC) and invasive edge (TI) (**Table 21**).

Table 21: Frequency of LYVE-1 positive lymphatic vessels within primary colorectal tumours

LYVE-1 positive vessels	**TS (%)**	**TC (%)**	**TI (%)**
Absent	19 (63)	25 (83)	21 (70)
Present	11 (37)	5 (17)	9 (30)

Lymphatic vessels were present in the TS (37%) and TI (30%) areas of the tumour more frequently than the TC (17%). The sites where lymphatic vessels were present within the superficial tumour were typically at the border between normal tissue and tumour, these lymphatics could conceivably have been pre-existing vessels which had been incorporated into the tumour margin. It was noteworthy that LYVE-1 positive intra-tumoural lymphatics were small with closed or narrow lumens in comparison to the larger lymphatic vessels identified outside the tumour boundaries.

3.2.10. LVD, MVD, clinicopathological variables and route of metastasis

The median LVD count was significantly lower than the median MVD count (median LVD, 1.125 (range 0-2.75) vs. median MVD 4.0 (1.5-7.375); Wilcoxon signed rank test, $p<0.001$) but the two scores correlated within individual primary tumours (Spearman's rank correlation coefficient, 0.41, $p=0.025$) (**Figure 31**). The relationship between LVD, MVD and clinicopathological variables is summarised in **Table 22**. No significant differences between LVD and any variable or route of metastasis was identified. In particular, no differences were observed for median LVD between tumours with and without lymph node involvement ($p=0.82$) or lymphatic invasion ($p=0.57$). MVD was higher in tumours of more advanced Dukes' stage (early disease vs. late disease: Mann-Whitney U test, $p=0.023$) (**Figure 32**), but no other significant differences between MVD and clinicopathological variables were noted.

Figure 31: Correlation of LVD and MVD in primary colorectal cancers

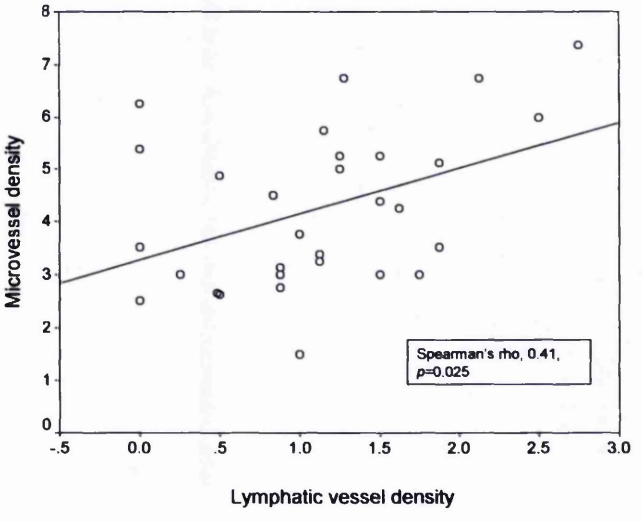


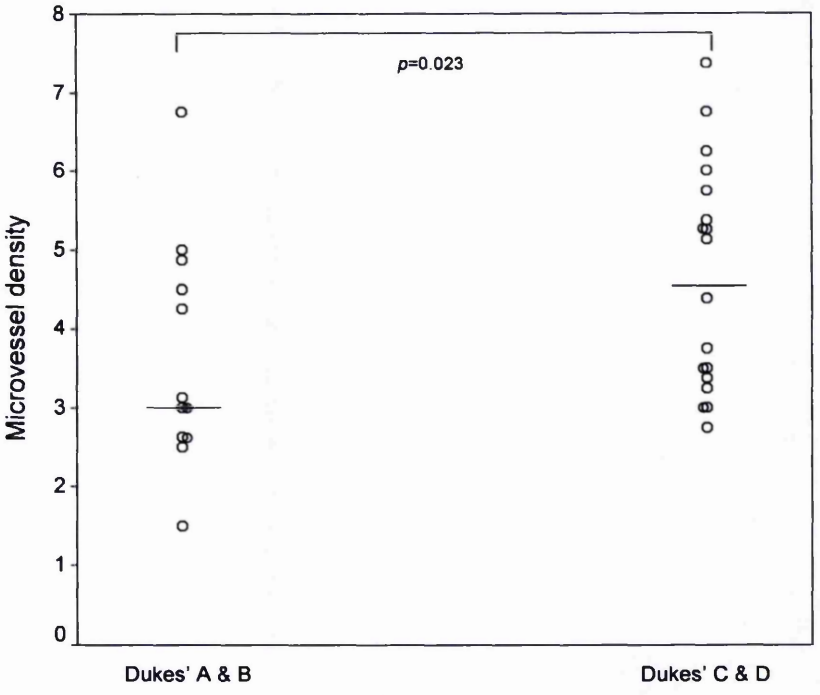
Table 22: LVD, MVD and clinicopathological variables

Parameter	Number of cases	LVD	*p*-value	MVD	*p*-value
Dukes' stage					
Dukes' A & B	12	1.0 (0-1.75)	0.58	3.0 (1.5-6.75)	0.023*
Dukes' C & D	18	1.125 (0-2.75)		4.5 (2.75-7.38)	
N stage					
N0	14	1.125 (0-1.88)	0.82	3.69 (1.5-6.75)	0.13
N1 & N2	16	1.125 (0-2.75)		4.06 (2.75-7.38)	
Differentiation					
Moderately differentiated	22	1.2 (0-2.13)	0.32	4.31 (1.5-6.75)	0.45
Poorly differentiated	8	0.69 (0-2.75)		3.0 (2.63-7.38)	
Lymphatic invasion					
Present	8	0.94 (0-2.75)	0.57	4.1 (2.5-7.38)	0.32
Absent	10	1.2 (0.5-1.88)		3.3 (1.5-5.25)	
Vascular invasion					
Present	9	0.875 (0-2.75)	0.65	4.375 (2.5-7.38)	0.19
Absent	12	1.125 (0.5-1.88)		3.19 (1.5-5.25)	
Liver metastases					
Present	10	1.06 (0-1.88)	0.45	4.68 (3.25-6.25)	0.18
Absent	20	1.14 (0-2.75)		3.25 (1.5-7.38)	
Route of metastasis§					
None	9	0.94 (0-1.88)	0.71§	2.875 (1.5-6.75)	0.098§
LN	11	1.33 (0-2.75)		4.81 (3-7.38)	
Liver	5	1.25 (0.5-1.63)		4.9 (4.25-5.25)	
LN & liver	5	1.0 (0-1.88)		3.75 (3.25-6.25)	

Values are median (range). Except where stated, all *p*-values relate to Mann-Whitney U tests. § Kruskall-Wallis test. LVD, lymphatic vessel density; MVD, microvessel density; N, nodal stage; LN, lymph node. Within each subgroup, the number of cases varied between 18 and 30, this was due to lack of some aspects of pathological data for all cases.

* denotes a significant *p* value of <0.05.

Figure 32: MVD and Dukes' stage



Horizontal lines represent the median values. MVD scores were significantly different between early and late disease; Mann-Whitney U test, p=0.023.

3.2.11. Lymphatic vessel presence, LVD, MVD & VEGF family expression

The relationship between lymphatic vessel presence, LVD, MVD and expression of VEGF-A, VEGF-C, VEGF-D and VEGFR2 at the TI site was explored using Spearman's rank correlation coefficients. Although statistically significant, albeit weak, correlations between the TI expression of VEGF-C and LVD (Spearman's rho, 0.36, p=0.048) and VEGF-A and MVD (Spearman's rho, -0.42, p=0.022) were found, the scatterplots showed wide variation that limited the interpretation of these findings (**Figures 33-34**).

To examine whether the presence of LYVE-1 positive lymphatic vessels in the deep part of the tumour was associated with particular patterns of growth factor expression, the group was assessed with respect to the presence or absence of these vessels (see **Table 21, page 174**). No differences were seen in TI expression of VEGF-A or VEGFR2 in relation to the presence of lymphatic vessels within the TI. Median TI VEGF-C expression was higher (Mann-Whitney U test, p=0.028) and VEGF-D expression was lower (p=0.011) in tumours with deep intra-tumoural lymphatic vessels present (**Figures 35-36**).

In the group as a whole, expression of both VEGF-C and VEGF-D increased towards the deep part of the tumour, the presence of deep intra-tumoural lymphatics however, was associated with lower VEGF-D and higher VEGF-C expression at the TI site.

Figure 33: VEGF-C expression at TI and LVD

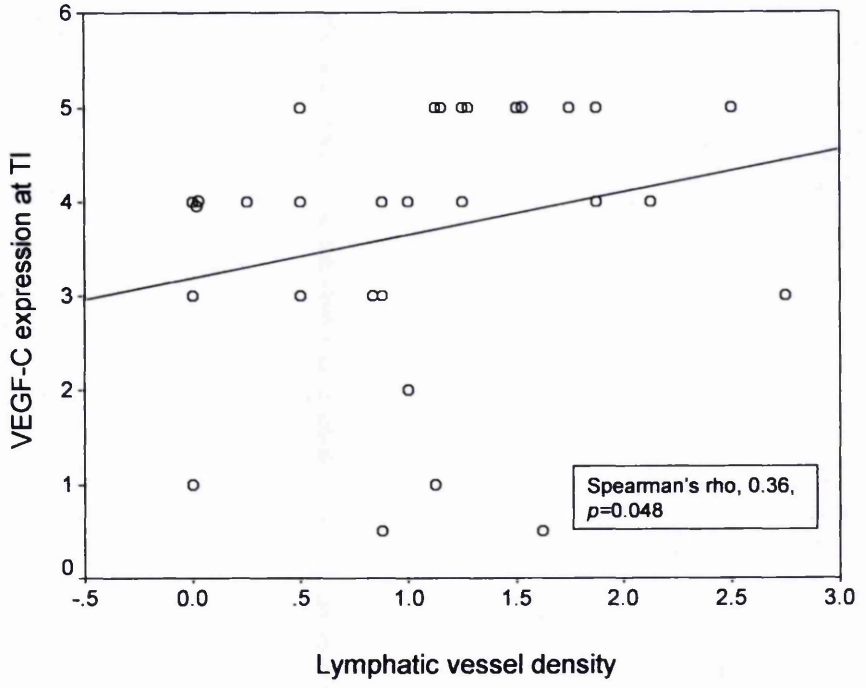


Figure 34: VEGF-A expression at TI and MVD

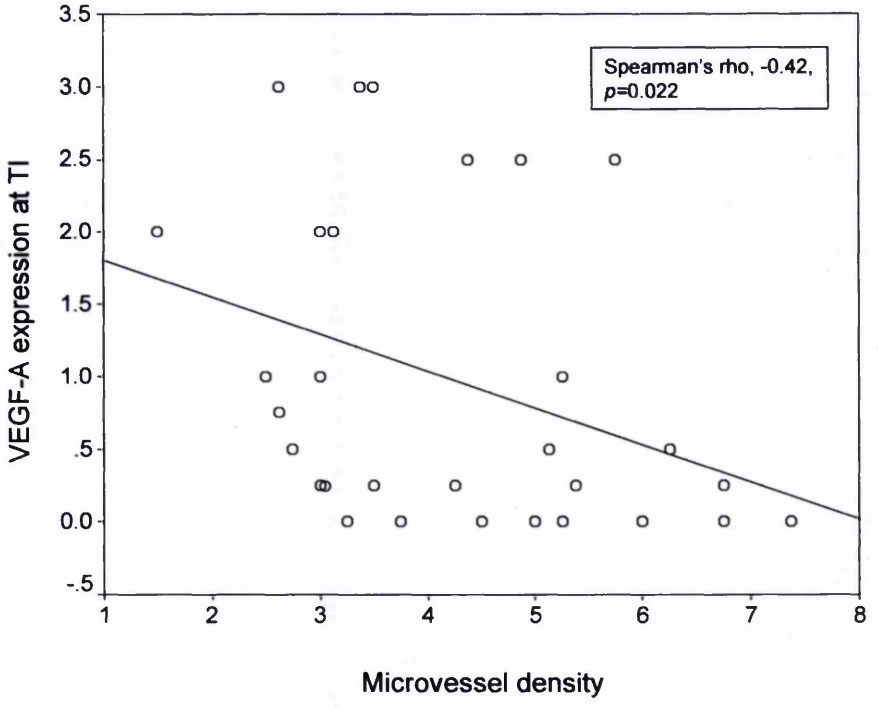
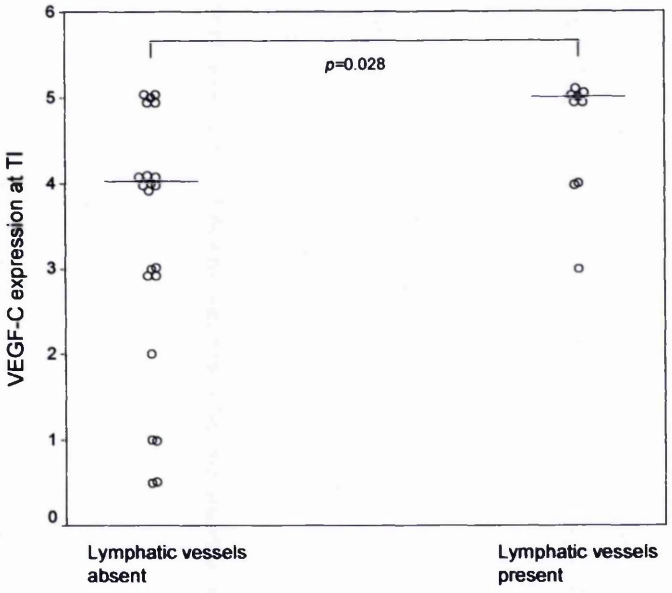
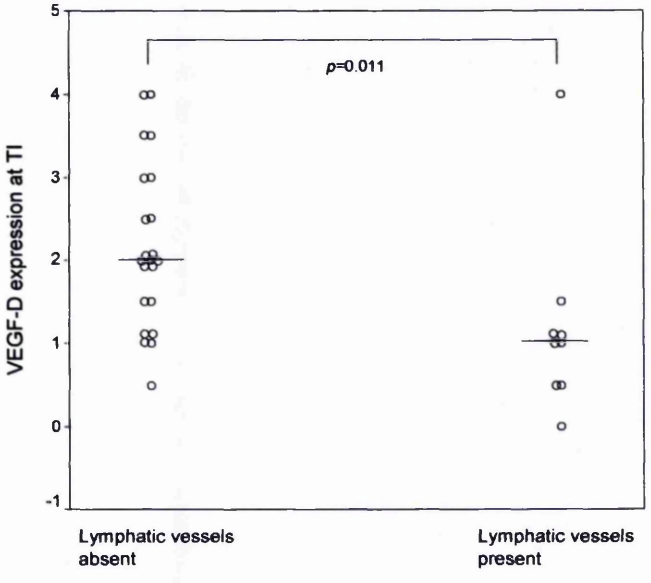


Figure 35: VEGF-C expression at TI and presence of deep intra-tumoural lymphatics



Horizontal lines represent the median values. Median VEGF-C expression at the TI was higher in the presence of deep intra-tumoural lymphatics; Mann-Whitney U test, p=0.028.

Figure 36: VEGF-D expression at TI and presence of deep intra-tumoural lymphatics



Horizontal lines represent the median values. Median VEGF-D expression at the TI was lower in the presence of deep intra-tumoural lymphatics; Mann-Whitney U test, p=0.011.

3.3. SUMMARY OF RESULTS

This series of immunohistochemical experiments demonstrated that:

i) the pattern of expression of the VEGF family members, VEGF-C, VEGF-D and VEGF-A, increased from normal tissue adjacent to colorectal cancer, through the junctional mucosa and throughout the tumour to a maximum at the invasive tumour edge.

ii) VEGFR2 was expressed in colorectal cancer cells and the pattern of expression reflected that of its ligands, increasing to a maximum at the invasive tumour edge.

iii) the expression of VEGF-C and VEGFR2 correlated with one another and co-localised throughout colorectal cancer and in metastatic lymph nodes but not in liver metastases.

iv) the expression of VEGF-C and VEGF-D did not correlate within colorectal cancer.

v) no differences in the pattern of expression of VEGF-C, VEGF-D, VEGF-A or VEGFR2 in primary tumours were seen in early or late colorectal cancers or in those cancers that spread to the lymph nodes.

vi) colorectal cancers that spread to the liver had a lower level of VEGF-C expression at the invasive tumour edge in the primary tumour in comparison to tumours that did not spread to the liver.

vii) VEGF-C and VEGFR2 expression in the invasive edge of primary colorectal cancers correlated with expression of the same antigens in metastatic lymph nodes.

viii) the expression of VEGFR2 was reduced in liver metastases in comparison to expression at the invasive edge of the primary tumour.

ix) LYVE-1 antibodies clearly stained lymphatic vessels in and around colorectal cancer specimens and LVD scores could be determined using this method of staining.

x) lymphatic vessels within colorectal cancers were an uncommon finding and the presence of deep intra-tumoural lymphatics was associated with increased expression of VEGF-C and decreased expression of VEGF-D in the invasive edge of the tumour.

xi) no associations between LVD and clinicopathological factors were identified.

4. RESULTS: IMMUNOASSAYS

4.1. DEVELOPMENT OF VEGF-C ELISA

The possibility of detection of VEGF-C in body fluids has only been addressed recently (Tamura & Ohta, 2003). However, in common with VEGF-A, VEGF-C is contained within platelets (Wartiovaara et al., 1998) and therefore may be detectable in serum and plasma. Since VEGF-C expression in colorectal cancer is associated with negative clinicopathological characteristics (Akagi et al., 2000; Furodoi et al., 2002), measurement of circulating levels of VEGF-C may provide a surrogate marker for these variables and predict patients with or at risk of lymphatic involvement. The following results describe the development and validation of an ELISA for VEGF-C (**Appendix II**, Duff et al., 2003c).

4.1.1. Detection of VEGF-C

The ELISA for VEGF-C was performed as described in **section 2.2.1, page 122**. The optimum signal detected was achieved with a coating antibody concentration of 1μg/ml and a detection antibody concentration of 0.5μg/ml. Use of the biotin-avidin system directed against the detection antibody doubled the light emission signal detected. The approximate sensitivity of the assay was 0.4 units/ml, which represented twice the background level and the linear detection range for VEGF-C was up to 100 units/ml.

4.1.2. Quality assessment

Intra-assay and inter-assay variation

The intra-assay variation was assessed using the same quantity of standard plasma in 22 wells on a single plate. The inter-assay variation was measured using the same quantity of plasma over eight separate experiments. The coefficient of variation (CV) was calculated by dividing the standard deviation by the mean and multiplying by 100. The intra-assay and inter-assay CV measured 5.2% and 7.7% respectively (**Table 23**). These CV values are comparable with those obtained for serum/plasma assays with commercially available ELISA kits eg. VEGF-A Quantikine® ELISA (R & D Systems), intra-assay CV up to 6.7% and inter-assay CV up to 8.8% and VEGF-D Quantikine® ELISA (R & D Systems), intra-assay CV up to 6.2% and inter-assay CV up to 8.0%.

Table 23: Intra- and inter-assay variations for the VEGF-C ELISA

	N	Mean ± SD (U/ml)	CV (%)
Intra-assay	22	33.57 ± 1.74	5.2
Inter-assay	8	25.91 ± 2.00	7.7

CV, coefficient of variation

Specificity

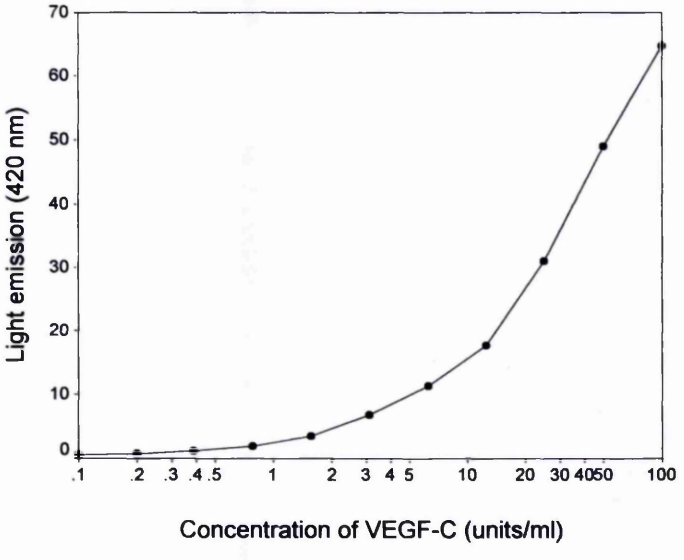
Specific binding of the antibodies to VEGF-C in standard plasma was demonstrated by substitution tests. Substitution of standard plasma by recombinant VEGF-A, VEGF-D, TGFβ1, TGFβ3 or IL-6 showed no detectable signal above background. Substitution of the capture antibody, goat anti-human VEGF-C, by alternative capture antibodies

(mouse monoclonal antibodies directed against human TGFβ1, TGFβ3, CD105, CD31 and CD34) resulted in no detectable signal above background.

4.1.3. Standard curve for VEGF-C

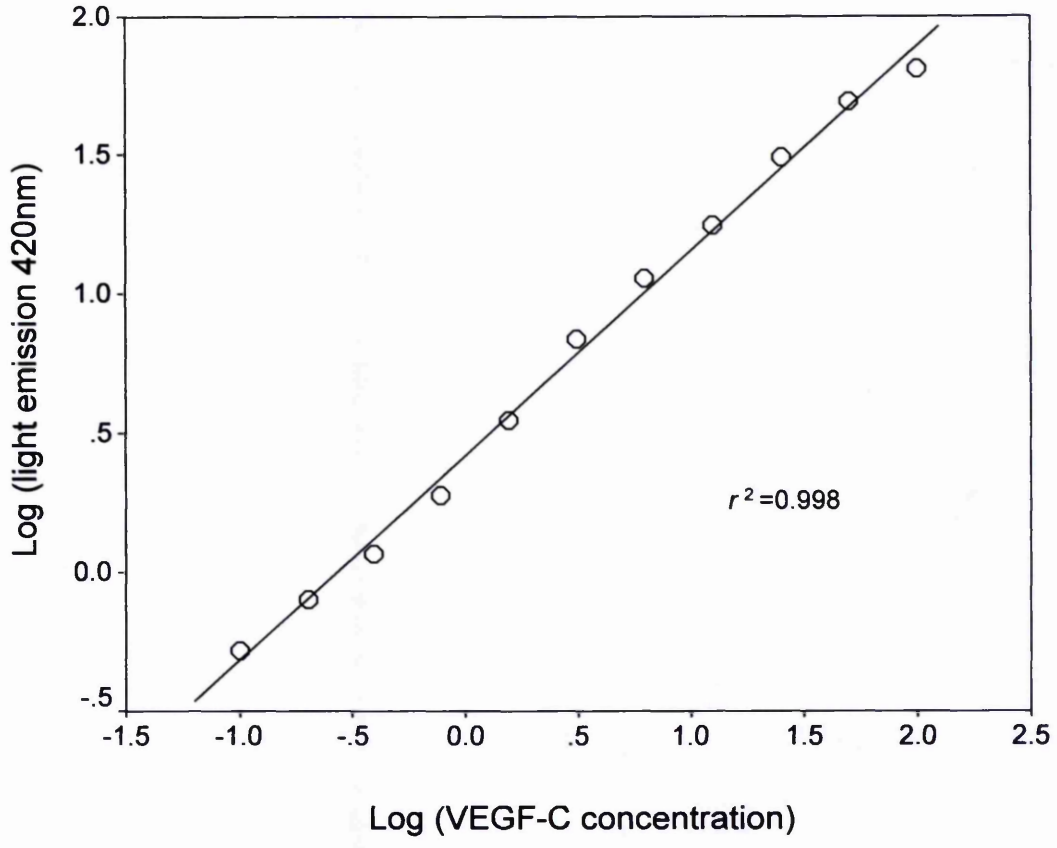
In order to calculate the concentrations of VEGF-C in test plasma, a standard curve was included on each plate by serial dilution of the standard plasma (**Figure 37**). In most of the assays, the curve fit was 100% and was never less than 99%. Logarithmic transformation of the concentration and optical emission data produced a straight line plot (**Figure 38**). From these reference curves, the measured values of light emission of the test samples were automatically converted into concentrations in units per milliliter using regression techniques by an in–house computer attached to the plate reader (courtesy of Dr Philip Wilson, Department of Immunology, St Mary's Hospital, Manchester).

Figure 37: Standard curve of VEGF-C concentration in standard plasma



Standard plasma (100 units/ml) was serially diluted and the curve of concentration against light emission plotted.

Figure 38: Standard curve linearised by $\log_{10}$ conversion



Conversion of the VEGF-C concentration and light emission variables by logarithmic transformation produced a straight line (Pearson's correlation coefficient, r^2=0.998, p<0.001). The mean of the duplicate light emissions of each sample was converted into units/ml according to the standard curve on each plate.

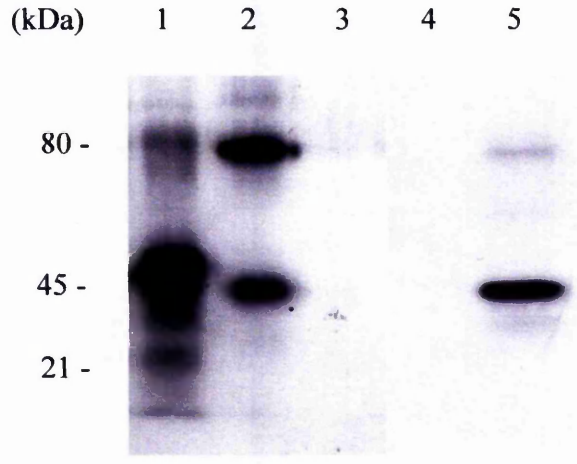
4.1.4. Molecular forms of VEGF-C in standard plasma

In order to increase the signal detected by the ELISA, a biotin-avidin amplification step was used. It was essential to use antibodies derived from different species for both capture and detection steps. As the detection antibody, rabbit anti-human VEGF-C IgG_1 was unable to detect the only currently available recombinant human VEGF-C peptide (R & D Systems, Abingdon, UK), it was not possible to create a standard curve on each plate using this peptide. To validate the use of standard plasma to create the standard curve, this plasma was subjected to immunoprecipitation and immunoblotting in an attempt to analyse the molecular forms of VEGF-C detected in plasma and ensure that these forms were recognised by both antibodies used in the ELISA.

Goat polyclonal anti-human VEGF-C antibody (the ELISA capture antibody) was used for immunoprecipitation. Negative controls employed goat anti-human IgG as the antibody for immunoprecipitation. Detection of the precipitated species on the gel was made by goat anti-human VEGF-C or rabbit anti-human VEGF-C antibodies. Recombinant human VEGF-C (R & D) was also run on the gel (as a positive control) to demonstrate reactivity with the goat but not the rabbit anti-human VEGF-C (further demonstrating the lack of effectiveness of recombinant human VEGF-C as a standard in this sandwich ELISA). The results of immunoprecipitation and immunoblotting with goat anti-human VEGF-C antibody are shown in **Figure 39**. Recombinant VEGF-C (lane 1), had a major band of ~45 kDa, and weaker bands of approximately 15, 23 and 80 kDa. Standard plasma from the immunoprecipitation experiment with goat anti-human VEGF-C antibody is seen in lane 2. Two bands had a molecular weight of 40 and 80 kDa, no bands were present in lane 3 where the negative control goat anti-human IgG was used as the antibody for immunoprecipitation. Lanes 4 and 5 show the results of immunoblotting with rabbit anti-human VEGF-C antibody, recombinant VEGF-C was not detectable in lane 4 (as this does not react with the rabbit antibody used in

detection) and standard plasma was seen in lane 5 as a band of approximately 40 kDa and a weaker band of ~80 kDa. These bands were identical in size to those detected using goat anti-human VEGF-C antibody for immunoprecipitation and detection (lane 2), demonstrating that both the goat and rabbit anti-human VEGF-C antibodies used in the indirect sandwich ELISA detect the same species of VEGF-C in plasma.

Figure 39: Immunoprecipitation and immunoblotting of VEGF-C in standard plasma



Samples were immunoprecipitated with goat anti-human VEGF-C antibody (lanes 2, 5) or negative control antibody (goat anti-human IgG, lane 3). Lanes 1-3, VEGF-C was recognised by goat anti-human VEGF-C antibody and revealed by rabbit anti-goat antibody conjugated to HRP. Lanes 4-5, VEGF-C was recognised by rabbit anti-human VEGF-C antibody and revealed by goat anti-rabbit antibody conjugated to HRP. The blots were developed with ECL and the exposure time was 15 seconds.

- Lane 1 - recombinant VEGF-C loaded separately onto the gel, with a main band of ~45 kDa, and weaker bands of ~15, 23 and 80 kDa.
- Lane 2 - standard plasma, VEGF-C is detected as 2 bands of ~40 and ~80 kDa.
- Lane 3 - standard plasma immunoprecipitated with goat anti-human IgG as a negative control, no bands are visualised.
- Lane 4 - recombinant VEGF-C loaded separately onto the gel, no bands are detected (as the rabbit anti-human VEGF-C antibody does not recognise the recombinant protein).
- Lane 5 - standard plasma, a strong band of ~40 kDa and a weaker band of ~80 kDa is detected.

This experiment confirmed the reactivity of the goat anti-human VEGF-C IgG (capture antibody) with the recombinant VEGF-C against which it was raised and showed that the rabbit anti-human VEGF-C IgG (detection antibody) failed to react with this protein (**Figure 39,** lanes 1 and 4). This is likely to be due to differences in protein structure between the native and recombinant forms that are required for antibody binding. However, both capture and detection antibodies recognised VEGF-C species of similar molecular weights in the standard plasma (**Figure 39,** lanes 2 and 5), thus validating its use for creation of a standard curve on the ELISA plate.

4.1.5. VEGF-C in serum and plasma from normal controls

The immunoassay was employed to measure the levels of VEGF-C in serum and plasma samples from healthy individuals. Plasma samples were available for 31 individuals and serum samples from 40 individuals. Matched pairs of plasma and serum were available in 19 cases. The results are shown in **Table 24.**

Table 24: Serum and plasma VEGF-C levels in normal controls

	Serum VEGF-C	**Plasma VEGF-C**
Number of cases	40	31
Median age (range)	39 (22-69)	37 (20-71)
Gender	13 male, 27 female	12 male, 19 female
Mean value U/ml (± S.D.)	28.2 (± 19.1)	12.2 (± 4.1) **
Median value U/ml (range)	22.7 (6.3-81.4)	11.5 (5.4-21.5)

** $p<0.001$, paired t-test

The values of serum and plasma VEGF-C obtained in controls fitted a normal distribution (Kolmogorov-Smirnov test, $Z=1.29$, $p=0.07$ and $Z=0.62$, $p=0.083$

respectively). Consequently, the difference in means within and between groups was made with the paired and independent samples t-tests and correlations between continuous data made with the Pearson correlation coefficient.

The mean levels of plasma VEGF-C were significantly lower than serum VEGF-C in matched pairs of samples (paired t-test, t = -4.318, df =18, $p<0.001$). There was no significant difference in mean levels of plasma or serum VEGF-C with gender (independent samples t-test, $p=0.486$ and $p=0.328$ respectively) and there was no correlation between plasma or serum VEGF-C levels and age ($p=0.277$ and $p=0.761$ respectively).

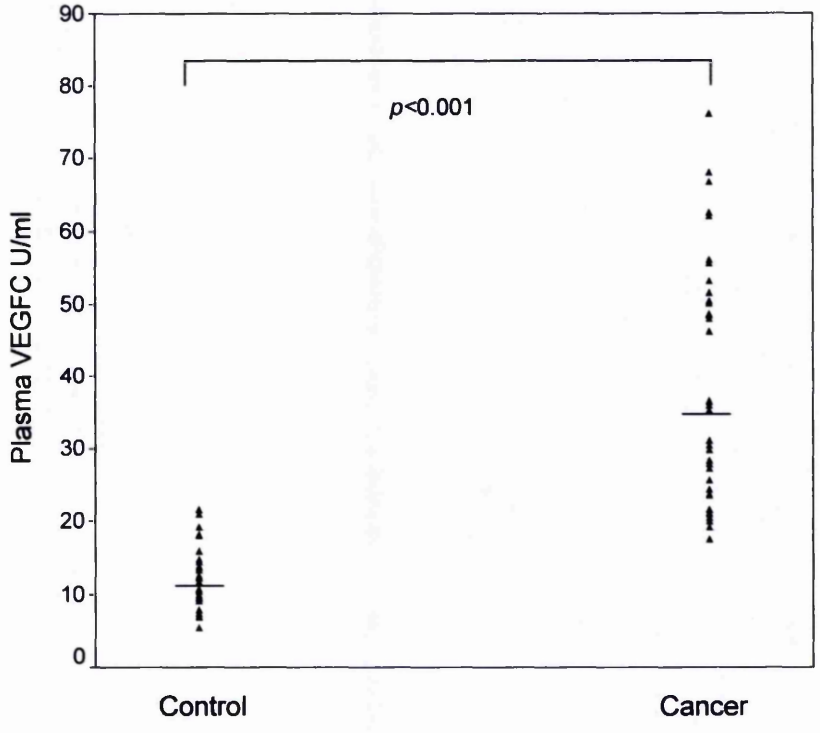
4.1.6. VEGF-C in plasma from cancer patients and normal controls

The immunoassay was employed to measure the levels of VEGF-C in plasma samples from 41 colorectal cancer patients and 31 healthy controls as a preliminary investigation of the assay. The values of plasma VEGF-C obtained in the group as a whole were not consistent with a normal distribution (Kolmogorov-Smirnov test, Z= 1.41, $p=0.039$). Consequently, differences between groups were examined with non-parametric tests. The Mann-Whitney U test was used to compare medians between two independent groups and the Kruskal-Wallis test to compare medians of more than two groups.

The median age of the colorectal cancer patients was 65 years (range, 36-87 years). There were 23 females and 18 males. The levels of VEGF-C were significantly elevated in plasma from colorectal cancer patients, 35.0 units/ml (17.4 – 75.9 units/ml) compared to normal controls 11.5 units/ml (5.4 – 21.5 units/ml) ($p<0.001$) (**Figure 40**). The colorectal cancer patients were divided into groups of early [n=14, Dukes' A (n=5) and B (n=9)] and advanced disease [n=27, Dukes' C (n=5) and D (n=22)]. The median VEGF-C levels increased from 24.25 U/ml in the early disease group to 35.8 U/ml in

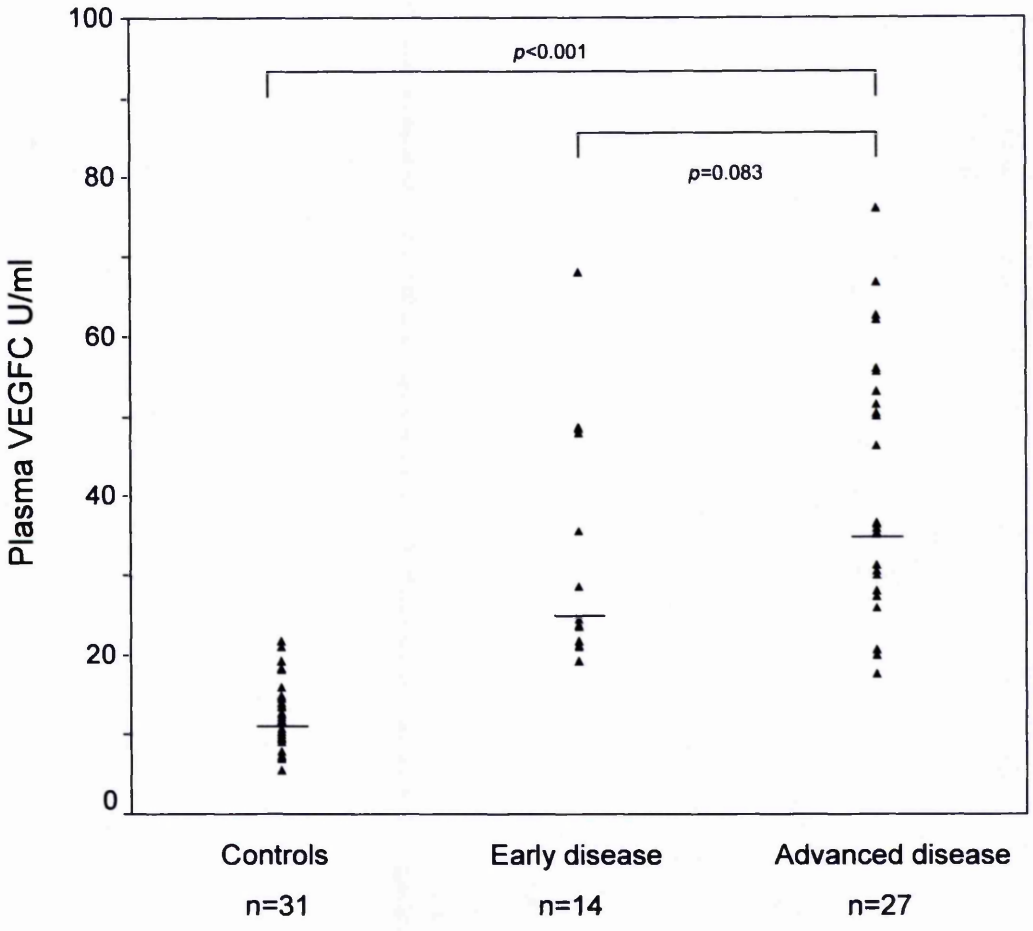
the advanced disease group, but this was not statistically significant (p=0.083) (**Figure 41**).

Figure 40: Plasma levels of VEGF-C in cancer patients and controls



Plasma levels of VEGF-C in normal individuals (n=31) and colorectal cancer patients (n=41) were determined by ELISA. Significantly elevated levels were found in cancer patients ($p<0.001$, Mann-Whitney U test). Horizontal lines indicate the medians.

Figure 41: Plasma levels of VEGF-C in controls and early and late stage colorectal cancer groups



Plasma levels of VEGF-C in normal individuals and colorectal cancer patients divided into early disease (Dukes' A and B, n=14) and advanced disease (Dukes' C and D, n=27). Significant differences were found between the controls and the cancer groups (Kruskall-Wallis test, $p<0.001$), but not between early vs. advanced disease (Mann-Whitney U test, $p=0.083$, NS. Horizontal lines indicate the medians.

4.1.7. Discussion - VEGF-C ELISA development

Development of ELISA

The sensitivity of the newly developed indirect ELISA was optimised by the use of an amplification step based on the avidin-biotin system and the enhanced chemiluminescence method substituted for conventional colorimetric ELISA. Intra- and inter-assay variations were minimal and compared well with other similar assays (Wang et al., 1994b; Li et al., 1998). Immunoprecipitation and immunoblotting confirmed the presence and detection of VEGF-C in standard plasma using the two antibodies employed in the ELISA system. The two main bands detected were likely to represent circulating dimers and tetramers of mature VEGF-C and partially processed forms of the parent molecule.

Plasma versus serum samples

Plasma levels of VEGF-C were found to be lower than their paired serum levels in a control group. This is analogous to the situation found in the measurement of VEGF-A, where serum levels of VEGF-A are consistently higher than plasma levels due to release from platelets and other cellular components (see **section 1.4.4, page 60**). The increased serum level of VEGF-C observed is also likely to be due to cytokine release secondary to platelet activation and degranulation (Wartiovaara et al., 1998).

Potential for clinical use

The potential clinical use of the VEGF-C ELISA was assessed in a small group of colorectal cancer patients and controls. Increased VEGF-C levels were found in the plasma of colorectal cancer patients in comparison to controls, although levels were not significantly different between groups of patients with early and advanced disease.

Problems associated with ELISA development

Problems were encountered with the absolute quantitation of cytokine levels, the comparability between groups and handling of samples.

The ELISA expressed levels of VEGF-C in relative rather than absolute units. The antibody combination used in the sandwich ELISA had to be derived from different species to allow the addition of an amplification step using a biotinylated secondary antibody to increase the sensitivity of detection. The only recombinant VEGF-C peptide available was that to which the goat anti-VEGF-C antibody used for capture in the ELISA was raised. This recombinant peptide was not recognised by the detection anti-VEGF-C antibody. Consequently, although the antibody pair recognised the same VEGF-C forms in the standard plasma, as demonstrated by immunoprecipitation and immunoblotting (see **Figure 39, page 188**), they were unable to recognise the recombinant peptide when used as a pair. The ELISAs therefore used standard plasma to create a standard curve and VEGF-C levels were expressed as units/ml.

In the control group, no significant correlations were seen between age and plasma or serum VEGF-C levels; nor were any significant differences found between the mean VEGF-C levels between males and females in this group. However, the control and cancer groups differed in important ways, which may have limited their comparability and relevance to the general population. The healthy controls had a median age of 39 years, whereas, the median age of the colorectal cancer patients was 65 years. The gender distribution of both groups was similar (61% female in control group, 56% in cancer group). However, this was not reflective of the general colorectal cancer population, which tends to have more males affected.

Sample handling was different between the groups. The colorectal cancer patient samples had been stored frozen at -80°C for 2 years prior to use and had been thawed a

variable number of times (ranging from 0 to 2). The influence that this may have had on the plasma levels of VEGF-C was not investigated.

Further evaluation and clinical assessment of VEGF-C ELISA

In view of the promising initial results observed for the measurement of VEGF-C in colorectal cancer patients and controls, a prospective study was planned. This study aimed to eliminate the confounding issues discussed of age and sex discrepancy between groups and differences in sample handling. Based on the pilot data above, a sample size calculation was undertaken for a prospective study (Medical Statistics Department, Christie Hospital). Assuming that the difference between patients and controls was as large as that seen in the pilot study, 50 controls were needed and to detect a difference in the $\log_{10}$ means between early and advanced cancers of 0.1 (common standard deviation 0.182), 120 patients would be required, split approximately into 1/3 early disease and 2/3 advanced disease. This number of patients would give the study an 80% power to detect such a difference between the means of early and advanced disease, in a two-tailed test at the 5% level of significance.

The prospective study selected plasma rather than serum as the optimal sample for VEGF-C level assessment. The rationale for this selection was that the preliminary pilot study had demonstrated differences between cancer patients and controls based on plasma samples, and that evaluation of serum levels would be complicated by the contribution of platelet VEGF-C to the overall level.

4.2. IMMUNOASSAYS IN COLORECTAL CANCER

The aim of this group of studies was to assess prospectively:

1) pre-operative plasma growth factor levels in primary colorectal cancer and controls as predictors of disease stage and clinicopathological characteristics, particularly lymphatic involvement.

2) pre-operative plasma growth factor levels in patients with hepatic colorectal cancer metastases and controls, any alteration with time in patients undergoing hepatic metastatectomy and the relationship between circulatory and tissue growth factor expression.

Immunoassays for plasma levels of VEGF-A, VEGF-C and VEGF-D were performed.

4.2.1. Pre-operative colorectal cancer patient/control group

One hundred and twenty colorectal cancer patients and 50 controls were recruited (see **section 4.1.7, page 195**). The patient and control groups were comparable in their age and gender distributions (**Table 25**).

Table 25: Demographics of pre-operative colorectal cancers and controls

	Control group	**Cancer group**	***p*-value**
Number of cases	50	120	
Mean age (range)	62 years (40-79)	65 years (25-84)	0.103 §
Gender	29 male, 21 female	81 male, 39 female	0.238 ^

§ independent samples t-test
^ χ^2 test

Referral patterns and types of radiotherapy received by cancer patients

The 120 colorectal cancer patients originated from 16 NHS Trusts, with a range of 1-18 patients per Trust (**Figure 42**). Of the 120 patients, 111 (93%) were referred to the clinical oncology services at the Christie Hospital NHS Trust for consideration of pre-operative short-course radiotherapy for rectal cancer. The remaining 9 patients were recruited from South Manchester University Hospitals NHS Trust prior to undergoing surgery for colon or rectal cancer and did not undergo radiotherapy (**Table 26**). The type of radiotherapy treatment that patients eventually received was dependent on the full results of staging investigations. Consequently, a few patients referred for short-course radiotherapy received long-course or palliative treatment, when their disease stage was found to be more advanced than that expected from the initial assessment.

Figure 42: The source of colorectal cancer patients

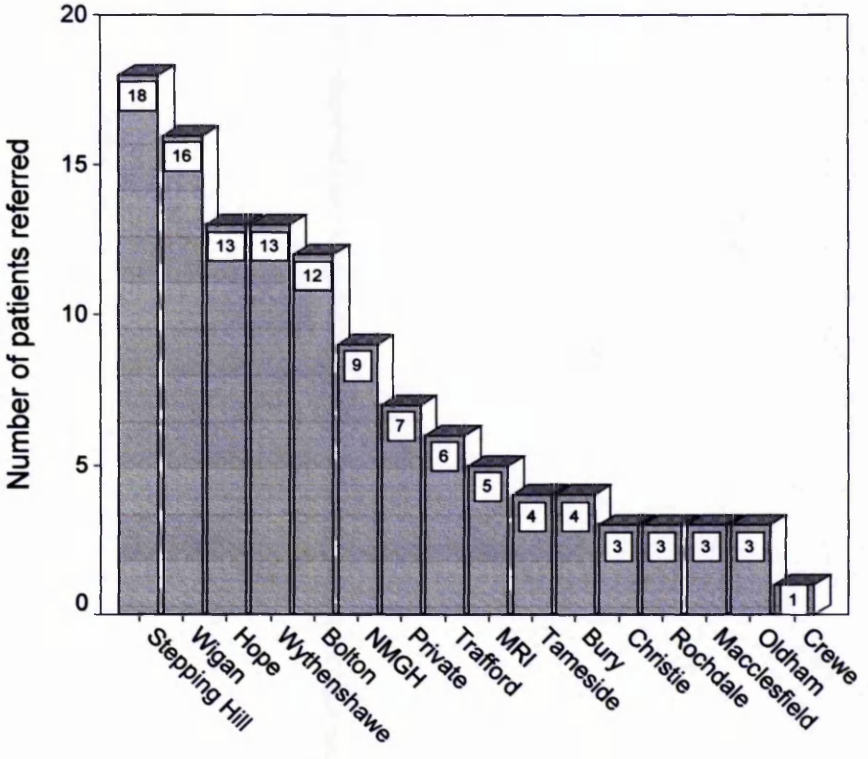


Table 26: Radiotherapy received by cancer patients

Type of radiotherapy	Number of cases (%)
Short-course radiotherapy	104 (86.7)
Long-course radiotherapy	4 (3.3)
Palliative radiotherapy	3 (2.5)
No radiotherapy	9 (7.5) - 3 rectal cancers - 3 hepatic flexure cancers - 3 sigmoid cancers

Short-course radiotherapy was given as a 4-field brick and consisted of a total dose of 20 Gy (4 fractions of 5 Gy on consecutive days) (Marsh et al., 1994). Long-course radiotherapy was administered in the same manner to a total dose of 45 Gy (25 fractions of 1.8 Gy each over 5 weeks, followed by a delay of at least 4 weeks until surgery). Palliative treatment was individually tailored to each patient but usually consisted of 8 consecutive fractions of 2 Gy each.

Surgical procedures undergone by colorectal cancer patients

Surgical procedures undergone by the 120 colorectal cancer patients are listed in **Table 27**. The majority of patients underwent anterior resections or abdominoperineal excisions of the rectum for rectal cancer.

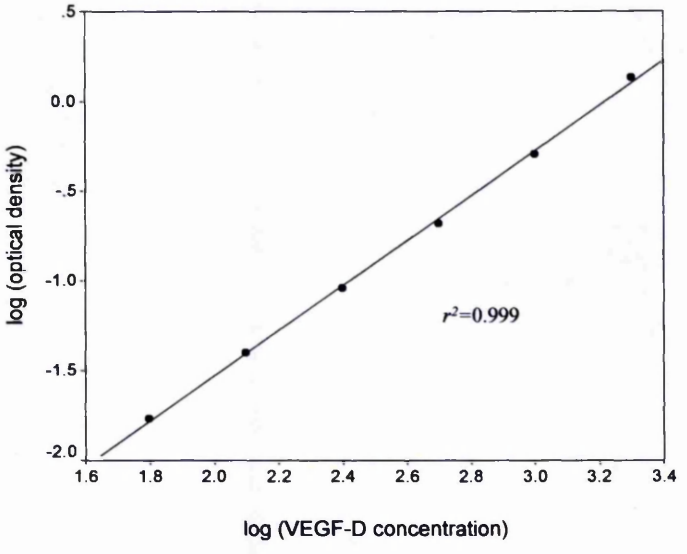
Table 27: Surgical procedures performed

Type of surgery	Number of procedures (%)
Anterior resection	70 (58.3)
Abdominoperineal excision of rectum	35 (29.2)
Hartmann's procedure	2 (1.7)
Sigmoid colectomy	1 (0.8)
Defunctioning colostomy	2 (1.7)
No surgery	4 (3.3)
Other	6 (5) - 2 subtotal colectomies - 2 right hemicolectomies - 1 panproctocolectomy - 1 bypass procedure
Total	120 (100)

Calculation of growth factor levels from standard curves

Calculation of the concentration of VEGF-C in the duplicate patient samples is described above in **section 4.1.3, page 185** and **Figures 37-38**. Levels of VEGF-D and VEGF-A were calculated using the standard curve created on each ELISA plate and linear regression analysis using SPSS 10.1 statistical software (**Figure 43**).

Figure 43: Data obtained from standard curve in VEGF-D ELISA kit



For each VEGF-D and VEGF-A ELISA kit used, the $\log_{10}$ values of the mean optical density readings of the duplicate standard samples, corrected for background signal, were plotted against the $\log_{10}$ converted standard sample concentrations.

For each data set on each ELISA plate, linear regression analysis was used to calculate the equation of the regression line for the logged data, using the formula for a straight line:

$y = a + b(x)$, where a is a linear constant and b is the regression coefficient (slope of the line).

For the data shown above in the figure, the relationship between the data sets is linear, r^2=0.9990, p<0.001. The equation of this line is:

$$\text{Log}_{10}\text{ VEGF-D} = 3.220 + 0.797\ (\log_{10}\text{ optical density})$$

Where a = 3.220 (t=240.7, p<0.001) and b = 0.797 (t=63.2, p<0.001)

Using this equation, the mean optical density readings of the duplicate standard samples, corrected for background signal, were used to calculate the $\log_{10}$ VEGF-D values, which were then converted back into values of VEGF-D.

Comparison of VEGF-C, VEGF-D and VEGF-A levels between groups

The values for plasma VEGF-C, VEGF-D and VEGF-A levels did not fit a normal distribution (Kolmogorov-Smirnov Z statistics, 2.52, 1,71, 2.04 and *p*-values, <0.001, 0.006 and <0.001, respectively), hence data were evaluated using non-parametric tests. Differences between median levels of VEGF-C, VEGF-D and VEGF-A in cancer and control groups and between the sexes were compared with the Mann-Whitney U test. Correlations between VEGF-C, VEGF-D and VEGF-A and age were examined with Spearman rank correlation coefficients.

The range of values was wider in the cancer group than the control group for all three growth factors. There were no significant differences in median values between cancer cases and controls for VEGF-D or VEGF-A, but the controls had a higher median VEGF-C level than cancer patients (**Table 28**).

Table 28: Plasma VEGF-C, VEGF-D and VEGF-A levels in control and cancer groups

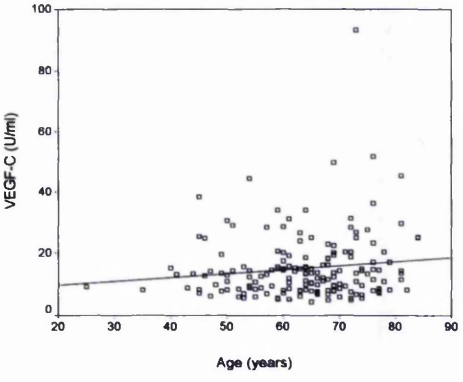
	Control group	**Cancer group**	***p*-value**
Number of cases	50	120	
VEGF-C (U/ml)	15.2 (5.2-34)	10.5 (4.4-93.4)	0.001 **
VEGF-D (pg/ml)	370 (75-926)	329.5 (0-1343)	0.093
VEGF-A (pg/ml)	36.5 (4-230)	39.0 (7-273)	0.988

Data are presented as median (range). ** Mann-Whitney U test

There were no significant differences in median VEGF-D or VEGF-A level between the sexes (p=0.179 and p=0.280, respectively). The median VEGF-C level was slightly lower in men than in women (12.0 vs.14.2, p=0.045).

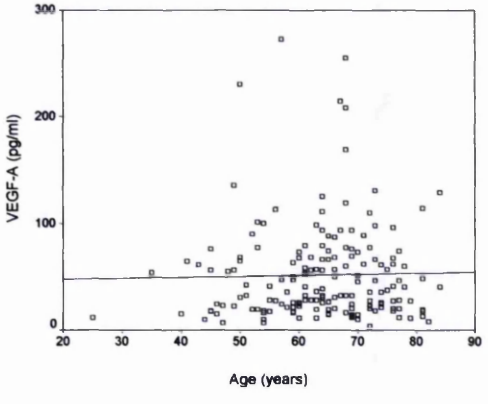
There were no significant correlations of VEGF-C or VEGF-A level with age (Spearman's rho, 0.087 and 0.03, p-values, 0.262 and 0.702, respectively) (**Figures 44-45**). A statistically significant, but weak, correlation was seen with VEGF-D and age (Spearman's rho, 0.181, p=0.018) (**Figure 46**).

Figure 44: Correlation of VEGF-C levels with age



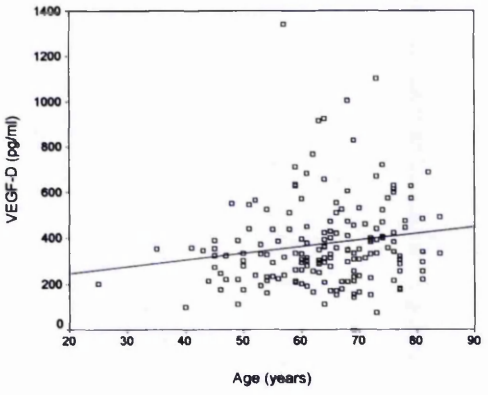
Spearman's rank correlation coefficient, r=0.087, p=0.262

Figure 45: Correlation of VEGF-A levels with age



Spearman's rank correlation coefficient, r=0.03, p=0.702

Figure 46: Correlation of VEGF-D levels with age



Spearman's rank correlation coefficient, r=0.181, p=0.018

Comparison between clinicopathological factors in cancer patients

The cancer patient group was separated by clinicopathological factors, and any differences in growth factor levels assessed in the subgroups. The variables assessed were T, N and M stage, histological grade, vascular invasion, lymph node involvement, marginal involvement, Dukes' stage and merged Dukes' stage (where early disease was defined as Dukes' A and B disease and late disease as Dukes' C & D disease) (**Table 29**).

No significant differences in median plasma levels were seen for VEGF-C, VEGF-D or VEGF-A with any clinicopathological variable assessed, with the exception of a statistically significant difference (p=0.041), of limited clinical relevance, in VEGF-A levels between different tumour T stages (**Table 29 & Figure 47**). In particular, no significant differences were found in median plasma VEGF-C or VEGF-D levels between patients with and without lymph node involvement (**Table 29, Figures 48-49**).

No correlation between plasma VEGF-C, VEGF-D or VEGF-A levels and the number of positive lymph nodes identified was found (Spearman's rank correlation p-values, 0.602, 0.731 and 0.460, respectively).

Table 29: VEGF-C, VEGF-D and VEGF-A and clinicopathological variables

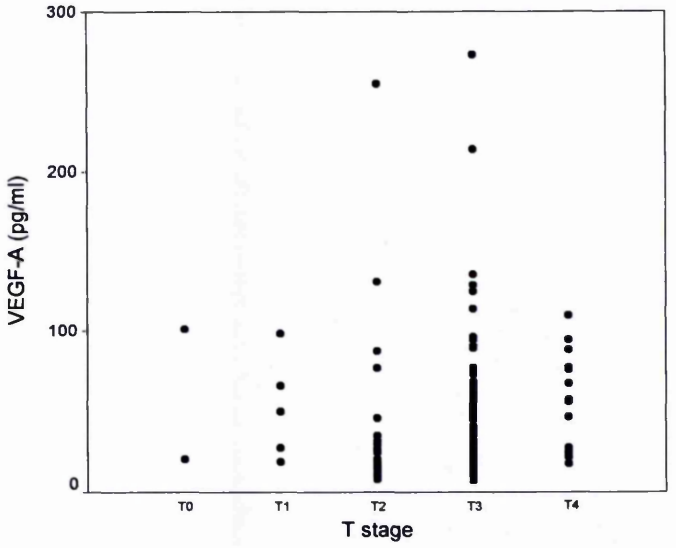
Parameter	Number of cases	*p*-values §		
		VEGF-C	VEGF-D	VEGF-A
T stage	116	0.053	0.18	0.041*
T0	2			
T1	5			
T2	25			
T3	69			
T4	15			
N stage	113	0.968	0.757	0.473
N0	57			
N1	31			
N2	25			
M stage	120	0.473	0.206	0.087
M0	111			
M1	9			
Histological grade	111	0.147	0.388	0.730
Well differentiated	8			
Moderately differentiated	77			
Poorly differentiated	23			
Other	3			
Vascular invasion	113	0.515	0.242	0.145
Absent	81			
Present	20			
Not known	12			
Lymph node involvement	113	0.895	0.675	0.410
Absent	57			
Present	56			
Marginal involvement	113	0.332	0.176	0.231
Negative	104			
Positive	9			
Dukes' stage	113	0.541	0.776	0.587
No tumour	2			
Dukes' A	15			
Dukes' B	39			
Dukes' C	52			
Dukes' D	5			
Merged Dukes' stage (Dukes' A & B vs. C & D)	118	0.912	0.699	0.206
Early disease	54			
Late disease	64			

§ Comparisons between two groups (M stage, lymph node involvement, marginal involvement, merged Dukes' stage) were made with the Mann-Whitney-U test. Comparisons between more than two groups (T stage, N stage, histological grade, vascular invasion, Dukes' stage) were made with the Kruskal-Wallis test.

Within each subgroup, the number of cases varied between 111 and 120, this reflects inability to complete surgical resection in all cases due to the extent of disease discovered at the time of surgery and consequent lack of pathological specimen for assessment.

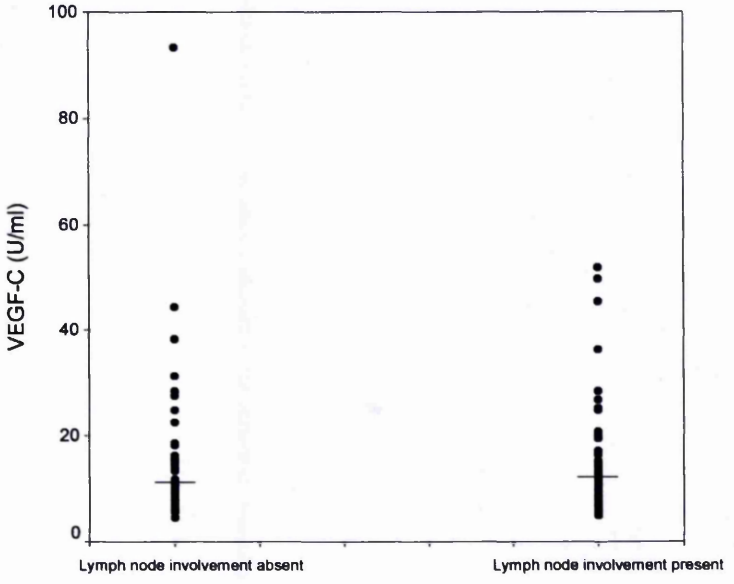
* Statistically significant, $p<0.05$.

Figure 47: Plasma levels of VEGF-A with different T stages



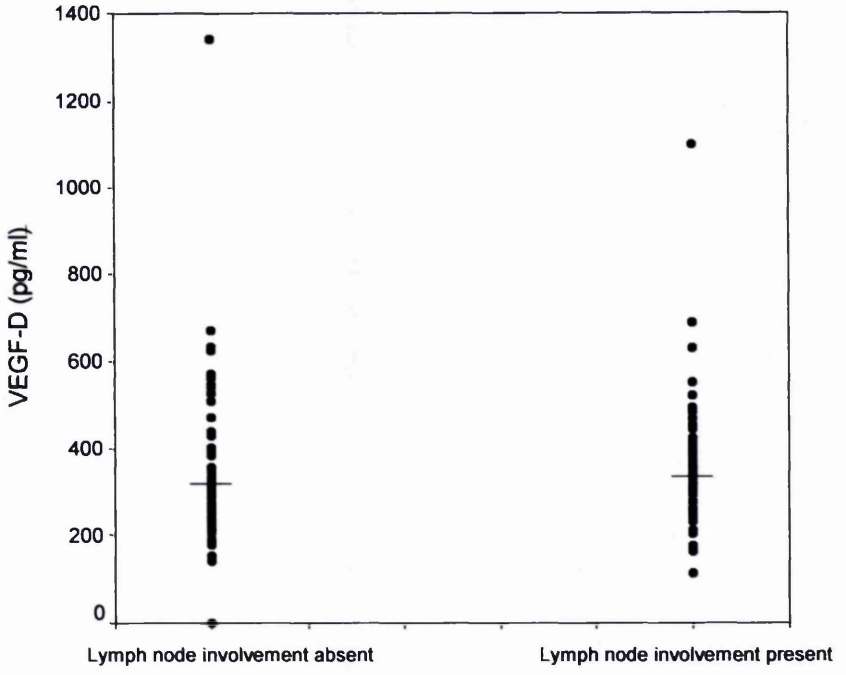
Differences between median plasma VEGF-A levels at different T stages were evaluated with the Kruskall-Wallis test, p=0.041.

Figure 48: Plasma levels of VEGF-C with lymph node status



Horizontal lines represent median values. Median VEGF-C value of lymph node negative group, 9.9 U/ml and median of lymph node positive group, 11.2 U/ml (Mann-Whitney U test, p=0.90)

Figure 49: Plasma levels of VEGF-D levels with lymph node status



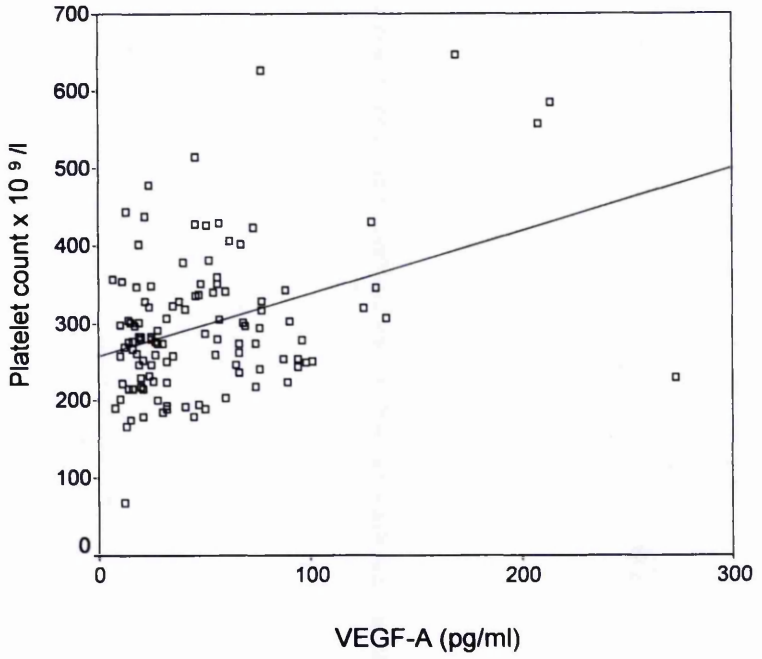
Horizontal lines represent medians. Median VEGF-D value of lymph node negative group, 316.5 pg/ml and median of lymph node positive group, 335 pg/ml (Mann-Whitney U test, p=0.68)

Correlations of growth factors, CEA and haematological variables.

Plasma levels of VEGF family members did not correlate with one another (Spearman's rank correlation p-values; VEGF-C with VEGF-D, p=0.956; VEGF-C with VEGF-A, p=0.09; VEGF-D with VEGF-A, p=0.855). Neither VEGF-C nor VEGF-D levels correlated with levels of the tumour marker CEA (p=0.08 and p=0.938, respectively). However, a correlation was identified between plasma VEGF-A levels and CEA level (Spearman's rho, r=0.206, p=0.039).

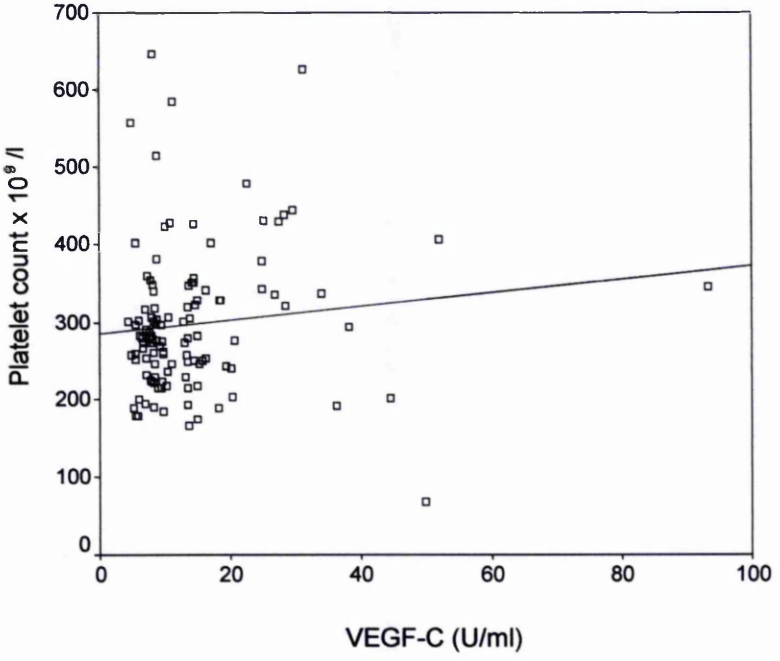
Plasma VEGF-A levels correlated with platelet count (Spearman's rho, r=0.25, p=0.007) (**Figure 50**), a possible trend towards a positive correlation was seen for VEGF-C (p=0.063) (**Figure 51**) but no correlation was observed between VEGF-D level and platelet count (p=0.793).

Figure 50: Correlation between plasma VEGF-A levels and platelet counts



Spearman's rank correlation coefficient, r=0.25, p=0.007

Figure 51: Correlation between plasma VEGF-C levels and platelet count



Spearman's rank correlation coefficient, r=0.174, p=0.063

Summary - immunoassays in colorectal cancer

In summary, despite the promising results of the pilot study illustrating significant differences in plasma VEGF-C levels between colorectal cancer and control patients and a trend towards higher values in advanced disease (see **section 4.1.6, page 190, and Appendix II,** Duff et al., 2003c), this was not confirmed in a larger prospective study. Plasma levels of VEGF-C, VEGF-D and VEGF-A were not higher in cancer patients than controls and analysis of growth factor levels by clinicopathological subgroups showed no extensive differences in circulating levels between groups.

4.2.2. Hepatic resection and control laparotomy patients

As well as their lymphangiogenic effects, VEGF-C and VEGF-D have angiogenic potential via their interaction with the endothelial cell receptor VEGFR2. Therefore, VEGF-C and VEGF-D may influence the haematogenous spread of cancer to the liver. It is not known whether VEGF-C and VEGF-D are expressed in the hepatic metastases of colorectal cancer, nor whether circulating levels of the growth factors are detectable and/or vary during and after surgical resection of metastases. Pre-operative, intra-operative and post-operative levels of plasma growth factors were determined in a group of patients undergoing resection of hepatic metastases (n=10) and compared with those undergoing laparotomy for non-malignant disease (n=10).

The cancer group consisted of 8 men and 2 women, with a median age of 58 (33-68) years. The control group consisted of 4 men and 6 women, with a median age of 56.5 (39-77) years. There was no significant difference in age (independent samples t-test, p=0.854).

The length of operations ranged from 60-280 minutes (mean, 122 minutes) in the control group and 90-320 minutes (mean, 181 minutes) in the cancer group and there was no significant difference between groups (independent samples t-test, p=0.106).

Table 30: Patient characteristics and operative details

	Gender	**Age**	**Surgical procedure**	**Length of operation (minutes)**
Control patients				
1	Female	55	Hepaticojejunostomy for biliary injury	75
2	Female	63	Aorto-bifemoral bypass graft	270
3	Female	68	Marsupialisation of liver cyst and cholecystectomy	60
4	Female	57	Right hemicolectomy for Crohn's disease	75
5	Male	63	Aorto-bifemoral bypass graft	280
6	Male	49	Choledochojejunostomy, cholecystectomy and Pustow's procedure for chronic pancreatitis	150
7	Female	33	Partial splenectomy	90
8	Male	50	Cystogastrostomy for pancreatic psuedocyst and cholecystectomy	80
9	Female	67	Marsupialisation of liver cyst	60
10	Male	57	Cystogastrostomy for pancreatic psuedocyst and cholecystectomy	75
Cancer patients				
1	Female	43	Extended left hemihepatectomy	320
2	Male	48	Right hemihepatectomy	240
3	Male	76	Right hemihepatectomy	180
4	Male	61	Left hemihepatectomy	210
5	Male	52	Right hemihepatectomy and cryotherapy to segment IV	150
6	Male	39	Left hemihepatectomy	90
7	Male	77	Right hemihepatectomy	140
8	Male	61	Right hemihepatectomy	150
9	Female	73	Extended right hemihepatectomy	150
10	Male	53	Right hemihepatectomy	180

Calculations of growth factor levels

Plasma samples were obtained and processed as described in **section 2.3.2, page 133.** ELISAs were performed on all samples in duplicate and the growth factor levels calculated as described in **sections 4.1.3, page 185** and **4.2.1, page 200 & Figure 43.**

Plasma VEGF-C levels between groups and alterations with time from surgery

Plasma VEGF-C levels were measured at 9 time points in each patient: pre-operatively, intra-operatively, at 1, 6, 12, 24, 48 and 96 hours and 7 days post-operatively and plotted against time (**Figures 52-53**). In the hepatic resection group, an unusual outlying value was found in one patient in their intra-operative measurement (86.7 U/ml, **Figure 52**) and a single control patient (control 2, see **Table 30**) had much higher levels than the rest of the group (**Figure 53**). These two samples were checked by repetition of the ELISA with similar results. Consequently, in order to eliminate undue influence from these patients, the VEGF-C values of each patient were normalised to their pre-operative baseline value and the intra-operative value of the single liver resection patient excluded. The mean of the normalised values for each group was then plotted against time (**Figure 54**).

Figure 52: Variations in plasma VEGF-C levels with time in hepatic metastasis

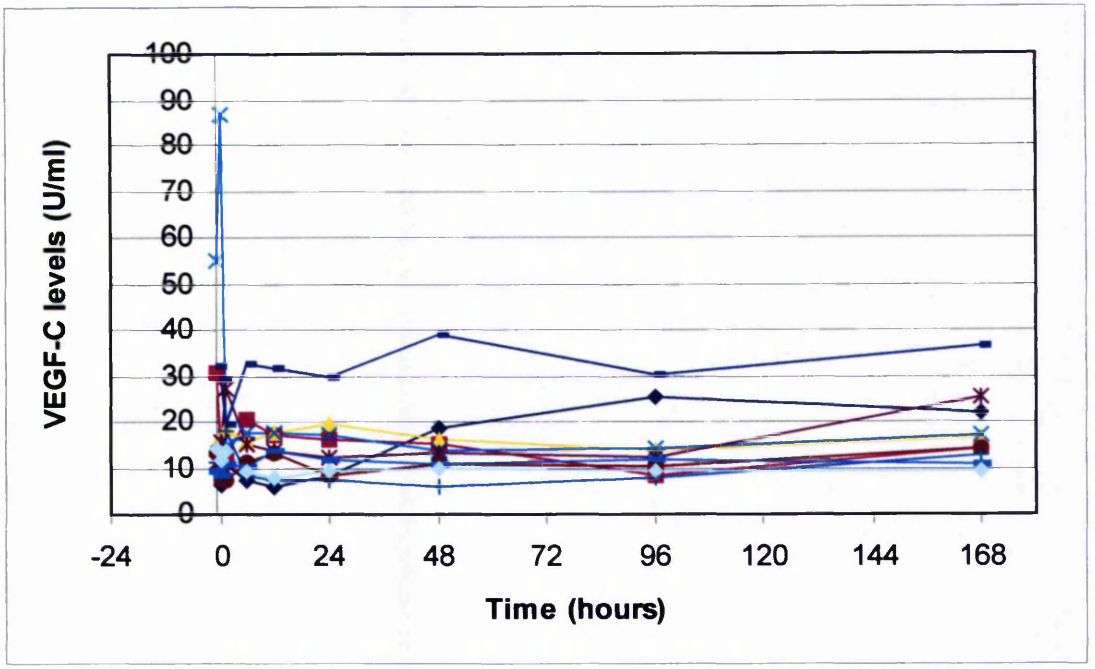


Figure 53: Variations in plasma VEGF-C levels with time in laparotomy controls

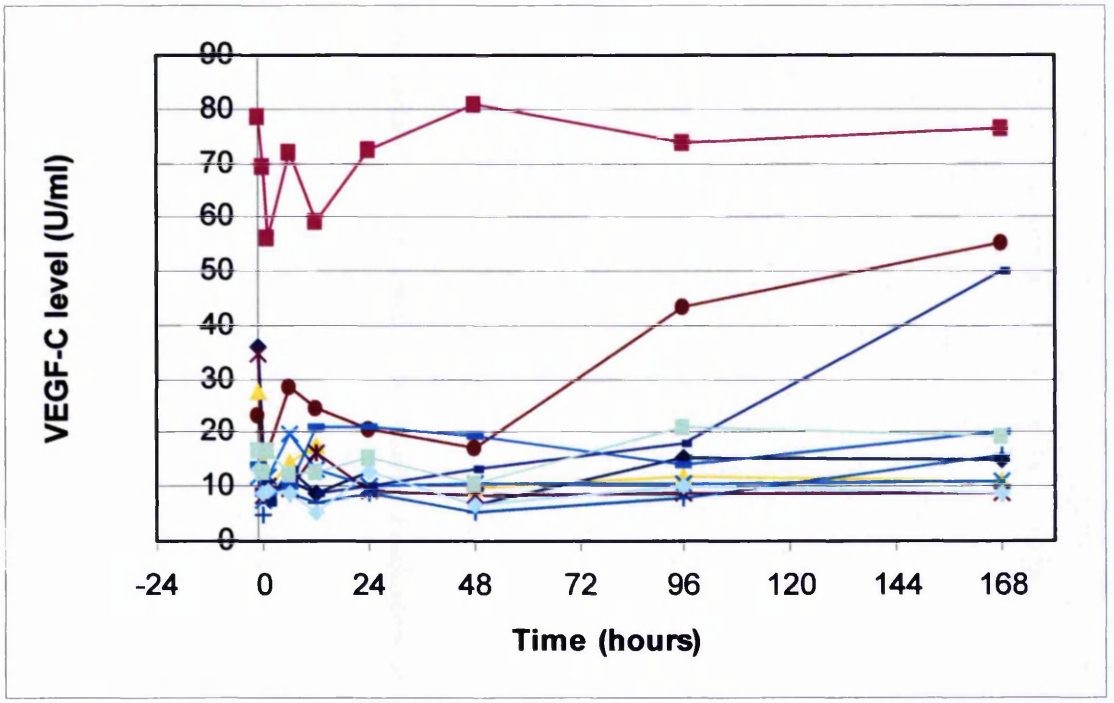
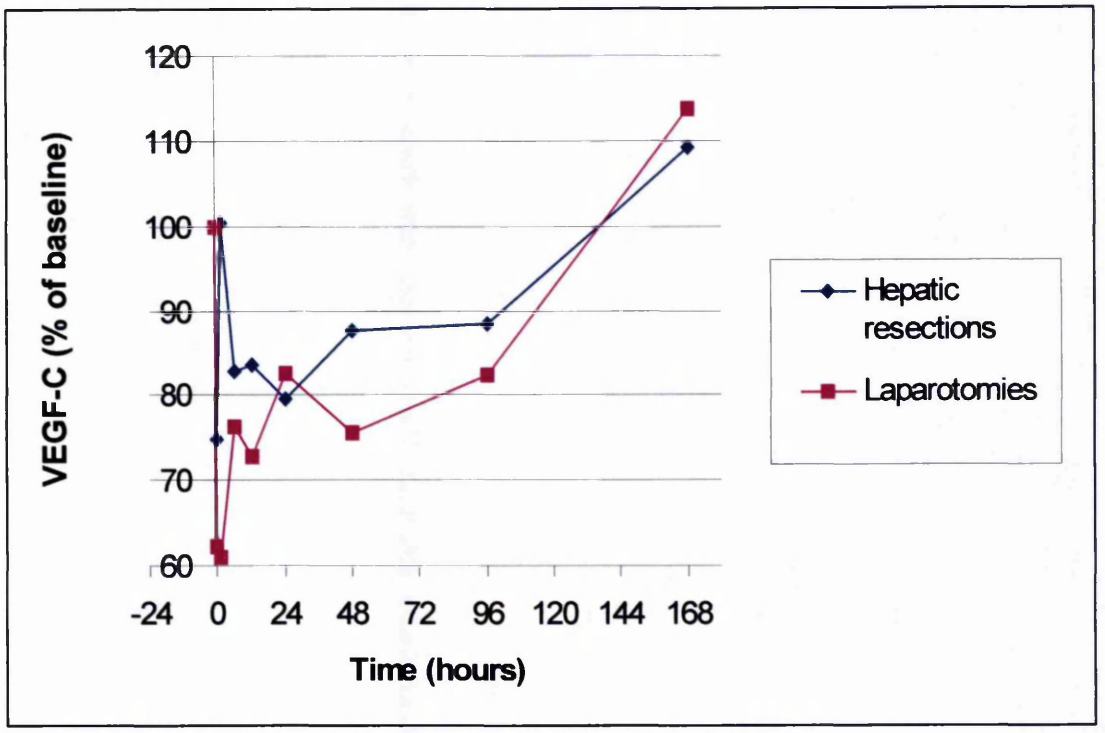


Figure 54: Normalised plasma VEGF-C levels over time



A tendency towards reduced VEGF-C levels in the peri-operative period in comparison with pre-operative baseline levels, followed by an increase at a week post-operatively was observed in both colorectal cancer liver resection patients and control laparotomies (**Figure 54**). However, there were no statistically significant differences in levels at different time points in the group as a whole (Friedman test, χ^2=13.2, p=0.106), nor when split into cancer and control groups (Friedman test, χ^2=6.5, p=0.59 and χ^2=14.9, p=0.06 respectively).

Plasma VEGF-D and VEGF-A levels between groups and changes over time from surgery

Plasma VEGF-D and VEGF-A levels were measured at two time points: pre-operatively and at 7 days post-operatively. A wide range of VEGF-D and VEGF-A levels were seen in each group of patients (**Table 31**).

Table 31: Comparison of plasma VEGF-D and VEGF-A levels between groups at different time points

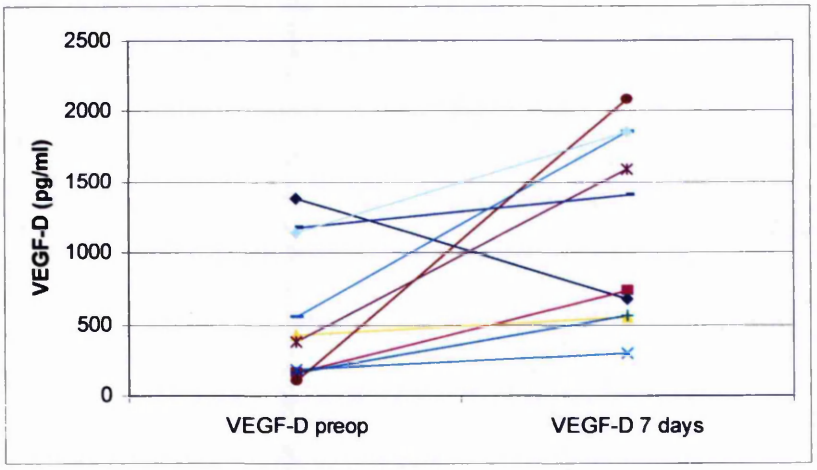
	Time point	VEGF-D (pg/ml)	*p*-value	VEGF-A (pg/ml)	*p*-value
Whole group	Pre-operative 7 days after surgery	557 (105-3537) 677 (288-2091)	0.47	26 (6-553) 117 (8-432)	0.018*
Hepatic metastasis group	Pre-operative 7 days after surgery	403 (105-1387) 1073 (294-2091)	0.028*	26 (6-119) 120 (8-415)	0.005**
Control laparotomy group	Pre-operative 7 days after surgery	1540 (117-3537) 547 (288-1828)	0.37	40 (15-553) 102 (20-432)	0.68

Values are median (range). * $p<0.05$, ** $p<0.01$, Wilcoxon signed rank tests.

In the group as a whole (both hepatic metastasis and control laparotomy patients), no significant differences were found between the pre-operative and seven day plasma VEGF-D level (Wilcoxon signed rank test, $p=0.47$), but plasma VEGF-A levels were elevated at seven days ($p=0.018$) (**Table 31**).

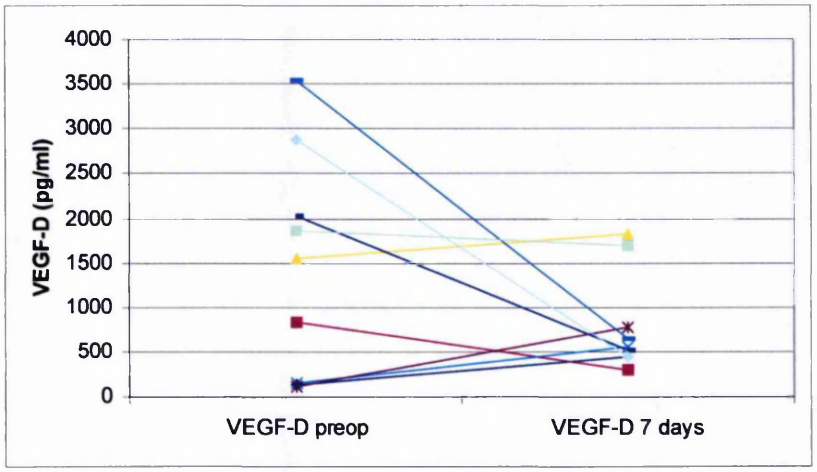
Alterations in VEGF-D and VEGF-A levels with time within each group are illustrated graphically in **Figures 55-58**. Changes in growth factor levels between the two time points were subject to individual variation in cancer and control groups; for a given individual, plasma levels of VEGF-D were seen to either increase or decrease at one week post-operatively (**Figures 55-56**). Similar variation was seen for plasma VEGF-A levels in the control patient group (**Figure 58**), but all cases in the hepatic metastasis resection group demonstrated elevated VEGF-A levels with time (**Figure 57**).

Figure 55: Pre-operative and 7 day post-operative plasma VEGF-D levels in hepatic metastasis



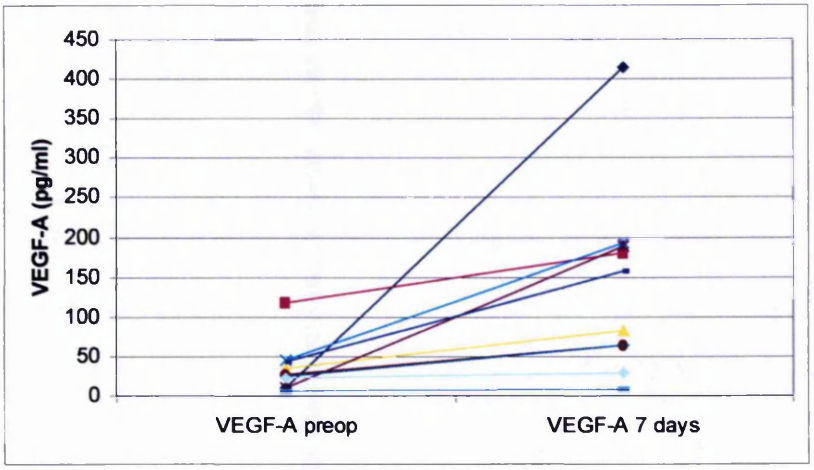
VEGF-D levels showed both increases and decreases at 7 days within individuals. The median pre-operative level was 403 pg/ml (range, 105-1387) and median 7 day level was 1073 pg/ml (294-2091). These levels were significantly different (Wilcoxon signed rank test, p=0.028, **Table 31**).

Figure 56: Pre-operative and 7 day post-operative plasma VEGF-D levels in laparotomy controls



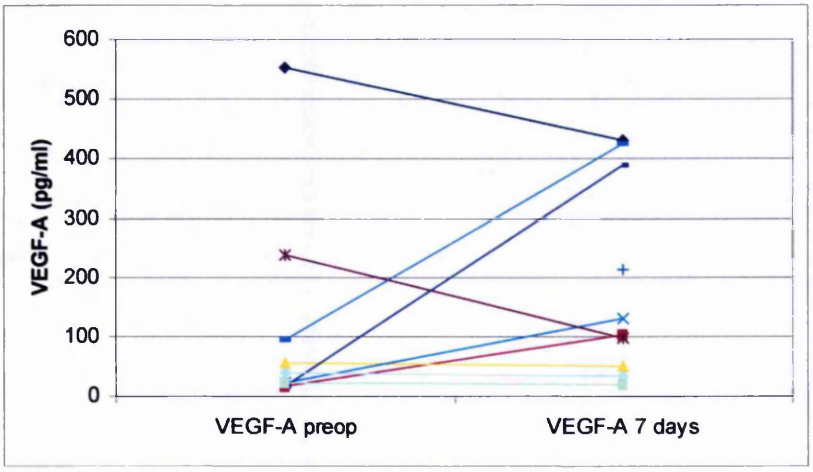
VEGF-D levels showed both increases and decreases at 7 days within individuals. The median pre-operative level was 1540 pg/ml (range, 115-3537) and median 7 day level was 547 pg/ml (288-1828). These levels were not significantly different (Wilcoxon signed rank test, p=0.37, **Table 31**).

Figure 57: Pre-operative and 7 day post-operative plasma VEGF-A levels in hepatic metastasis



VEGF-A levels all increased at 7 days within individuals. The median pre-operative level was 26 pg/ml (range, 6-119) and median 7 day level was 120 pg/ml (8-415). These levels were significantly different (Wilcoxon signed rank test, p=0.005, **Table 31**).

Figure 58: Pre-operative and 7 day post-operative plasma VEGF-A levels in laparotomy controls



VEGF-A levels showed both increases and decreases at 7 days within individuals. The median pre-operative level was 40 pg/ml (range, 16-553) and median 7 day level was 102 pg/ml (20-432). These levels were not significantly different (Wilcoxon signed rank test, p=0.68, **Table 31**).

In order to eliminate the effects of the intraindividual variation noted previously and to identify the direction of any systematic differences in plasma growth factor levels with time between groups, the difference in plasma values at the two different time points was calculated for each individual and the median values of each group compared (Mann-Whitney-U test) (**Table 32, Figures 59-60**).

Table 32: Differences in VEGF-D and VEGF-A levels with time

	Hepatic metastasis group	**Control laparotomy group**	***p*-value**
Median difference in VEGF-D values (pre-operative – 7 day value)	-492 (-1986-710)	169 (-660-2896)	0.043*
Median difference in VEGF-A values (pre-operative – 7 day value)	-54 (-404-(-2))	3 (-372-140)	0.243

Values are medians (range). * $p<0.05$, statistically significant result

An increase in plasma VEGF-D levels at 7 days post-operatively was seen in the cancer group over the controls in comparison to the pre-operative values (hence producing a negative value for the difference between the observations at different time points, $p=0.043$, **Table 32**) but no significant systematic differences were observed in plasma VEGF-A levels between groups with time from surgery ($p=0.243$, **Table 32**). Increased VEGF-A levels at one week post-operatively were observed in both cancer and control groups (although the median level was not significantly increased in the control laparotomy group, **Table 31**).

Figure 59: Difference in pre-operative vs. 7 day post-operative plasma VEGF-D levels

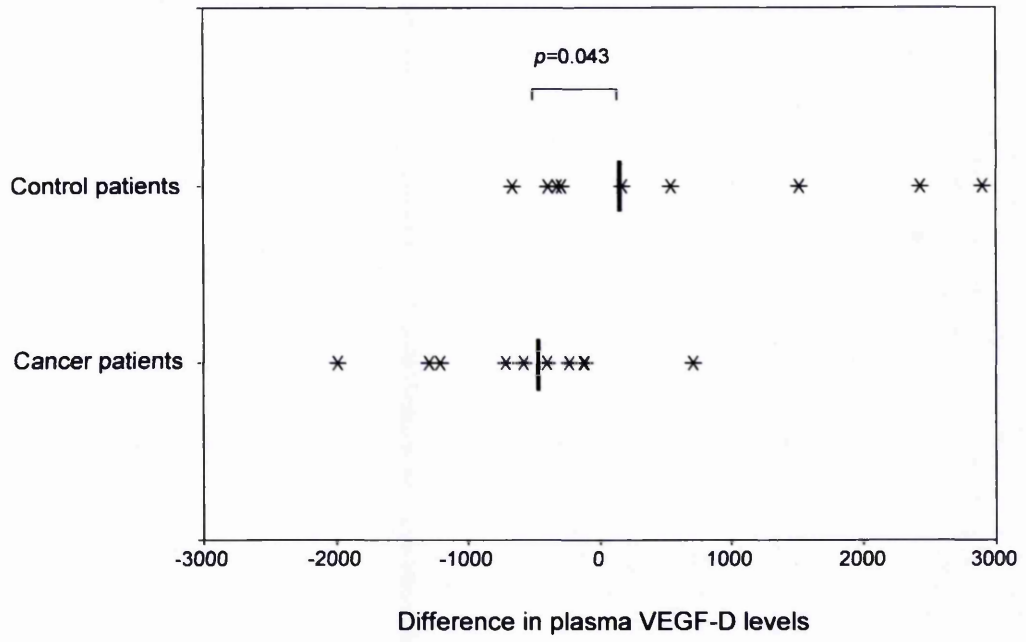
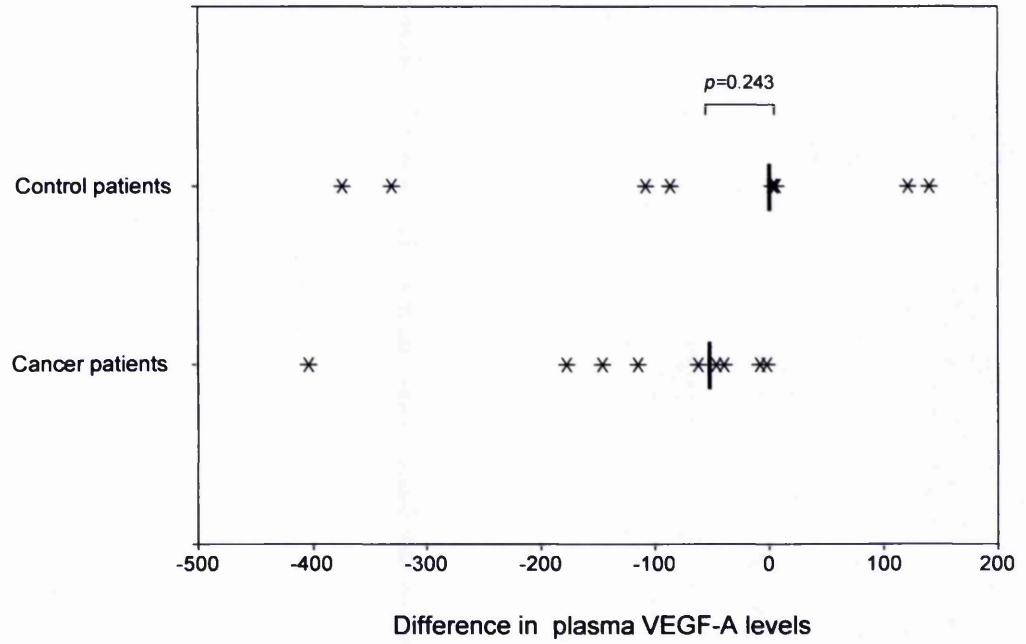


Figure 60: Difference in pre-operative vs. 7 day post-operative plasma VEGF-A levels



To summarise, no significant differences in pre-operative plasma VEGF-C levels were identified between hepatic metastasis and control laparotomy patients, nor were any changes observed with time from surgery. Plasma VEGF-A levels but not VEGF-D levels increased to 7 days post-operatively in both groups. A small systematic difference was detected for plasma VEGF-D levels between groups, which increased at one week post-operatively in cancer patients compared to controls.

Correlation of plasma growth factor levels with immunohistochemically detected tissue growth factor expression

Pre-operative plasma levels of VEGF-C, VEGF-D and VEGF-A did not correlate with immunohistochemically detected expression of the corresponding growth factors in the resected liver metastasis (Spearman rank correlations coefficients, -0.025, 0.127 and -0.361, with *p*-values of 0.95, 0.74 and 0.34 respectively) (see **section 3.2.7, page 168**).

Summary - immunoassays in hepatic resection and control laparotomy patients

In this small study of hepatic metastasis resection and control laparotomy patients, no differences were found in plasma VEGF-C levels between patient groups or at different time points from surgical resection. Similarly, no differences in plasma VEGF-D levels were seen in pre-operative compared with 7 day post-operative samples in the study group as a whole, although on subgroup assessment cancer patients had elevated 7 day post-operative levels in comparison with controls. An overall increase in plasma VEGF-A levels was seen in both groups, but no differences were observed between the groups. Plasma growth factor levels did not reflect the expression pattern in the liver metastasis detected immunohistochemically.

4.3. SUMMARY OF RESULTS

An ELISA for measurement of VEGF-C was developed, validated and evaluated in a pilot study as described in **section 4.1.7, page 193**. Prospective studies were undertaken to investigate the clinical potential of the VEGF-C ELISA in conjunction with ELISAs for VEGF-D and VEGF-A.

This series of experiments demonstrated that:

i) in preoperative colorectal cancer patients,

- plasma levels of VEGF-C, VEGF-D and VEGF-A were not higher in cancer patients than controls
- no extensive differences in growth factor levels in plasma were identified between different clinicopathological subgroups of patients

ii) in hepatic metastasis resection and control laparotomy groups,

- no significant differences in pre-operative plasma VEGF-C levels were identified between groups, nor were any changes observed with time from surgery.
- plasma VEGF-A levels increased to 7 days post-operatively in both groups, plasma VEGF-D levels increased at one week post-operatively in cancer patients compared to controls.
- plasma growth factor levels did not reflect the pattern of expression in the liver metastasis.

5. DISCUSSION

In order to investigate and clarify the roles played by the VEGF family members and lymphangiogenesis in colorectal cancer, the studies described in this thesis formed two groups: immunohistochemical experiments examining the expression of VEGF family members and receptors in the primary and metastatic sites of colorectal cancer and immunoassay experiments assessing the role of circulating VEGF-C and VEGF-D in the prediction of tumour behaviour (see **section 1.8, page 102**).

5.1. EXPRESSION OF THE VEGF FAMILY AND RECEPTORS IN PRIMARY AND METASTATIC SITES OF COLORECTAL CANCER

The findings to emerge from the immunohistochemical experiments will be considered under three headings:

i) the expression and relationships between VEGF family members and receptors in primary colorectal cancer

ii) the role of the VEGF family and its receptors in the development of colorectal cancer metastasis

iii) lymphangiogenesis and LVD in colorectal cancer

In vitro and *in vivo* tumour models have limitations; both are artificial situations in which cells are deprived of their natural tissue microenvironment and may behave in unrepresentative ways. In animal tumour models (particularly those producing tumours

in ectopic sites) the tumour microenvironment may only partially reflect the true condition in human tumours. As the expression of growth factors is influenced greatly by microenvironmental conditions, such as the concurrent presence of other cytokines and growth factors, the level of inflammation, hypoxia, extracellular matrix molecules and cell-cell interactions (Fidler, 2001; Liotta & Kohn, 2001), the observed expression of growth factors and receptors in animal models may be misleading. Consequently, examination of human tumour tissue is able to identify accurately how and where growth factors and their receptors are expressed within a tumour and detect the presence of confounding variables such as inflammation.

5.1.1. Expression and relationships between VEGF family members and receptors in primary colorectal cancer

In primary colorectal cancer:

- the pattern of expression of the VEGF family members, VEGF-C, VEGF-D and VEGF-A, increased from normal tissue adjacent to colorectal cancer, through the junctional mucosa and throughout the tumour to a maximum at the invasive tumour edge.
- VEGFR2 was expressed in colorectal cancer cells and the pattern of expression reflected that of its ligands, increasing to a maximum at the invasive tumour edge.
- the expression of VEGF-C and VEGFR2 correlated with one another and co-localised throughout colorectal cancer
- the expression of VEGF-C and VEGF-D did not correlate within colorectal cancer.
- the pattern of expression of VEGF-C, VEGF-D, VEGF-A and VEGFR2 in primary tumours was similar in early and late colorectal cancers.

Most studies addressing the relationship between tumour-expressed VEGF-A and prognosis in colorectal cancer fail to assess the intra-tumoural heterogeneity of growth factor expression (**section 1.4.3, page 51**). Cells at the invading edge of the tumour are considered to have the highest malignant potential in comparison to other parts of the tumour (Oh-e et al., 2001). Studies from Japan also examined the topographical distribution of VEGF-A and VEGF-C throughout primary colorectal cancers (Furodoi et al., 2002; Kaio et al., 2003) and have shown that growth factor expression increased throughout the tumour and TI expression correlated with MVD and poorer outcome. These topographical results are confirmed and extended by the work described in this thesis. Expression patterns of VEGF-A, VEGF-C and VEGF-D were similar, increasing to a maximum towards the TI site. For this reason, TI growth factor expression was considered in relation to the clinicopathological variables.

The expression of VEGF receptors by various malignant cell types has been discussed in **section 1.4.3, page 57**. In the context of colorectal cancer, increased VEGFR2 and VEGFR3 expression have been noted in malignant epithelial cells (Amaya et al., 1997; Witte et al., 2002; Kawakami et al., 2003). Here, the expression of VEGFR2 in colorectal cancer cells was confirmed and its pattern of distribution throughout the primary tumour found to mirror that of the VEGF family members, increasing to a maximum at the TI site. This important finding was confirmed by the use of two different antibodies (**Figure 19, page 160**). The clinical relevance of VEGR2 expression and its ligands in colorectal cancer is in the identification of potential overlapping intra-tumoural autocrine and paracrine circuits, which may influence tumour response to novel anti-VEGF treatments. Consequently, a VEGF-A expressing colorectal cancer may show initial response to anti-VEGF-A treatments eg. bevacizumab (Avastin™) (Hurwitz et al., 2003) but co-expression of VEGFR2 and

VEGF-C or VEGF-D would allow the tumour to escape from, and develop resistance to, this form of therapy. Anti-VEGF-A treatments may have beneficial anti-tumour effects (both directly and by enhancement of tumour response to conventional adjuvant treatments) in addition to their anti-angiogenic effects (Harmey & Bouchier-Hayes, 2002). However, existence of overlapping VEGF family ligand/receptor circuits within colorectal cancer has implications for anti-VEGF strategies, which may require targeting of several different ligands/receptors to achieve sustained clinical response.

A novel finding of this thesis was the close topographical relationship between VEGF-C and VEGFR2 expression within primary colorectal cancer (summarised in **Figure 61, page 232**). This was exemplified by the correlating expression between the growth factor and its receptor at multiple sites within the primary tumour and co-localisation within malignant epithelial cells (**Figures 20-21, pages 162-3**). The importance of this finding is the possibility of an autocrine/paracrine circuit between the mature growth factor and the receptor, potentially enabling VEGF-C to mediate tumour growth, proliferation, motility and invasion and allow the development of resistance to anti-VEGF-A treatments as mentioned above.

Despite the similar overall distribution pattern of primary tumour VEGF-C and VEGF-D expression, these related growth factors did not correlate with one another and nor did VEGF-D and VEGFR2 expression correlate. This may reflect a less important role for VEGF-D and the potential VEGF-D/VEGFR2 loop in the progression of malignant colorectal disease (George et al., 2001b; Hanrahan et al., 2003). Both George (2001b) and Hanrahan (2003) have demonstrated reduced VEGF-D mRNA levels in colorectal tumours in comparison to adenomas and normal mucosa. However, the relationship between reduced mRNA and subsequent protein expression within the tumour is unclear (George et al., 2001b). The authors of these studies have postulated the concept of VEGF-D as a competitive agonist to VEGF-A and VEGF-C, consequently reduced

VEGF-D levels in tumours would allow increased access of VEGF-A and VEGF-C to their receptors (George et al., 2001b) or that VEGF-D may only be important in a subset of tumours (Hanrahan et al., 2003). Expression of the VEGF family members alters throughout the adenoma-carcinoma sequence and the balance between the expressed cytokines is likely to be relevant for tumour progression and subsequent behaviour (Niki et al., 2000; George et al., 2001b; Hanrahan et al., 2003).

The topographical localisation pattern of the VEGF family and VEGFR2 expression was similar in colorectal cancers of early and late Dukes' stage. By contrast, MVD was increased in tumours of advanced Dukes' stage, a finding that has been reported previously (**section 1.2.2, page 32**). This is likely to be due to the concurrent increase in expression of additional angiogenic growth factors in advanced tumours, as tumour MVD is not solely determined by VEGF expression but depends on the overall balance of angiogenic growth factor and inhibitor expression within the tumour microenvironment.

Immunohistochemical studies suffer from limitations, which are often due to the heterogeneity of human cancer and the possibility that non-representative tissue has been sampled. Immunohistochemical techniques also tend to be subjective and semi-quantitative. Further criticism of these immunohistochemical experiments and the findings described could focus on the number of cases studied, the antigens chosen for assessment, vessel counting and assessment methodology.

These studies only employed small numbers of cases, with the consequence of a skewed spread in terms of tumour stage and histology, the tumours examined were mainly later T stages (T1 and T2, n=5; T3 and T4, n=25) and were all moderately or poorly differentiated (moderately differentiated, n=22; poorly differentiated, n=8). Furodoi et al. (2002) have demonstrated that TI expression of VEGF-C is increased in colorectal tumours of poorer histological grade. Through the use of multiple assessments at

VEGF-D levels in tumours would allow increased access of VEGF-A and VEGF-C to their receptors (George et al., 2001b) or that VEGF-D may only be important in a subset of tumours (Hanrahan et al., 2003). Expression of the VEGF family members alters throughout the adenoma-carcinoma sequence and the balance between the expressed cytokines is likely to be relevant for tumour progression and subsequent behaviour (Niki et al., 2000; George et al., 2001b; Hanrahan et al., 2003).

The topographical localisation pattern of the VEGF family and VEGFR2 expression was similar in colorectal cancers of early and late Dukes' stage. By contrast, MVD was increased in tumours of advanced Dukes' stage and in those that developed metastatic disease, a finding that has been reported previously (**section 1.2.2, page 32**). This is likely to be due to the concurrent increase in expression of additional angiogenic growth factors in advanced tumours, as tumour MVD is not solely determined by VEGF expression but depends on the overall balance of angiogenic growth factor and inhibitor expression within the tumour microenvironment.

Immunohistochemical studies suffer from limitations, which are often due to the heterogeneity of human cancer and the possibility that non-representative tissue has been sampled. Immunohistochemical techniques also tend to be subjective and semi-quantitative. Further criticism of these immunohistochemical experiments and the findings described could focus on the number of cases studied, the antigens chosen for assessment, vessel counting and assessment methodology.

These studies only employed small numbers of cases, with the consequence of a skewed spread in terms of tumour stage and histology, the tumours examined were mainly later T stages (T1 and T2, n=5; T3 and T4, n=25) and were all moderately or poorly differentiated (moderately differentiated, n=22; poorly differentiated, n=8). Furodoi et al. (2002) have demonstrated that TI expression of VEGF-C is increased in colorectal tumours of poorer histological grade. Through the use of multiple assessments at

different areas within individual tissue sections, it was possible to make a detailed critical assessment of the intra-tumoural patterns of receptor and growth factor tissue expression. However, the observations reported may reflect the situation in the tissue microenvironment encountered predominantly in more advanced tumours of poorer histological grades and clearly require confirmation in larger patient populations, which should encompass tumours of all T stages and histological grades.

The choice of VEGFR2 for histological study was made due to its importance in mediating the angiogenic effects of VEGF-A (Karkkainen & Petrova, 2000). Study of VEGFR3 expression was attempted in order to determine whether malignant cellular expression could be confirmed (Witte et al., 2002) and to assess the potential role of VEGFR3 as a lymphatic vessel marker in colorectal cancer. However, the lack of specificity of the available polyclonal antibodies meant that this attempt had to be abandoned (see **section 3.1.4, page 147**). The only study to demonstrate VEGFR3 expression in colorectal cancer cells also demonstrated an association with reduced survival and employed an in-house rabbit polyclonal antibody, which is not generally available (Witte et al., 2002). Should more specific antibodies become available, it will be important to examine the tissue expression of VEGFR3 in colorectal cancer, in particular, investigation of the pattern of expression throughout the tumour and its relationship to the other VEGFRs, ligands and clinicopathological characteristics would be desirable.

Chalkley point counting was used to quantify MVD as this method is reproducible and accurate in colorectal cancer (Abdalla et al., 1996; Li et al., 2003). CD34 antibody was chosen as the best endothelial marker in paraffin embedded colorectal tissue after assessment of the results of immunostaining with CD34, CD31 and CD105 antibodies (**section 3.1.1, page 135**). The choice of CD34 agrees with the findings of others in colorectal tissue (Abdalla et al., 1996).

Direct comparison of the levels of expression between VEGF-C, VEGF-D, VEGF-A and VEGFR2 at different tumour sites was not performed although the assessment of immunostaining was made on the same scale and protocols were optimised to include the same spectrum of immunostaining intensities for each of the antigens. The reason for this was that although within-group comparisons and rank correlations are valid, between-group comparisons of relative levels of antigen expression could be criticised as being the consequence of antibody affinity rather than due to actual differences in antigen expression.

To summarise, in primary colorectal cancers, expression of VEGF family members and VEGFR2 is increased throughout the tumour to a maximum at the TI, VEGFR2 is expressed in colorectal cancer cells and a close relationship exists between VEGF-C/VEGFR2. Potential overlapping autocrine and paracrine circuits have been identified between the VEGF ligands and VEGFR2, which may explain the clinical response seen to anti-VEGF-A treatments and why the responses observed are likely to be of limited duration.

5.1.2. Role of the VEGF family and its receptors in the development of colorectal cancer metastasis

In the development of colorectal cancer metastases:

- VEGF-C and VEGFR2 expression at the invasive edge of primary colorectal cancers correlated with their expression in metastatic lymph nodes.
- the expression of VEGF-C and VEGFR2 correlated with one another and co-localised in metastatic lymph nodes but not in liver metastases.
- the pattern of expression of VEGF-C, VEGF-D, VEGF-A and VEGFR2 in primary tumours was similar in those cancers that did and did not spread to lymph nodes.

- colorectal cancers that spread to the liver had a lower level of VEGF-C expression at the invasive tumour edge in the primary tumour in comparison to tumours that did not develop haematogenous metastasis.
- the expression of VEGFR2 was reduced in liver metastases in comparison to expression at the invasive edge of the primary tumour.

Akagi et al. (2000) examined metastatic lymph node expression of VEGF-C in 18 cases of colorectal cancer and commented that nodal VEGF-C expression was 'fairly consistent with expression in the primary tumour'. In their study, a 'positive' case was defined as one in which greater than 10% of the malignant cells were immunoreactive to VEGF-C, no account was taken of the intensity of VEGF-C expression or location of the ligand within the tumour (Akagi et al., 2000). In the current studies, VEGF-C expression in metastatic lymph nodes correlated with the TI expression of VEGF-C, emphasising the importance of the invading edge of the tumour in determining metastatic behaviour. Similar findings were seen between TI and metastatic lymph node expression of VEGFR2. The correlating expression of VEGF-C, VEGFR2 and the ligand-receptor pair at the TI and in lymph node metastases suggests that their interaction is important in metastatic nodal spread. This proposal is further supported by the correlation observed between levels of colorectal tumour VEGFR2 mRNA and lymph node metastasis by Hanrahan et al. (2003). The association between VEGF-C expression and lymph node metastasis has been observed in multiple tumours (see **section 1.7, page 90**), but the precise mechanism of how tumour growth factor expression influences metastasis in lymph nodes remains obscure. VEGF-C may promote tumour lymphangiogenesis and angiogenesis. Interaction between VEGF-C and VEGFR3 on lymphatic vessels could enhance lymphatic vessel invasion and lymph node metastasis. Alternatively, VEGFR2 or VEGFR3 expression on angiogenic vessels

provides a route for VEGF-C to influence angiogenesis and haematogenous spread. The finding of VEGFR2 expression on colorectal epithelial cells also opens the possibility that VEGF-C expression may be involved in the upregulation of the receptor and the production of autocrine-induced tumour cell survival and invasion.

To date, colorectal cancer liver metastases have not been examined for expression of VEGF-C or VEGF-D, although some years ago expression of VEGF-A and VEGFR2 mRNA in this metastatic site was investigated by Warren et al. (1995) who found high levels of VEGF-A mRNA in neoplastic cells and that VEGFR2 mRNA was confined to endothelial cells. Here, VEGFR2 expression was detected immunohistochemically in malignant cells within hepatic metastases, but at a much lower expression than in lymph node metastases or in the primary sites (**Table 20, page 168 & Figure 26, page 169**). The reduced level of VEGFR2 expression in liver metastases may be related to conditions within the local tissue microenvironment, in particular the oxygen tension found at this metastatic site. The relatively high levels of oxygen in hepatic parenchyma mean that VEGF-A is less of an angiogenic driver at this site than in the primary tumour, this is supported by the lower levels of VEGF-A seen in liver metastases in comparison to metastases at other intra-abdominal sites (Cascinu et al., 2001). Lower levels of VEGF-A within the hepatic metastasis may in turn result in reduced VEGFR2 expression (see **section 1.4.1, page 48 & section 1.4.3, page 51**). The clinical relevance of this finding is that new approaches to cancer therapeutics involving tyrosine kinase inhibitors may have limited effectiveness in the treatment of colorectal cancer hepatic metastases in comparison to other metastatic sites.

VEGF-C and VEGF-D were expressed in the hepatic metastases of colorectal cancer, albeit at a lower intensity than primary TI expression and in the liver metastases expression did not correlate with that at the TI of the primary tumour. Consequently, the interaction between VEGF-C and VEGFR2 appeared to be less influential on metastatic

spread to the liver than on lymph node metastasis, as evidenced by the lack of correlation between expression of the growth factor/receptor in this metastatic site. Growth factor/receptor expression by the liver metastasis did not reflect TI expression unlike the situation seen in the lymph node metastasis. This is not unexpected, as lymph node spread tends to occur earlier in the natural history of colorectal cancer than hepatic metastasis, fewer molecular genetic events separate a primary tumour from a lymph node metastasis than the primary tumour and its subsequent liver metastasis (Sleeman, 2000). Consequently, a lymph node metastasis is more likely to reflect the phenotypic characteristics of the primary tumour than a subsequent liver metastasis from the same primary (Sleeman, 2000).

A similar pattern of growth factor/receptor expression at the TI was seen for tumours with and without lymph node metastases but cases that subsequently developed liver metastases showed a lower level of TI VEGF-C expression compared with those that did not develop hepatic disease, although the numbers involved were small (n=10). This finding appears to conflict with that reported by Furodoi et al. (2002) who examined VEGF-C expression at the TI in 152 cases of colorectal cancer, finding that the number of cases showing positive TI VEGF-C expression was increased in those who subsequently developed liver metastasis. The reason for this apparent discrepancy is probably due to the difference in detection and assessment methodology. Furodoi et al. classified a VEGF-C positive case as one in which greater than 10% of malignant cells were immunoreactive for VEGF-C (Furodoi et al., 2002), whereas the studies reported in this thesis examined the intensity of VEGF-C expression at multiple sites throughout the tumour. Consequently, all the cases examined here showed some level of TI VEGF-C expression (**Table 18, page 155**) and would all have been classified 'positive' by the critieria of Furodoi et al., but assessment by this method would have obscured the subtle differences between tumours spreading by different metastatic routes. This apparent

discrepancy due to methodological differences highlights one of the difficulties of comparison between immunohistochemical studies as discussed in **section 1.7.1, page 90**. Although VEGF-C has angiogenic effects, it is less potent in this regard than VEGF-A (Joukov et al., 1997b), so the reduction in VEGF-C expression intensity at the TI site in primary tumours spreading via the haematogenous route may simply reflect the lesser role that VEGF-C plays in this mode of metastasis in this patient group. However, the number of cases examined was small and this finding requires confirmation in larger numbers of specimens.

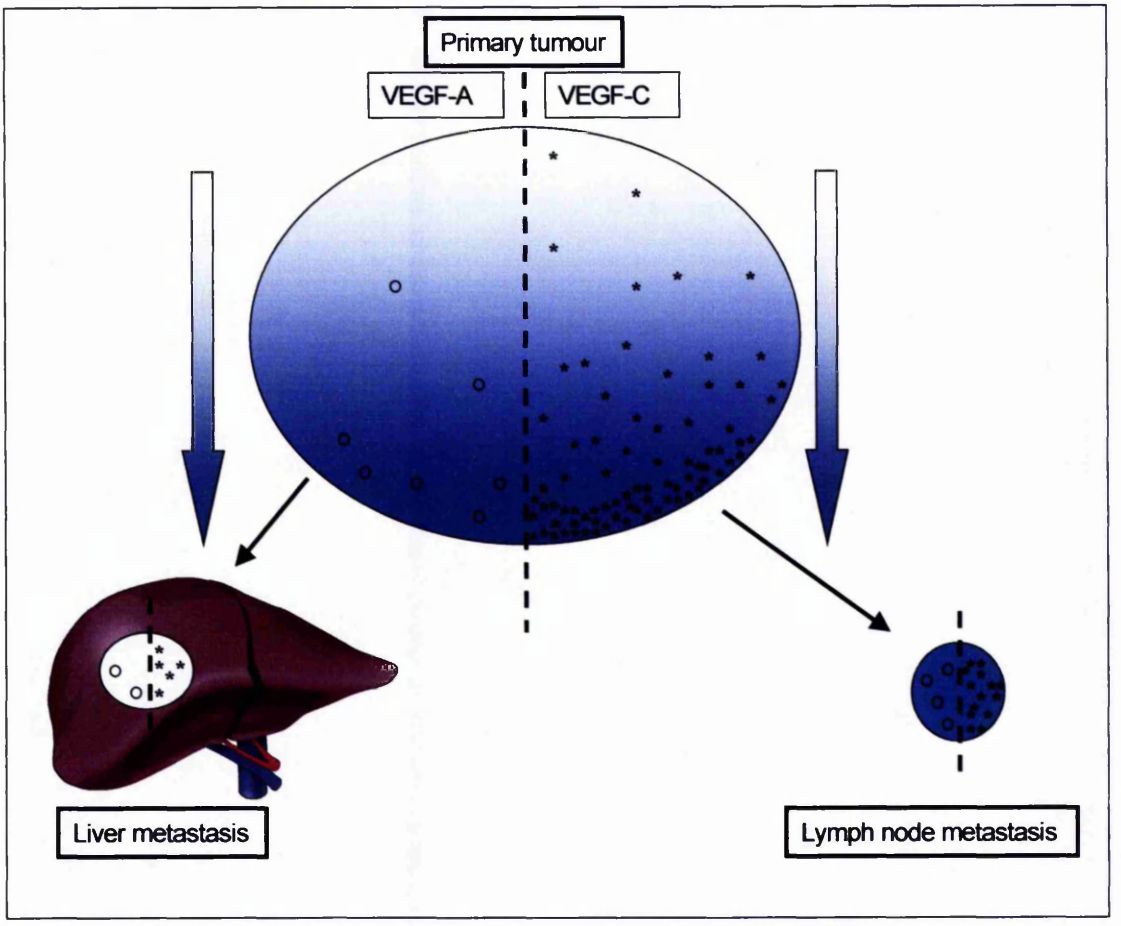
It is important to note that the cases of colorectal cancer that subsequently developed hepatic metastases and the metastases themselves that were examined immunohistochemically in this thesis, only represent resectable hepatic disease and consequently may not be representative of the whole spectrum of colorectal cancer or liver metastases. Tumour biology may differ between patients who have resectable and irresectable liver metastases. It is possible that observed alterations in expression of certain components of the VEGF family seen in resectable liver metastases (here, reduced VEGFR2 expression in liver metastases in comparison to lymph node and TI expression and reduced TI VEGF-C expression) may be relevant in terms of tumour growth control and in determining resectability. If this is the case, tumour VEGF/R profiles may give insight into future tumour behaviour and inhibitors of different components of the VEGF axis may have a potential role in the control of liver metastases. It is conceivable that VEGF/R profiles may guide selection of adjuvant anti-VEGF therapies for patients undergoing resection of metastases, allowing treatment strategies to be customised to promote resectability of disease within an individual.

To summarise, in the development of colorectal cancer metastasis, VEGF-C/VEGFR2 interactions are important in metastatic nodal spread, altered VEGF-C profile at the TI may influence future haematogenous metastasis, VEGF-C and VEGF-D are expressed

in the liver metastases of colorectal cancer and VEGFR2 expression is lower in hepatic metastases, which is likely to be due to tumour microenvironmental influences. VEGF/R profiles of primary tumours may provide insights into future tumour behaviour and response to new therapies, although reduced VEGFR2 expression by liver metastases implies there may only be a limited role for tyrosine kinase inhibition in treatment of the hepatic metastases of colorectal cancer.

The relationships between expression of the VEGF family of growth factors and receptors in primary colorectal cancer and secondary sites is simplified and summarised in **Figure 61**.

Figure 61: The relationship between primary tumour and secondary site expression of VEGF-C, VEGF-A and VEGFR2



The large oval shape represents a primary colorectal cancer, the smaller oval illustrates a lymph node metastasis. The primary tumour and secondary sites are divided by vertical dashed lines. The open circles to the left of the line represent VEGF-A expression, the asterisks to the right of the dashed line represent VEGF-C expression. The blue background colour of the primary and secondary sites represents the level of intensity of expression of VEGFR2.
The blue arrows illustrate how the expression intensity of VEGFR2 increases from the superficial to the deep tumour and this is paralleled by the increased expression of VEGF-C in the primary tumour.
In lymph node metastases, VEGFR2 expression remains high, as does the expression of VEGF-C (and that of VEGF-A to a lesser extent). In liver metastases, VEGFR2 expression is very low, VEGF-C and VEGF-A expression is present but at a lower level than in lymph node metastases or expression at the edge of the invading tumour.

5.1.3. Lymphangiogenesis and LVD in colorectal cancer

The following observations were made with regard to LVD in colorectal cancer:

- LYVE-1 antibodies clearly stained lymphatic vessels in and around colorectal cancer specimens and LVD scores could be determined by LYVE-1 positive vessel counting.
- lymphatic vessels within colorectal cancers were uncommon and the presence of deep intra-tumoural lymphatics was associated with increased expression of VEGF-C and decreased expression of VEGF-D in the invasive edge of the tumour.
- no associations were observed between LVD and clinicopathological factors

LYVE-1 antibody was found to be a sensitive and specific marker for lymphatic vessels in colorectal cancer, which concurs with the results of other groups examining a variety of malignant human tissues (Beasley et al., 2002; Mattila et al., 2002; Dadras et al., 2003; Maula et al., 2003; Straume et al., 2003; Williams et al., 2003). As discussed in **section 1.6.1, page 82,** numerous methods have been used to examine the lymphatic vasculature, antibodies to LYVE-1 have been developed recently as specific lymphatic markers (Jackson et al., 2001; Jackson, 2003) and show great promise in immunohistochemical and *in vitro* studies (**section 1.6.2, page 87** and Kriehuber et al., 2001; Podgrabinska et al., 2002). However, studies utilising LYVE-1 antibodies will be limited in the assessment of lymphatics in intra-hepatic pathology because sinusoidal endothelium is strongly positive for LYVE-1 (Carreira et al., 2001). In the studies reported here, Chalkley point counting was used to quantify LYVE-1 positive vessels to determine the LVD, the reason to select Chalkley point counting was that this method is reproducible, accurate and correlates with MVD measurements in colorectal cancer (Abdalla et al., 1996; White et al., 2002; Li et al., 2003). However, unlike MVD,

methods for the quantification of LVD have not been rigorously investigated in terms of either the optimal antibody or the best vessel counting method. Comparisons are currently hampered by the lack of availability of the lymphatic vessel specific antibodies (**Table 11, page 86**); hence collaborative studies between the research groups who have developed the antibodies would be a helpful advance.

This is the first report to demonstrate a relative lack of intra-tumoural lymphatics in colorectal cancer and is similar to the findings in breast cancer using antibodies to LYVE-1 (Williams et al., 2003). These results of few lymphatic vessels within colorectal cancer are indirectly supported by the gene expression experiments of Hanrahan et al. (Hanrahan et al., 2003) who found a reduced level of VEGFR3 mRNA in tumour tissue in comparison to normal colorectal mucosa, which may be considered a surrogate marker of intra-tumoural lymphatic vessels.

Various tumour types have been investigated for the presence of lymphatics and LVD using a variety of markers including antibodies directed against LYVE-1, podoplanin, VEGFR3, enzyme histochemistry and double staining techniques (Pepper et al., 2003). Each tumour type has particular behavioural characteristics, so it is likely that tumours that metastasise by different routes employ distinct signalling pathways to differing degrees. Consequently, the results of studies investigating one tumour type may not be translatable to other tumours. The finding that intra-tumoural lymphatics are sparse in colorectal cancer is a case in point. Whilst the spread of colorectal cancer to lymph nodes is well recognised as a crucial prognostic factor, LVD did not differ between subgroups displaying different clinicopathological characteristics, in particular, early and late disease, different nodal stages or tumours with and without lymphatic invasion. Pre-existing lymphatic vessels however, were commonly found in the submucosa and throughout the muscle layers of the bowel wall (**Figure 7b, page 140**). As a colorectal tumour progresses through the muscle layers it will naturally come into close contact

with pre-existing lymphatic vessels, at this stage expression of the VEGF family members may theoretically 'activate' the endothelial cells composing the vessel wall and promote entry of tumour cells. LVD, therefore, may not be a useful prognostic marker in tumour tissue where lymphatic vessels are already prominent and in close proximity to the invading tumour edge, whereas LVD may provide information of prognostic value for tumours located in tissues with more sparsely distributed lymphatics, possibly being more reflective in this situation of true tumour lymphangiogenesis.

The balance of VEGF-C and VEGF-D in the TI of colorectal cancer may be relevant in the promotion of the development of intra-tumoural lymphatic vessels. Increased VEGF-C and decreased VEGF-D expression intensity at the TI site was associated with the presence of deep intra-tumoural lymphatic vessels. This altered balance of VEGF-C and VEGF-D expression has been noted in colorectal cancer previously (George et al., 2001b; Hanrahan et al., 2003), although these earlier studies only described the altered VEGF-C/VEGF-D balance in terms of mRNA expression levels. Although the generation of intra-tumoural lymphatic vessels may enhance tumour cell metastasis, this is not an essential requirement, as lymphatic spread can clearly occur using the pre-existing lymphatic vessels (**section 1.5.6-1.5.7, page 77**). The production of lymphatic vessels within a colorectal tumour may be inconsequential if the new vessels are poorly functional as their contribution to metastatic spread may then be limited. The production of intra-tumoural lymphatic vessels in colorectal cancer may simply reflect the balance of growth factors expressed at TI and its effect on the peri-tumoural stromal cells, rather than being an essential requirement for lymphatic spread. Analogies to the complicated relationship between VEGF-A expression and MVD can be made for the relationship between LVD and tumour growth factor expression, which is unlikely to be straightforward. Various growth factors (eg. VEGF family members, bFGF,

chemokines) as well as other variables (eg. tissue inflammation, site/organ involved, location within the tumour) will influence the overall state of activation of lymphangiogenic pathways and the extent of lymphangiogenesis. Furthermore, the role of chemokines and their receptors in lymph node metastasis and lymphangiogenesis is only just beginning to be explored. Lymphatic endothelial cells secrete chemokines from their abluminal surfaces, which promote chemotaxis of activated antigen-presenting cells towards the vessels and initiation of the immune response (Saeki et al., 1999; Kriehuber et al., 2001). It had been thought that tumour cell entry into lymphatic vessels (whether pre-existing or new) was a passive process but the expression of chemokine receptors by malignant cells may represent tumour exploitation of this physiological pathway to allow active migration of tumour cells towards lymphatics (Forster et al., 2001; Muller et al., 2001; Wiley et al., 2001; Bachelder et al., 2002; Mashino et al., 2002). The extent of expression of chemokine receptors is likely to differ between varying tumour types and with microenvironmental conditions and may also influence the relative importance of LVD and intra-tumoural lymphatics as a requirement for lymphatic metastasis in differing tumours.

To summarise, in regard to lymphangiogenesis and LVD in colorectal cancer, antibodies to LYVE-1 clearly identify lymphatic vessels and provide a method for quantification of LVD, intra-tumoural lymphatics are relatively sparse within colorectal cancer and the presence of lymphatic vessels within the TI is associated with an alteration in the balance between VEGF-C/VEGF-D. The relative importance of tumour lymphangiogenesis versus pre-existing tissue lymphatics in colorectal cancer progression is an area for future research.

5.2. ROLE OF CIRCULATING VEGF-C AND VEGF-D IN THE PREDICTION OF TUMOUR BEHAVIOUR

Immunoassay experiments were performed to assess the role of circulating VEGF-C and VEGF-D in the prediction of tumour behaviour. A prospective study using 120 pre-operative colorectal cancer patients and 50 controls failed to confirm the initial results of a pilot study, illustrating increased plasma VEGF-C levels in colorectal cancer (Duff et al., 2003c). In the larger study, no significant differences in plasma VEGF-D and VEGF-A were found in cancer patients compared with controls and median plasma VEGF-C levels were elevated in control patients. No differences were found between patients with early and advanced disease, or patients with and without lymph node involvement.

Given the multitude of studies reporting elevated serum and plasma levels of VEGF-A in cancer patients (**section 1.4.4, page 60 & Table 9, page 63**), it is surprising that no such difference was detected in this study. This may relate to three factors; the site of the primary tumour, the choice of plasma for analysis and the nature of the control group. Firstly, the majority of tumours in this prospective study were rectal cancers (114/120, 95%). Werther et al. (2000; 2002) demonstrated in a group of 614 colorectal cancer patients that both serum and plasma VEGF-A levels were increased more in colonic than rectal cancers and this difference was reflected in the predictive power of elevated serum VEGF-A. Serum levels of VEGF-A above the 95th percentile of a healthy control group were predictive of reduced survival on multivariate analysis for the whole study group (Hazard ratio (HR) 1.44, 95% CI: 1.13-1.82, p=0.003). However, while this predictive value was increased on assessing the colonic cancer group alone (HR 1.7, 95% CI:1.2-2.3, p<0.0001), it lost predictive power for the subgroup of rectal

cancers (HR 1.4, 95% CI: 0.8-1.9, p=0.28) (Werther et al., 2000). Similar findings relating to plasma VEGF-A levels were illustrated in a subsequent study, with elevated plasma VEGF-A levels predicting reduced survival in colon cancers (p=0.01) but not rectal cancers (p=0.93) on univariate analysis (Werther et al., 2002). This may be a genuine reflection of biological differences between colonic and rectal tumours in the production of angiogenic growth factors and needs to be taken into consideration in the planning of future studies. Secondly, controversy persists regarding the optimum sample for the best reflection of biological activity (see **section 1.4.4, page 60**). In this study, a significant correlation was observed between plasma VEGF-A level and platelet count, with a trend towards a correlation for VEGF-C and platelet count. Correlations have previously been reported between serum VEGF-A and platelet count (Salgado et al., 2001; Bachelot et al., 2003; Poon et al., 2003). The choice of plasma sample for this study was partly to try and minimise the influence of platelet release of the growth factor but the weak correlation seen between platelet count and plasma growth factor level may reflect the occurrence of partial platelet degranulation in these samples. Thirdly, the choice of control group may have influenced the results. Control groups of published studies have used self-reported 'healthy' volunteers, who often differ in age and sex distribution from the cancer population and whether they have undiagnosed disease is unknown. This study aimed to correct the deficiencies of the pilot study and avoid these biases by using controls of similar age and sex distribution to the cancer patients and to exclude patients with colonic disease. In order to achieve this in practice, patients were recruited from those undergoing lower gastrointestinal endoscopy with normal results and it is possible that the prior bowel preparation necessary for the investigation could have influenced the circulating growth factor levels. Although no statistically significant difference existed between the gender proportions of cancer and control groups, there were relatively more males in the cancer

group (cancer group: 81 male, 39 female; control group: 29 male, 21 female, χ^2 test, p=0.238, **Table 25, page 196**). However, this is unlikely to have influenced the results unduly because no gender differences in either plasma or serum VEGF-C levels were found between control subjects in the pilot study (see **section 4.1.5, page 189**).

In patients undergoing resection of colorectal cancer liver metastases, circulating plasma levels of VEGF family members showed no relation to the immunohistochemically detected expression pattern seen in the liver metastasis. Consequently, plasma measurement of VEGF family members is of no value in the prediction of growth factor expression by the liver metastasis. Changes in plasma growth factor levels with time following surgical resection were insignificant for both VEGF-C and VEGF-D. The observed increase in plasma VEGF-A levels with time in both groups is likely to reflect the angiogenic potential of the healing surgical wound.

To summarise, an immunoassay was developed for the measurement of circulating VEGF-C. However, the immunoassay experiments failed to show clinical utility for the pre-operative measurement of circulating plasma levels of VEGF family members in the prediction of clinicopathological characteristics of primary colorectal cancers nor did they show ability to reflect the growth factor expression profile of hepatic metastases. The immunohistochemical studies illustrated that interactions between tumour expressed growth factors and receptors are important at the paracrine and autocrine level. This may explain why the measurement of circulating plasma levels of VEGF-C and VEGF-D failed to provide useful clinical information as circulating levels of the growth factors do not reflect the dynamic situation of the tumour invasive edge.

6. FUTURE DIRECTIONS

In order to confirm and build on the results of these studies, several areas require further exploration and clarification:

a) The relationship between VEGF family members and receptor expression in primary colorectal cancer:

- *in vitro* experiments are necessary to explore the relationship between colorectal cancer cell VEGF-C/VEGFR2 expression, controlling factors and influences behind such expression at a cellular level, the functional state of tumour-expressed VEGFR2, the importance of ligand/receptor expression to malignant cell survival, invasion and response to novel anti-VEGF treatments.
- similar immunohistochemical experiments are required in larger patient groups encompassing cases of all T stages and histological grades to confirm and extend the relationships observed in this thesis.
- the relationship between VEGFR3 expression, the VEGF ligands and VEGFR2 requires confirmation should specific antibodies become available.

b) The role of the VEGF family and its receptors in the development of colorectal cancer metastasis:

- *in vitro* and *in vivo* experiments are needed to clarify the mechanisms underlying reduced VEGFR2 expression observed in liver metastases.
- similar immunohistochemical experiments are required in larger groups of cases including representative samples of primary tumour and metastatic sites, to ideally

include recurrent disease, varying metastatic sites and irresectable liver metastases to assess whether the findings described are applicable to all cases.

- prospective studies to determine the immunohistochemical VEGF/VEGFR profile of primary colorectal tumours are essential to investigate the potential role of such profiling in determining tumour metastatic behaviour and response to novel therapies.

c) To further clarify the role of lymphangiogenesis and LVD in colorectal cancer:

- confirmation of the results of this thesis are needed using larger numbers of cases of primary colorectal cancer encompassing all T stages and histological grades.

- LYVE-1 immunostaining for lymphatic vessels may represent a means to determine zones of active lymphangiogenesis within and around tumours where the VEGF-C/VEGFR3 axis has been activated. In order to confirm this, double-staining for LYVE-1 and a marker of proliferation such as Ki67 could be used to pinpoint proliferating lymphatics (Beasley et al., 2002) in conjunction with serial section staining for VEGF-C.

- comparative immunohistochemical studies are required to determine the optimal antibody or antibody combination for lymphatic vessel discrimination and the best methodology for quantification of LVD, which should result in the production of guidelines to aid future research.

- the role of LVD as a prognostic factor in colorectal cancer and other malignancies should be examined further. In tumour types in which LVD is predictive of outcome the relationship between LVD, VEGF/VEGFR expression and topographical distribution, clinicopathological factors and chemokine/chemokine receptor expression can be investigated in more detail. Where LVD is prognostic, a LVD score or index combining

LVD with other independent prognostic factors may be useful to stratify patient risk and select for adjuvant treatment.

d) To investigate the role of circulating VEGF-C and VEGF-D in the prediction of tumour behaviour:

- clarification is required as to whether platelet VEGF-C level increases in malignancy, analogous to the situation with VEGF-A, and if platelets contain VEGF-D.
- the clinical utility of circulating plasma VEGF-C and VEGF-D levels requires further evaluation in colonic cancer as opposed to rectal cancer with careful selection of age and gender matched controls.

REFERENCES

Abdalla SA, Haboubi NY & Kumar S (1996) Quantification of microvessel density in human tumours: The effects of various pre-treatment methods and endothelial antibodies. *Int J Oncol* 9: 923-926.

Abdalla SA, Behzad F, Bsharah S, Kumar S, Amini S, O'Dwyer ST & Haboubi NY (1999) Prognostic relevance of microvessel density in colorectal tumours. *Oncol Rep* 6: 839-842.

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K & Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* 95: 548-553.

Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y & Sugimachi K (2000) Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* 83: 887-891.

Akagi K, Ikeda Y, Sumiyoshi Y, Kimura Y, Kinoshita J, Miyazaki M & Abe T (2002) Estimation of angiogenesis with anti-CD105 immunostaining in the process of colorectal cancer development. *Surgery* 131: S109-113.

Akbulut H, Altuntas F, Akbulut KG, Ozturk G, Cindoruk M, Unal E & Icli F (2002) Prognostic role of serum vascular endothelial growth factor, basic fibroblast growth factor and nitric oxide in patients with colorectal carcinoma. *Cytokine* 20: 184-190.

Amaya H, Tanigawa N, Lu C, Matsumura M, Shimomatsuya T, Horiuchi T & Muraoka R (1997) Association of vascular endothelial growth factor expression with tumor angiogenesis, survival and thymidine phosphorylase/platelet-derived endothelial cell growth factor expression in human colorectal cancer. *Cancer Lett* 119: 227-235.

American Society of Clinical Oncology (1998) 1997 update of recommendations for the use of tumor markers in breast and colorectal cancer. *J Clin Oncol* 16: 793-795.

Amioka T, Kitadai Y, Tanaka S, Haruma K, Yoshihara M, Yasui W & Chayama K (2002) Vascular endothelial growth factor-C expression predicts lymph node metastasis of human gastric carcinomas invading the submucosa. *Eur J Cancer* 38: 1413-1419.

Andre T, Kotelevets L, Vaillant J-C, Coudray AM, Weber L, Prevot S, Parc R, Gespach C & Chastre E (2000) VEGF, VEGF-B, VEGF-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. *Int J Cancer* 86: 174-181.

Arinaga M, Noguchi T, Takeno S, Chujo M, Miura T & Uchida Y (2003) Clinical significance of vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 in patients with nonsmall cell lung carcinoma. *Cancer* 97: 457-464.

Asano T & McLeod RS (2002) Dietary fibre for the prevention of colorectal adenomas and carcinomas. *Cochrane Database Syst Rev*: CD003430.

Astler VB & Coller FA (1954) The prognostic significance of direct extension of carcinoma of the colon and rectum. *Ann Surg* 139: 846-849.

Atkin WS, Morson BC & Cuzick J (1992) Long-term risk of colorectal cancer after excision of rectosigmoid adenomas. *N Engl J Med* 326: 658-662.

Atkin WS, Cuzick J, Northover JM & Whynes DK (1993) Prevention of colorectal cancer by once-only sigmoidoscopy. *Lancet* 341: 736-740.

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Shibuya M, Collen D, Conway EM & Carmeliet P (2003) Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 9: 936-943.

Auvinen MI, Sihvo EIT, Ruohtula T, Salminen JT, Koivistoinen A, Siivola P, Ronnholm R, Ramo JO, Bergman M & Salo JA (2002) Incipient angiogenesis in Barrett's epithelium and lymphangiogenesis in Barrett's adenocarcinoma. *J Clin Oncol* 20: 2971-2979.

Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G & Mercurio AM (2001) Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res* 61: 5736-5740.

Bachelder RE, Wendt MA & Mercurio AM (2002) Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res* 62: 7203-7206.

Bachelot T, Ray-Coquard I, Menetrier-Caux C, Rastkha M, Duc A & Blay J-Y (2003) Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. *Br J Cancer* 88: 1721-1726.

Balza E, Castellani P, Zulstra A, Neri D, Zardi L & Siri A (2001) Lack of specificity of endoglin expression for tumor blood vessels. *Int J Cancer* 94: 579-585.

Banerji S, Ni J, Wang S-X, Clasper S, Su J, Tammi R, Jones M & Jackson DG (1999) LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 144: 789-801.

Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C & Selby PJ (1998) Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. *Br J Cancer* 77: 956-964.

Banner BF, Whitehouse R, Baker SP & Swanson RS (1998) Tumor angiogenesis in stage II colorectal carcinoma: association with survival. *Am J Clin Pathol* 109: 733-737.

Barozzi C, Ravaioli M, D'Errico A, Grazi GL, Poggioli G, Cavrini G, Mazziotti A & Grigioni WF (2002) Relevance of biologic markers in colorectal carcinoma. A comparative study of a broad panel. *Cancer* 94: 647-657.

Bast RC, Jr., Ravdin P, Hayes DF, Bates S, Fritsche H, Jr., Jessup JM, Kemeny N, Locker GY, Mennel RG & Somerfield MR (2001) 2000 update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin Oncol* 19: 1865-1878.

Bates DO, Cui T-G, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D & Harper SJ (2002) VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 62: 4123-4131.

Beasley NJ, Prevo R, Banerji S, Leek RD, Moore J, van Trappen P, Cox G, Harris AL & Jackson DG (2002) Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res* 62: 1315-1320.

Berglund A, Molin D, Larsson A, Einarsson R & Glimelius B (2002) Tumour markers as early predictors of response to chemotherapy in advanced colorectal carcinoma. *Ann Oncol* 13: 1430-1437.

Bernatchez PN, Soker S & Sirois MG (1999) Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent. *J Biol Chem* 274: 31047-31054.

Berney CR, Yang J-L, Fisher RJ, Russell PJ & Crowe PJ (1998) Vascular endothelial growth factor expression is reduced in liver metastasis from colorectal cancer and correlates with urokinase-type plasminogen activator. *Anticancer Res* 18: 973-977.

Bingham SA, Day NE, Luben R, Ferrari P, Slimani N, Norat T, Clavel-Chapelon F, Kesse E, Nieters A, Boeing H, Tjonneland A, Overvad K, Martinez C, Dorronsoro M, Gonzalez CA, Key TJ, Trichopoulou A, Naska A, Vineis P, Tumino R, Krogh V, Bueno-de-Mesquita HB, Peeters PHM, Berglund G, Hallmans G, Lund E, Skeie G, Kaaks R & Riboli E (2003) Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet* 361: 1496-1501.

Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Kowalski H & Oberhuber G (2000) Lymphatic microvessel density in epithelial ovarian cancer: its impact on prognosis. *Anticancer Res* 20: 2981-2986.

Birner P, Obermair A, Schindl M, Kowalski H, Breitenecker G & Oberhuber G (2001a) Selective immunohistochemical staining of blood and lymphatic vessels reveals independent prognostic influence of blood and lymphatic vessel invasion in early-stage cervical cancer. *Clin Cancer Res* 7: 93-97.

Birner P, Schindl M, Obermair A, Breitenecker G, Kowalski H & Oberhuber G (2001b) Lymphatic microvessel density as a novel prognostic factor in early-stage invasive cervical cancer. *Int J Cancer* 95: 29-33.

Boardman KC & Swartz MA (2003) Interstitial flow as a guide for lymphangiogenesis. *Circ Res* 92: 801-808.

Boocock CA, Charnock-Jones DS, Sharkey AM, McLaren J, Barker PJ, Wright KA, Twentyman PR & Smith SK (1995) Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst* 87: 506-516.

Borg J-P, deLapeyriere O, Noguchi T, Rottapel R, Dubreuil P & Birnbaum D (1995) Biochemical characterization of two isoforms of FLT4, a VEGF receptor-related tyrosine kinase. *Oncogene* 10: 973-984.

Bossi P, Viale G, Lee AKC, Alfano R, Coggi G & Bosari S (1995) Angiogenesis in colorectal tumors: microvessel quantitation in adenomas and carcinomas with clinicopathological correlations. *Cancer Res* 55: 5049-5053.

Breiteneder-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G & Kerjaschki D (1997) Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. *Am J Pathol* 151: 1141-1152.

Brewer CA, Setterdahl JJ, Li MJ, Johnston JM, Mann JL & Mcasey ME (2000) Endoglin expression as a measure of microvessel density in cervical cancer. *Obstet Gynecol* 96: 224-228.

Broll R, Erdmann H, Duchrow M, Oevermann E, Schwandner O, Markert U, Bruch HP & Windhovel U (2001) Vascular endothelial growth factor (VEGF)--a valuable serum tumour marker in patients with colorectal cancer? *Eur J Surg Oncol* 27: 37-42.

Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR & Dvorak HF (1993) Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 53: 4727-4735.

Burkitt DP, Walker AR & Painter NS (1972) Effect of dietary fibre on stools and the transit-times, and its role in the causation of disease. *Lancet* 2: 1408-1412.

Cancer Research UK. (2002). Bowel Cancer Factsheet. Cancer Research UK.

Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, Qi J-H, Claesson-Welsh L & Alitalo K (1998) Vascular endothelial growth factor C induces angiogenesis in vivo. *Proc Natl Acad Sci USA* 95: 14389-14394.

Carreira CM, Nasser SM, di Tomaso E, Padera TP, Boucher Y, Tomarev SI & Jain RK (2001) LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancers and cirrhosis. *Cancer Res* 61: 8079-8084.

Cascinu S, Staccioli MP, Gasparini G, Giordani P, Catalano V, Ghiselli R, Rossi C, Baldelli AM, Graziano F, Saba V, Muretto P & Catalano G (2000) Expression of vascular endothelial growth factor can predict event-free survival in stage II colon cancer. *Clin Cancer Res* 6: 2803-2807.

Cascinu S, Graziano F, Catalano V, Staccioli MP, Barni S, Giordani P, Rossi MC, Baldelli AM, Muretto P, Valenti A & Catalano G (2001) Differences of vascular endothelial growth factor (VEGF) expression between liver and abdominal metastases from colon cancer. Implications for the treatment with VEGF inhibitors. *Clin Exp Met* 18: 651-655.

Caspari R, Friedl W, Mandl M, Moslein G, Kadmon M, Knapp M, Jacobasch KH, Ecker KW, Kreissler-Haag D, Timmermanns G & Propping P (1994) Familial

adenomatous polyposis: mutation at codon 1309 and early onset of colon cancer. *Lancet* 343: 629-632.

Cawthorn SJ, Gibbs NM & Marks CG (1986) Clearance technique for the detection of lymph nodes in colorectal cancer. *Br J Surg* 73: 58-60.

Chalkley HW (1943) Method for the quantitative morphologic analysis of tissues. *J Natl Cancer Inst* 4: 47-53.

Charames GS, Millar AL, Pal T, Narod S & Bapat B (2000) Do MSH6 mutations contribute to double primary cancers of the colorectum and endometrium? *Hum Genet* 107: 623-629.

Chilov D, Kukk E, Taira S, Jeltsch M, Kaukonen J, Palotie A, Joukov V & Alitalo K (1997) Genomic organization of human and mouse genes for vascular endothelial growth factor C. *J Biol Chem* 272: 25176-25183.

Chin K-F, Greenman J, Gardiner E, Kumar H, Topping K & Monson J (2000) Pre-operative serum vascular endothelial growth factor can select patients for adjuvant treatment after curative resection in colorectal cancer. *Br J Cancer* 83: 1425-1431.

Choi HJ, Hyun MS, Jung GJ, Kim SS & Hong SH (1998) Tumor angiogenesis as a prognostic predictor in colorectal carcinoma with special reference to mode of metastasis and recurrence. *Oncology* 55: 575-581.

Clarijs R, Ruiter DJ & de Waal RM (2001a) Lymphangiogenesis in malignant tumours: Does it occur? *J Pathol* 193: 143-146.

Clarijs R, Schalkwijk L, Ruiter DJ & de Waal RMW (2001b) Lack of lymphangiogenesis despite coexpression of VEGF-C and its receptor Flt-4 in uveal melanoma. *Invest Ophthalmol Vis Sci* 42: 1422-1428.

Clarijs R, Schalkwijk L, Hofmann UB, Ruiter DJ & De Waal RMW (2002) Induction of vascular endothelial growth factor receptor-3 expression on tumor microvasculature as a new progression marker in human cutaneous melanoma. *Cancer Res* 62: 7059-7065.

Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J & Risau W (1996) The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 271: 17629-17634.

Colorectal Cancer Colloborative Group (2001) Adjuvant radiotherapy for rectal cancer: a systematic overview of 8507 patients from 22 randomised trials. *Lancet* 358: 1291-1304.

Commission for Health Improvement/Audit Commission. (2001). National Service Framework Assessments No.1. NHS Cancer Care in England and Wales pp. 7-8: London.

Dadras SS, Paul T, Bertoncini J, Brown LF, Muzikansky A, Jackson DG, Ellwanger U, Garbe C, Mihm MC & Detmar M (2003) Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. *Am J Pathol* 162: 1951-1960.

Daniels CB, Lewis BC, Tsopelas C, Munns SL, Orgeig S, Baldwin ME, Stacker SA, Achen MG, Chatterton BE & Cooter RD (2003) Regenerating lizard tails: A new model for investigating lymphangiogenesis. *FASEB J* 17: 479-481.

Davies MM, Jonas SK, Kaur S & Allen-Mersh TG (2000) Plasma vascular endothelial but not fibroblast growth factor levels correlate with colorectal liver metastasis vascularity and volume. *Br J Cancer* 82: 1004-1008.

de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N & Williams LT (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255: 989-991.

de Waal RM, van Altena MC, Erhard H, Weidle UH, Nooijen PT & Ruiter DJ (1997) Lack of lymphangiogenesis in human primary cutaneous melanoma. Consequences for the mechanism of lymphatic dissemination. *Am J Pathol* 150: 1951-1957.

Deans GT, Heatley M, Anderson N, Patterson CC, Rowlands BJ, Parks TG & Spence RAJ (1994) Jass' classification revisited. *J Am Coll Surg* 179: 11-17.

Diep CB, Thorstensen L, Meling GI, Skovlund E, Rognum TO & Lothe RA (2003) Genetic tumor markers with prognostic impact in Dukes' stages B and C colorectal cancer patients. *J Clin Oncol* 21: 820-829.

Dirix LY, Vermeulen PB, Hubens G, Benoy I, Martin M, De Pooter C & Van Oosterom AT (1996) Serum basic fibroblast growth factor and vascular endothelial growth factor and tumour growth kinetics in advanced colorectal cancer. *Ann Oncol* 7: 843-848.

Dirix LY, Vermeulen PB, Pawinski A, Prove A, Benoy I, De Pooter C, Martin M & Van Oosterom AT (1997) Elevated levels of the angiogenic cytokines basic fibroblast growth factor and vascular endothelial growth factor in sera of cancer patients. *Br J Cancer* 76: 238-243.

Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L & Rougier P (2000) Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 355: 1041-1047.

Duff SE & Jayson GC. (2003). Vascular endothelial growth factor C and vascular endothelial growth factor D: biology, functions and role in malignancy. *In press*. In *VEGF and Cancer*, Harmey, JH (ed). Kluwer Academic Press and Landes Bioscience.

Duff SE, Li C, Garland JM & Kumar S (2003a) CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 17: 984-992.

Duff SE, Li C, Jeziorska M, Kumar S, Saunders MP, Sherlock D, O'Dwyer ST & Jayson GC (2003b) Vascular endothelial growth factors C and D and lymphangiogenesis in gastrointestinal tract malignancy. *Br J Cancer* 89: 426-430.

Duff SE, Li C, Renehan A, O'Dwyer ST & Kumar S (2003c) Immunodetection and molecular forms of plasma vascular endothelial growth factor-C. *Int J Oncol* 22: 339-343.

Dukes CE (1932) The classification of cancer of the rectum. *J Pathol* 35: 323-332.

Dukes CE (1949) The surgical pathology of rectal cancer. *J Clin Pathol* 2: 95-99.

Dukes CE & Bussey HJR (1958) The spread of rectal cancer and its effect on prognosis. *Br J Cancer* 12: 309-320.

Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, Breitman M & Alitalo K (1998) Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 282: 946-949.

Dunk C & Ahmed A (2001) Vascular endothelial growth factor receptor-2-mediated mitogenesis is negatively regulated by vascular endothelial growth factor receptor-1 in tumor epithelial cells. *Am J Pathol* 158: 265-273.

Duque JL, Loughlin KR, Adam RM, Kantoff PW, Zurakowski D & Freeman MR (1999) Plasma levels of vascular endothelial growth factor are increased in patients with metastatic prostate cancer. *Urology* 54: 523-527.

Dvorak HF, Brown LF, Detmar M & Dvorak AM (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146: 1029-1039.

Dvorak HF (2002) Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 20: 4368-4380.

Ekbom A, Helmick C, Zack M & Adami H-O (1990a) Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med* 323: 1228-1233.

Ekbom A, Helmick C, Zack M & Adami H-O (1990b) Increased risk of large-bowel cancer in Crohn's disease with colonic involvement. *Lancet* 336: 357-359.

Eliceiri BP, Puente XS, Hood JD, Stupack DG, Schlaepfer DD, Huang XZ, Sheppard D & Cheresh DA (2002) Src-mediated coupling of focal adhesion kinase to integrin $\alpha v\beta 5$ in vascular endothelial growth factor signaling. *J Cell Biol* 157: 149-160.

Engel CJ, Bennett ST, Chambers AF, Doig GS, Kerkvliet N & O'Malley FP (1996) Tumor angiogenesis predicts recurrence in invasive colorectal cancer when controlled for Dukes staging. *Am J Surg Pathol* 20: 1260-1265.

Enholm B, Paavonen K, Ristimaki A, Kumar V, Gunji Y, Klefstrom J, Kivinen L, Laiho M, Olofsson B, Joukov V, Eriksson U & Alitalo K (1997) Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene* 14: 2475-2483.

Fearnhead NS, Wilding JL & Bodmer WF (2002) Genetics of colorectal cancer: hereditary aspects and overview of colorectal tumorigenesis. *Br Med Bull* 64: 27-43.

Fearon ER & Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* 61: 759-767.

Ferrara N & Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161: 851-858.

Ferrara N & Davis-Smyth T (1997) The biology of vascular endothelial growth factor. *Endocr Rev* 18: 4-25.

Ferrara N (1999) Molecular and biological properties of vascular endothelial growth factor. *J Mol Med* 77: 527-543.

Fidler IJ (2001) Angiogenic heterogeneity: regulation of neoplastic angiogenesis by the organ microenvironment. *Journal of the National Cancer Institute* 93: 1040-1041.

Fielding LP, Phillips RK, Fry JS & Hittinger R (1986) Prediction of outcome after curative resection for large bowel cancer. *Lancet* 2: 904-907.

Fitz LJ, Morris JC, Towler P, Long A, Burgess P, Greco R, Wang J, Gassaway R, Nickbarg E, Kovacic S, Ciarletta A, Giannotti J, Finnerty H, Zollner R, Beier DR, Leak LV, Turner KJ & Wood CR (1997) Characterization of murine Flt4 ligand/VEGF-C. *Oncogene* 15: 613-618.

Folkman J (1995) Seminars in Medicine of the Beth Israel Hospital, Boston: Clinical applications of research on angiogenesis. *N Engl J Med* 333: 1757-1763.

Fong G-H, Rossant J, Gertsenstein M & Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376: 66-70.

Forster R, Ohl L & Henning G (2001) Lessons learned from lymphocytes: CC chemokine receptor-7 involved in lymphogenic metastasis of melanoma. *J Natl Cancer Inst* 93: 1588-1589.

Fournier E, Dubreuil P, Birnbaum D & Borg J-P (1995) Mutation at tyrosine residue 1337 abrogates ligand-dependent transforming capacity of the FLT4 receptor. *Oncogene* 11: 921-931.

Fox SB, Leek RD, Weekes MP, Whitehouse RM, Gatter KC & Harris AL (1995) Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. *J Pathol* 177: 275-283.

Fox SH, Whalen GF, Sanders MM, Burleson JA, Jennings K, Kurtzman S & Kreutzer D (1998) Angiogenesis in normal tissue adjacent to colon cancer. *J Surg Oncol* 69: 230-234.

Frank RE, Saclarides TJ, Leurgans S, Speziale NJ, Drab EA & Rubin DB (1995) Tumor angiogenesis as a predictor of recurrence and survival in patients with node-negative colon cancer. *Ann Surg* 222: 696-699.

Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA & Cheresh DA (1995) Definition of two angiogenic pathways by distinct alpha v integrins. *Science* 270: 1500-1502.

Fuhrmann-Benzakein E, Ma MN, Rubbia-Brandt L, Mentha G, Ruefenacht D, Sappino A-P & Pepper MS (2000) Elevated levels of angiogenic cytokines in the plasma of cancer patients. *Int J Cancer* 85: 40-45.

Fujii T, Otsuki T, Moriya T, Sakaguchi H, Kurebayashi J, Yata K, Uno M, Kobayashi T, Kimura T, Jo Y, Kinugawa K, Furukawa Y, Morioka M, Ueki A & Tanaka H (2002) Effect of hypoxia on human seminoma cells. *Int J Oncol* 20: 955-962.

Fujisaki K, Mitsuyama K, Toyonaga A, Matsuo K & Tanikawa K (1998) Circulating vascular endothelial growth factor in patients with colorectal cancer. *Am J Gastroenterol* 93: 249-252.

Fukumura D, Yuan F, Monsky WL, Chen Y & Jain RK (1997) Effect of host microenvironment on the microcirculation of human colon adenocarcinoma. *Am J Pathol* 151: 679-688.

Fukuzawa M, Sugiura H, Koshinaga T, Ikeda T, Hagiwara N & Sawada T (2002) Expression of vascular endothelial growth factor and its receptor Flk-1 in human neuroblastoma using in situ hybridization. *J Pediatr Surg* 37: 1747-1750.

Funaki H, Nishimura G, Harada S-i, Ninomiya I, Terada I, Fushida S, Tani T, Fujimura T, Kayahara M, Shimizu K, Ohta T & Miwa K (2003) Expression of vascular endothelial growth factor D is associated with lymph node metastasis in human colorectal carcinoma. *Oncology* 64: 416-422.

Furodoi A, Tanaka S, Haruma K, Kitadai Y, Yoshihara M, Chayama K & Shimamoto F (2002) Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. *Oncology* 62: 157-166.

Gabriel WB, Dukes CE & Bussey HJR (1935) Lymphatic spread in cancer of the rectum. *Br J Surg* 23: 395-413.

Gabrilovich D, Ishida T, Oyama T, Ran S, Kravtsov V, Nadaf S & Carbone DP (1998) Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* 92: 4150-4166.

Gabrilovich DI, Ishida T, Nadaf S, Ohm JE & Carbone DP (1999) Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. *Clin Cancer Res* 5: 2963-2970.

Galindo Gallego M, Fernandez Acenero MJ, Sanz Ortega J & Alijama A (2000) Vascular enumeration as a prognosticator for colorectal carcinoma. *Eur J Cancer* 36: 55-60.

Gaudry M, Bregerie O, Andrieu V, El Benna J, Pocidalo MA & Hakim J (1997) Intracellular pool of vascular endothelial growth factor in human neutrophils. *Blood* 90: 4153-4161.

George ML, Eccles SA, Tutton MG, Abulafi AM & Swift RI (2000) Correlation of plasma and serum vascular endothelial growth factor levels with platelet count in colorectal cancer: clinical evidence of platelet scavenging? *Clin Cancer Res* 6: 3147-3152.

George ML, Dzik-Jurasz ASK, Padhani AR, Brown G, Tait DM, Eccles SA & Swift RI (2001a) Non-invasive methods of assessing angiogenesis and their value in predicting response to treatment in colorectal cancer. *Br J Surg* 88: 1628-1636.

George ML, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA & Swift RI (2001b) VEGF-A, VEGF-C and VEGF-D in colorectal cancer progression. *Neoplasia* 3: 420-427.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V & Ferrara N (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273: 30336-30343.

Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N & Ferrara N (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J Biol Chem* 276: 3222-3230.

Gitay-Goren H, Halaban R & Neufeld G (1993) Human melanoma cells but not normal melanocytes express vascular endothelial growth factor receptors. *Biochem Biophys Res Commun* 190: 702-708.

Gunsilius E, Tschmelitsch J, Eberwein M, Schwelberger H, Spizzo G, Kahler CM, Stockhammer G, Lang A, Petzer AL & Gastl G (2002) In vivo release of vascular endothelial growth factor from colorectal carcinomas. *Oncology* 62: 313-317.

Haboubi NY, Clark P, Kaftan SM & Schofield PF (1992) The importance of combining xylene clearance and immunohistochemistry in the accurate staging of colorectal carcinoma. *J R Soc Med* 85: 386-388.

Haboubi NY, Abdalla SAA, Amini S, Clark P, Dougal M, Dube A & Schofield P (1998) The novel combination of fat clearance and immunohistochemistry improves prediction of the outcome of patients with colorectal carcinomas: a preliminary study. *Int J Colorect Dis* 13: 99-102.

Hanahan D & Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86: 353-364.

Hanrahan V, Currie MJ, Gunningham SP, Morrin HR, Scott PA, Robinson BA & Fox SB (2003) The angiogenic switch for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in the adenoma-carcinoma sequence during colorectal cancer progression. *J Pathol* 200: 183-194.

Harada Y, Ogata Y & Shirouzu K (2001) Expression of vascular endothelial growth factor and its receptor KDR (kinase domain-containing receptor)/Flk-1 (fetal liver kinase-1) as prognostic factors in human colorectal cancer. *Int J Clin Oncol* 6: 221-228.

Hardcastle JD, Chamberlain JO, Robinson MHE, Moss SM, Amar SS, Balfour TW, James PD & Mangham CM (1996) Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. *Lancet* 348: 1472-1477.

Harmey JH & Bouchier-Hayes D (2002) Vascular endothelial growth factor (VEGF), a survival factor for tumour cells: implications for anti-angiogenic therapy. *Bioessays* 24: 280-283.

He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B & Kinzler KW (1998) Identification of c-MYC as a target of the APC pathway. *Science* 281: 1509-1512.

Heald RJ, Husband EM & Ryall RDH (1982) The mesorectum in rectal cancer surgery--the clue to pelvic recurrence? *Br J Surg* 69: 613-616.

Hirakawa S, Hong Y-K, Harvey N, Schacht V, Matsuda K, Libermann T & Detmar M (2003) Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol* 162: 575-586.

Hurwitz H, Fehrenbacher L, Cartwright T, Hainsworth J, Helm W, Berlin J, Griffing S, Novotny W, Holmgren E & Kabbinavar F. (2003). Bevacizumab (a monoclonal antibody to vascular endothelial growth factor) prolongs survival in first-line colorectal cancer (CRC): Results of a phase III trial of bevacizumab in combination with bolus IFL (irinotecan, 5-fluorouracil, leucovorin) as first-line therapy in subjects with metastatic CRC. In *ASCO meeting*: Chicago, Illinois, USA.

Hyodo I, Doi T, Endo H, Hosokawa Y, Nishikawa Y, Tanimizu M, Jinno K & Kotani Y (1998) Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. *Eur J Cancer* 34: 2041-2045.

Ichikura T, Tomimatsu S, Ohkura E & Mochizuki H (2001) Prognostic significance of the expression of vascular endothelial growth factor (VEGF) and VEGF-C in gastric carcinoma. *J Surg Oncol* 78: 132-137.

Inoshima N, Nakanishi Y, Minami T, Izumi M, Takayama K, Yoshino I & Hara N (2002) The influence of dendritic cell infiltration and vascular endothelial growth factor expression on the prognosis of non-small cell lung cancer. *Clin Cancer Res* 8: 3480-3486.

Ionov Y, Peinado MA, Malkhosyan S, Shibata D & Perucho M (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363: 558-561.

Irrthum A, Karkkainen MJ, Devriendt K, Alitalo K & Vikkula M (2000) Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. *Am J Hum Genet* 67: 295-301.

Ishibashi T, Kimura H, Shikama Y, Uchida T, Kariyone S, Hirano T, Kishimoto T, Takatsuki F & Akiyama Y (1989) Interleukin-6 is a potent thrombopoietic factor in vivo in mice. *Blood* 74: 1241-1244.

Ishigami SI, Arii S, Furutani M, Niwano M, Harada T, Mizumoto M, Mori A, Onodera H & Imamura M (1998) Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. *Br J Cancer* 78: 1379-1384.

Ishikawa M, Kitayama J, Kazama S & Nagawa H (2003) Expression of vascular endothelial growth factor C and D (VEGF-C and -D) is an important risk factor for lymphatic metastasis in undifferentiated early gastric carcinoma. *Jpn J Clin Oncol* 33: 21-27.

Jaakkola P, Mole DR, Tian Y-M, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW & Ratcliffe PJ (2001) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* 292: 468-472.

Jackson DG, Prevo R, Clasper S & Banerji S (2001) LYVE-1, the lymphatic system and tumor lymphangiogenesis. *Trends Immunol* 22: 317-321.

Jackson DG (2003) The lymphatics revisited. New perspectives from the hyaluronan receptor LYVE-1. *Trends Cardiovasc Med* 13: 1-7.

Jackson MW, Roberts JS, Heckford SE, Ricciardelli C, Stahl J, Choong C, Horsfall DJ & Tilley WD (2002) A potential autocrine role for vascular endothelial growth factor in prostate cancer. *Cancer Res* 62: 854-859.

Jain RK & Fenton BT (2002) Intratumoral lymphatic vessels: a case of mistaken identity or malfunction? *J Natl Cancer Inst* 94: 417-421.

Jass JR, Love SB & Northover JM (1987) A new prognostic classification of rectal cancer. *Lancet* 1: 1303-1306.

Jelkmann W (2001) Pitfalls in the measurement of circulating vascular endothelial growth factor. *Clin Chem* 47: 617-623.

Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK & Alitalo K (1997) Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* 276: 1423-1425.

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N & Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 15: 290-298.

Joukov V, Kaipainen A, Jeltsch M, Pajusola K, Olofsson B, Kumar V, Eriksson U & Alitalo K (1997a) Vascular endothelial growth factors VEGF-B and VEGF-C. *J Cell Physiol* 173: 211-215.

Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N & Alitalo K (1997b) Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* 16: 3898-3911.

Jussila L, Valtola R, Partanen TA, Salven P, Heikkila P, Matikainen M-T, Renkonen R, Kaipainen A, Detmar M, Tschachler E, Alitalo R & Alitalo K (1998) Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res* 58: 1599-1604.

Kabashima A, Maehara Y, Kakeji Y & Sugimachi K (2001) Overexpression of vascular endothelial growth factor C is related to lymphogenous metastasis in early gastric carcinoma. *Oncology* 60: 146-150.

Kaio E, Tanaka S, Kitadai Y, Sumii M, Yoshihara M, Haruma K & Chayama K (2003) Clinical significance of angiogenic factor expression at the deepest invasive site of advanced colorectal carcinoma. *Oncology* 64: 61-73.

Kaipainen A, Korhonen J, Pajusola K, Aprelikova O, Persico MG, Terman BI & Alitalo K (1993) The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells. *J Exp Med* 178: 2077-2088.

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M & Alitalo K (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* 92: 3566-3570.

Kang S-M, Maeda K, Chung Y-S, Onoda N, Ogawa Y, Takatsuka S, Ogawa M, Sawada T, Nakata B, Nishiguchi Y, Ikehara T, Okuno M & Sowa M (1997a) Vascular endothelial growth factor expression correlates with hematogenous metastasis and prognosis in colorectal carcinoma. *Oncol Rep* 4: 381-384.

Kang S-M, Maeda K, Onoda N, Chung Y-S, Nakata B, Nishiguchi Y & Sowa M (1997b) Combined analysis of p53 and vascular endothelial growth factor expression in colorectal carcinoma for determination of tumor vascularity and liver metastasis. *Int J Cancer* 74: 502-507.

Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJT, Pahlman L, Glimelius B, van Krieken JHJM, Leer JWH & van de Velde CJH (2001) Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 345: 638-646.

Kapiteijn E, Putter H & van de Velde CJH (2002) Impact of the introduction and training of total mesorectal excision on recurrence and survival in rectal cancer in The Netherlands. *Br J Surg* 89: 1142-1149.

Karayiannakis AJ, Syrigos KN, Zbar A, Baibas N, Polychronidis A, Simopoulos C & Karatzas G (2002) Clinical significance of preoperative serum vascular endothelial growth factor levels in patients with colorectal cancer and the effect of tumor surgery. *Surgery* 131: 548-555.

Karkkainen MJ, Ferrell RE, Lawrence EC, Kimak MA, Levinson KL, McTigue MA, Alitalo K & Finegold DN (2000) Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat Genet* 25: 153-159.

Karkkainen MJ & Petrova TV (2000) Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene* 19: 5598-5605.

Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K, Bueler H, Eichmann A, Kauppinen R, Kettunen MI, Yla-Herttuala S, Finegold DN, Ferrell RE & Alitalo K (2001) A model for gene therapy of human hereditary lymphedema. *Proc Natl Acad Sci USA* 98: 12677-12682.

Karpanen T & Alitalo K (2001) Lymphatic vessels as targets of tumor therapy? *J Exp Med* 194: F37-42.

Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M & Alitalo K (2001) Vasular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 61: 1786-1790.

Kawakami M, Furuhata T, Kimura Y, Yamaguchi K, Hata F, Sasaki K & Hirata K (2003) Quantification of vascular endothelial growth factor-C and its receptor-3 messenger RNA with real-time quantitative polymerase chain reaction as a predictor of lymph node metastasis in human colorectal cancer. *Surgery* 133: 300-308.

Kendall RL & Thomas KA (1993) Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci USA* 90: 10705-10709.

Keyt BA, Nguyen HV, Berleau LT, Duarte CM, Park J, Chen H & Ferrara N (1996) Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *J Biol Chem* 271: 5638-5646.

Khorana AA, Ryan CK, Cox C, Eberly S & Sahasrabudhe DM (2003) Vascular endothelial growth factor, CD68, and epidermal growth factor receptor expression and survival in patients with Stage II and Stage III colon carcinoma. *Cancer* 97: 960-968.

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS & Ferrara N (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362: 841-844.

Kirklin JW, Dockerty MB & Waugh JM (1949) The role of the peritoneal reflection in the prognosis of carcinoma of the rectum and sigmoid colon. *Surg Gynecol Obstet* 88: 326-331.

Kitadai Y, Amioka T, Haruma K, Tanaka S, Yoshihara M, Sumii K, Matsutani N, Yasui W & Chayama K (2001) Clinicopathological significance of vascular endothelial growth factor (VEGF)-C in human esophageal squamous cell carcinomas. *Int J Cancer* 93: 662-666.

Knudson AG (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 68: 820-823.

Kondo Y, Arii S, Furutani M, Isigami S-I, Mori A, Onodera H, Chiba T & Imamura M (2000a) Implication of vascular endothelial growth factor and p53 status for angiogenesis in noninvasive colorectal carcinoma. *Cancer* 88: 1820-1827.

Kondo Y, Arii S, Mori A, Furutani M, Chiba T & Imamura M (2000b) Enhancement of angiogenesis, tumor growth, and metastasis by transfection of vascular endothelial growth factor into LoVo human colon cancer cell line. *Clin Cancer Res* 6: 622-630.

Koolwijk P, Peters E, van der Vecht B, Hornig C, Weich HA, Alitalo K, Hicklin DJ, Wu Y, Witte L & van Hinsbergh VWM (2001) Involvement of VEGFR-2 (kdr/flk-1) but not VEGFR-1 (flt-1) in VEGF-A and VEGF-C-induced tube formation by human microvascular endothelial cells in fibrin matrices *in vitro*. *Angiogenesis* 4: 53-60.

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

Kriehuber E, Breiteneder-Geleff S, Groeger M, Soleiman A, Schoppmann SF, Stingl G, Kerjaschki D & Maurer D (2001) Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med* 194: 797-808.

Kronborg O, Fenger C, Olsen J, Jorgensen OD & Sondergaard O (1996) Randomised study of screening for colorectal cancer with faecal-occult-blood test. *Lancet* 348: 1467-1471.

Kubo H, Fujiwara T, Jussila L, Hashi H, Ogawa M, Shimizu K, Awane M, Sakai Y, Takabayashi A, Alitalo K, Yamaoka Y & Nishikawa SI (2000) Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. *Blood* 96: 546-553.

Kubo H, Cao R, Brakenhielm E, Makinen T, Cao Y & Alitalo K (2002) Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. *Proc Natl Acad Sci USA* 99: 8868-8873.

Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V & Alitalo K (1996) VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* 122: 3829-3837.

Kumar H, Heer K, Lee PWR, Duthie GS, MacDonald AW, Greenman J, Kerin MJ & Monson JRT (1998) Preoperative serum vascular endothelial growth factor can predict stage in colorectal cancer. *Clin Cancer Res* 4: 1279-1285.

Kumar H, Heer K, Duthie GS & Monson JR (2002a) Kinetics of postoperative serum vascular endothelial growth factor (VEGF) - can it be used to predict curative resections in colorectal cancer? *Anticancer Res* 22: 3717-3722.

Kumar H, Heer K, Greenman J, Kerin MJ & Monson JRT (2002b) Soluble FLT-1 is detectable in the sera of colorectal and breast cancer patients. *Anticancer Res* 22: 1877-1880.

Kune GA, Kune S, Field B, White R, Brough W, Schellenberger R & Watson LF (1990) Survival in patients with large-bowel cancer. A population-based investigation from the Melbourne Colorectal Cancer Study. *Dis Colon Rectum* 33: 938-946.

La Rosa S, Uccella S, Finzi G, Albarello L, Sessa F & Capella C (2003) Localization of vascular endothelial growth factor and its receptors in digestive endocrine tumors: correlation with microvessel density and clinicopathologic features. *Hum Pathol* 34: 18-27.

Landriscina M, Cassano A, Ratto C, Longo R, Ippoliti M, Palazzotti B, Crucitti F & Barone C (1998) Quantitative analysis of basic fibroblast growth factor and vascular endothelial growth factor in human colorectal cancer. *Br J Cancer* 78: 765-770.

Lee J, Gray A, Yuan J, Luoh SM, Avraham H & Wood WI (1996) Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA* 93: 1988-1992.

Lee J-C, Chow N-H, Wang S-T & Huang S-M (2000a) Prognostic value of vascular endothelial growth factor expression in colorectal cancer patients. *Eur J Cancer* 36: 748-753.

Lee J-K, Hong Y-J, Han C-J, Hwang D-Y & Hong S-I (2000b) Clinical usefulness of serum and plasma vascular endothelial growth factor in cancer patients: Which is the optimal specimen? *Int J Oncol* 17: 149-152.

Lennard-Jones JE, Melville DM, Morson BC, Ritchie JK & Williams CB (1990) Precancer and cancer in extensive ulcerative colitis: findings among 401 patients over 22 years. *Gut* 31: 800-806.

Leu AJ, Berk DA, Lymboussaki A, Alitalo K & Jain RK (2000) Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res* 60: 4324-4327.

Li C, Gardy R, Seon BK, Duff SE, Abdalla SA, Renehan A, O'Dwyer ST, Haboubi N & Kumar S (2003) Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis. *Br J Cancer* 88: 1424-1431.

Li CG, Wilson PB, Bernabéu C, Raab U, Wang JM & Kumar S (1998) Immunodetection and characterisation of soluble CD105-TGFß complexes. *J Immunol Meth* 218: 85-93.

Lindmark G, Gerdin B, Sundberg C, Påhlman L, Bergström R & Glimelius B (1996) Prognostic significance of the microvascular count in colorectal cancer. *J Clin Oncol* 14: 461-466.

Liotta LA & Kohn EC (2001) The microenvironment of the tumour-host interface. *Nature* 411: 375-379.

Liu B, Nicolaides NC, Markowitz S, Willson JKV, Parsons RE, Jen J, Papadopolous N, Peltomaki P, de la Chapelle A, Hamilton SR, Kinzler KW & Vogelstein B (1995) Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nat Genet* 9: 48-55.

Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, Dunlop M, Wyllie A, Peltomaki P, de la Chapelle A, Hamilton SR, Vogelstein B & Kinzler KW (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat Med* 2: 169-174.

Longo R, Sarmiento R, Fanelli M, Capaccetti B, Gattuso D & Gasparini G (2002) Anti-angiogenic therapy: rationale, challenges and clinical studies. *Angiogenesis* 5: 237-256.

Lothe RA, Peltomaki P, Meling GI, Aaltonen LA, Nystrom-Lahti M, Pylkkanen L, Heimdal K, Andersen TI, Moller P, Rognum TO, Fossa SD, Haldorsen T, Langmark F, Brogger A, de la Chapelle A & Borresen A-L (1993) Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. *Cancer Res* 53: 5849-5852.

Lymboussaki A, Partanen TA, Olofsson B, Thomas-Crusells J, Fletcher CD, de Waal RMW, Kaipainen A & Alitalo K (1998) Expression of the vascular endothelial growth factor C receptor VEGFR-3 in lymphatic endothelium of the skin and in vascular tumors. *Am J Pathol* 153: 395-403.

Lynch HT, Watson P, Kriegler M, Lynch JF, Lanspa SJ, Marcus J, Smyrk T, Fitzgibbons RJ, Jr. & Cristofaro G (1988) Differential diagnosis of hereditary nonpolyposis colorectal cancer (Lynch syndrome I and Lynch syndrome II). *Dis Colon Rectum* 31: 372-377.

MacFarlane JK, Ryall RDH & Heald RJ (1993) Mesorectal excision for rectal cancer. *Lancet* 341: 457-460.

Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, Kauppinen R, Jackson DG, Kubo H, Nishikawa S, Yla-Herttuala S & Alitalo K (2001a) Inhibition

of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* 7: 199-205.

Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG & Alitalo K (2001b) Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J* 20: 4762-4773.

Maloney JP, Silliman CC, Ambruso DR, Wang J, Tuder RM & Voelkel NF (1998) In vitro release of vascular endothelial growth factor during platelet aggregation. *Am J Physiol* 275: H1054-1061.

Mandel JS, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM & Ederer F (1993) Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N Engl J Med* 328: 1365-1371.

Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G & Pepper MS (2001) Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 20: 672-682.

Marconcini L, Marchio S, Morbidelli L, Cartocci E, Albini A, Ziche M, Bussolino F & Oliviero S (1999) c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. *Proc Natl Acad Sci USA* 96: 9671-9676.

Marks CG, Reid FDA, Lewis CE, Jackson PA & Cook MG (2000) What determines the outcome after total mesorectal excision for rectal carcinoma - 15 years experience of a specialist surgical unit. *Colorectal Dis* 2: 270-276.

Marsh PJ, James RD & Schofield PF (1994) Adjuvant preoperative radiotherapy for locally advanced rectal carcinoma. Results of a prospective, randomized trial. *Dis Colon Rectum* 37: 1205-1214.

Mashino K, Sadanaga N, Yamaguchi H, Tanaka F, Ohta M, Shibuta K, Inoue H & Mori M (2002) Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res* 62: 2937-2941.

Masood R, Cai J, Zheng T, Smith DL, Hinton DR & Gill PS (2001) Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. *Blood* 98: 1904-1913.

Mattila MM-T, Ruohola JK, Karpanen T, Jackson DG, Alitalo K & Harkonen PL (2002) VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. *Int J Cancer* 98: 946-951.

Maula S-M, Luukkaa M, Grenman R, Jackson D, Jalkanen S & Ristamaki R (2003) Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res* 63: 1920-1926.

McArdle CS, Hole D, Hansell D, Blumgart LH & Wood CB (1990) Prospective study of colorectal cancer in the west of Scotland: 10-year follow-up. *Br J Surg* 77: 280-282.

McColl BK, Baldwin MA, Roufail S, Freeman C, Moritz RL, Simpson RJ, Alitalo K, Stacker SA & Achen MG (2003) Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J Exp Med* 198: 863-868.

McLeod HL & Murray GI (1999) Tumour markers of prognosis in colorectal cancer. *Br J Cancer* 79: 191-203.

Miao H-Q, Lee P, Lin H, Soker S & Klagsbrun M (2000) Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. *FASEB J* 14: 2532-2539.

Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NPH, Risau W & Ullrich A (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72: 835-846.

Minagawa N, Nakayama Y, Hirata K, Onitsuka K, Inoue Y, Nagata N & Itoh H (2002) Correlation of plasma level and immunohistochemical expression of vascular endothelial growth factor in patients with advanced colorectal cancer. *Anticancer Res* 22: 2957-2964.

Moertel CG, Fleming TR, Macdonald JS, Haller DG, Laurie JA, Tangen CM, Ungerleider JS, Emerson WA, Tormey DC, Glick JH, Veeder MH & Mailliard JA (1995) Fluorouracil plus levamisole as effective adjuvant therapy after resection of stage III colon carcinoma: a final report. *Ann Intern Med* 122: 321-326.

Mohle R, Green D, Moore MAS, Nachman RL & Rafii S (1997) Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. *Proc Natl Acad Sci USA* 94: 663-668.

Moller B, Lindblom B & Olovsson M (2002) Expression of the vascular endothelial growth factors B and C and their receptors in human endometrium during the menstrual cycle. *Acta Obstet Gynecol Scand* 81: 817-824.

Mulcahy HE, Farthing MJ & O'Donoghue DP (1997) Screening for asymptomatic colorectal cancer. *BMJ* 314: 285-291.

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E & Zlotnik A (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410: 50-56.

Nakata S, Ito K-i, Fujimori M, Shingu K, Kajikawa S, Adachi W, Matsuyama I, Tsuchiya S-i, Kuwano M & Amano J (1998) Involvement of vascular endothelial growth factor and urokinase-type plasminogen activator receptor in microvessel invasion in human colorectal cancers. *Int J Cancer* 79: 179-186.

Nakayama Y, Sako T, Shibao K, Onitsuka K, Nagashima N, Hirata K, Nagata N & Itoh H (2000) Relationship between plasma levels of vascular endothelial growth factor and serum levels of interleukin-12 in patients with colorectal cancer. *Anticancer Res* 20: 4097-4102.

Nakayama Y, Sako T, Shibao K, Okazaki K, Rempo N, Onitsuka K, Minagawa N, Akahane K, Nagashima N, Nagata N & Itoh H (2002) Prognostic value of plasma vascular endothelial growth factor in patients with colorectal cancer. *Anticancer Res* 22: 2437-2442.

Nanashima A, Ito M, Sekine I, Naito S, Yamaguchi H, Nakagoe T & Ayabe H (1998) Significance of angiogenic factors in liver metastatic tumors originating from colorectal cancers. *Dig Dis Sci* 43: 2634-2640.

Nanni O, Volpi A, Frassineti GL, De Paola F, Granato AM, Dubini A, Zoli W, Scarpi E, Turci D, Oliverio G, Gambi A & Amadori D (2002) Role of biological markers in the clinical outcome of colon cancer. *Br J Cancer* 87: 868-875.

Nerlich AG & Schleicher E (1991) Identification of lymph and blood capillaries by immunohistochemical staining for various basement membrane components. *Histochemistry* 96: 449-453.

Neuchrist C, Erovic BM, Handisurya A, Steiner GE, Rockwell P, Gedlicka C & Burian M (2001) Vascular endothelial growth factor receptor 2 (VEGFR2) expression in squamous cell carcinomas of the head and neck. *Laryngoscope* 111: 1834-1841.

Neuchrist C, Erovic BM, Handisurya A, Fischer MB, Steiner GE, Hollemann D, Gedlicka C, Saaristo A & Burian M (2003) Vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 expression in squamous cell carcinomas of the head and neck. *Head and Neck* 25: 464-474.

Neufeld G, Cohen T, Gengrinovitch S & Poltorak Z (1999) Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 13: 9-22.

NICE. (2002). Guidance on the use of irinotecan, oxaliplatin and ralitrexed for the treatment of advanced colorectal cancer - Technology Appraisal Guidance No. 33. National Institute for Clincal Excellence: London.

Nielsen HJ, Werther K, Mynster T & Brunner N (1999) Soluble vascular endothelial growth factor in various blood transfusion components. *Transfusion* 39: 1078-1083.

Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y & Hirohashi S (2000) Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res* 6: 2431-2439.

Niki T, Iba S, Yamada T, Matsuno Y, Enholm B & Hirohashi S (2001) Expression of vascular endothelial growth factor receptor 3 in blood and lymphatic vessels of lung adenocarcinoma. *J Pathol* 193: 450-457.

Noguchi T, Takeno S, Shibata T, Uchida Y, Yokoyama S & Muller W (2002) VEGF-C expression correlates with histological differentiation and metastasis in squamous cell carcinoma of the esophagus. *Oncol Rep* 9: 995-999.

Northover JMA (1997) Staging and management of colorectal cancer. *World J Surg* 21: 672-677.

O'Byrne KJ, Dobbs N & Harris AL (2000) Serum vascular endothelial growth factor load and interleukin-6 in cancer patients. *Br J Cancer* 82: 1895-1896.

O'Dwyer ST, Haboubi NY, Johnson JS & Gardy R (2001) Detection of lymph node metastases in colorectal carcinoma. *Colorectal Dis* 3: 288-294.

Oh SJ, Jeltsch MM, Birkenhager R, McCarthy JE, Weich HA, Christ B, Alitalo K & Wilting J (1997) VEGF and VEGF-C: specific induction of angiogenesis and

lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev Biol* 188: 96-109.

Oh-e H, Tanaka S, Kitadai Y, Shimamoto F, Yoshihara M & Haruma K (2001) Angiogenesis at the site of deepest penetration predicts lymph node metastasis of submucosal colorectal cancer. *Dis Colon Rectum* 44: 1129-1136.

Ohta Y, Shridhar V, Bright RK, Kalemkerian GP, Du W, Carbone M, Watanabe Y & Pass HI (1999) VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. *Br J Cancer* 81: 54-61.

Ono T & Miki C (2000) Factors influencing tissue concentration of vascular endothelial growth factor in colorectal carcinoma. *Am J Gastroenterol* 95: 1062-1067.

Orlandini M, Marconcini L, Ferruzzi R & Oliviero S (1996) Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. *Proc Natl Acad Sci USA* 93: 11675-11680.

Orlandini M & Oliviero S (2001) In fibroblasts *Vegf-D* expression is induced by cell-cell contact mediated by cadherin-11. *J Biol Chem* 276: 6576-6581.

Paavonen K, Puolakkainen P, Jussila L, Jahkola T & Alitalo K (2000) Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol* 156: 1499-1504.

Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL & Jain RK (2002) Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 296: 1883-1886.

Pajusola K, Aprelikova O, Armstrong E, Morris S & Alitalo K (1993) Two human FLT4 receptor tyrosine kinase isoforms with distinct carboxy terminal tails are produced by alternative processing of primary transcripts. *Oncogene* 8: 2931-2937.

Pajusola K, Aprelikova O, Pelicci G, Weich H, Claesson-Welsh L & Alitalo K (1994) Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene* 9: 3545-3555.

Partanen TA, Alitalo K & Miettinen M (1999) Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumors. *Cancer* 86: 2406-2412.

Partanen TA, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker SA, Achen MG & Alitalo K (2000) VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J* 14: 2087-2096.

Partanen TA & Paavonen K (2001) Lymphatic versus blood vascular endothelial growth factors and receptors in humans. *Microsc Res Tech* 55: 108-121.

Parums DV, Cordell JL, Micklem K, Heryet AR, Gatter KC & Mason DY (1990) JC70: a new monoclonal antibody that detects vascular endothelium associated antigen on routinely processed tissue sections. *J Clin Pathol* 43: 752-757.

Paul P, Letteboer T, Gelbert L, Groden J, White R & Coppes MJ (1993) Identical APC exon 15 mutations result in a variable phenotype in familial adenomatous polyposis. *Hum Mol Genet* 2: 925-931.

Pepper MS, Mandriota SJ, Jeltsch M, Kumar V & Alitalo K (1998) Vascular endothelial growth factor (VEGF)-C synergizes with basic fibroblast growth factor and VEGF in the induction of angiogenesis in vitro and alters endothelial cell extracellular proteolytic activity. *J Cell Physiol* 177: 439-452.

Pepper MS (2001) Lymphangiogenesis and tumor metastasis: myth or reality? *Clin Cancer Res* 7: 462-468.

Pepper MS, Tille J-C, Nisato R & Skobe M (2003) Lymphangiogenesis and tumor metastasis. *Cell Tissue Res*. Eprint ahead of publication. DOI 10.1007

Peters U, Sinha R, Chatterjee N, Subar AF, Ziegler RG, Kulldorff M, Bresalier R, Weissfeld JL, Flood A, Schatzkin A & Hayes RB (2003) Dietary fibre and colorectal adenoma in a colorectal cancer early detection programme. *Lancet* 361: 1491-1495.

Pinedo HM, Verheul HMW, D'Amato RJ & Folkman J (1998) Involvement of platelets in tumor angiogenesis? *Lancet* 352: 1775-1777.

Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, Jackson DG & Skobe M (2002) Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci USA* 99: 16069-16074.

Poon RT-P, Fan S-T & Wong J (2001) Clinical implications of circulating angiogenic factors in cancer patients. *J Clin Oncol* 19: 1207-1225.

Poon RT-P, Lau CP-Y, Cheung S-T, Yu W-C & Fan S-T (2003) Quantitative correlation of serum levels and tumor expression of vascular endothelial growth factor in patients with hepatocellular carcinoma. *Cancer Res* 63: 3121-3126.

Potter JD (1999) Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 91: 916-932.

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B & Kinzler KW (1992) APC mutations occur early during colorectal tumorigenesis. *Nature* 359: 235-237.

Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M & Ferrara N (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 57: 4593-4599.

Pugh CW & Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 9: 677-684.

QUASAR Collaborative Group (2000) Comparison of fluorouracil with additional levamisole, higher-dose folinic acid, or both, as adjuvant chemotherapy for colorectal cancer: a randomised trial. *Lancet* 355: 1588-1596.

Quirke P, Durdey P, Dixon MF & Williams NS (1986) Local recurrence of rectal adenocarcinoma due to inadequate surgical resection. Histopathological study of lateral tumour spread and surgical excision. *Lancet* 2: 996-999.

Ristimaki A, Narko K, Enholm B, Joukov V & Alitalo K (1998) Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J Biol Chem* 273: 8413-8418.

Rocchigiani M, Lestingi M, Luddi A, Orlandini M, Franco B, Rossi E, Ballabio A, Zuffardi O & Oliviero S (1998) Human FIGF: cloning, gene structure, and mapping to chromosome Xp22.1 between the PIGA and the GRPR genes. *Genomics* 47: 207-216.

Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perucho M, Smyrk T, Sobin L & Srivastava S (1997) A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: meeting highlights and Bethesda guidelines. *J Natl Cancer Inst* 89: 1758-1762.

Ryan TJ (1989) Structure and function of lymphatics. *J Invest Dermatol* 93: 18S-24S.

Saclarides TJ, Speziale NJ, Drab E, Szeluga DJ & Rubin DB (1994) Tumor angiogenesis and rectal carcinoma. *Dis Colon Rectum* 37: 921-926.

Saeki H, Moore AM, Brown MJ & Hwang ST (1999) Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol* 162: 2472-2475.

Saito H, Tsujitani S, Ikeguchi M, Maeta M & Kaibara N (1998) Relationship between the expression of vascular endothelial growth factor and the density of dendritic cells in gastric adenocarcinoma tissue. *Br J Cancer* 78: 1573-1577.

Salgado R, Vermeulen PB, Benoy I, Weytjens R, Huget P, Van Marck E & Dirix LY (1999) Platelet number and interleukin-6 correlate with VEGF but not with bFGF serum levels of advanced cancer patients. *Br J Cancer* 80: 892-897.

Salgado R, Benoy I, Weytjens R, Vermeulen PB & Dirix LY (2000) Serum vascular endothelial growth factor load and interleukin-6 in cancer patients - reply. *Br J Cancer* 82: 1896.

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

Salgado R, Benoy I, Weytjens R, Van Bockstaele D, Van Marck E, Huget P, Hoylaerts M, Vermeulen P & Dirix LY (2002) Arterio-venous gradients of IL-6, plasma and serum VEGF and D-dimers in human cancer. *Br J Cancer* 87: 1437-1444.

Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N, Elfring GL & Miller LL (2000) Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med* 343: 905-914.

Salven P, Manpaa H, Orpana A, Alitalo K & Joensuu H (1997) Serum vascular endothelial growth factor is often elevated in disseminated cancer. *Clin Cancer Res* 3: 647-651.

Salven P, Lymboussaki A, Heikkila P, Jaaskela-Saari H, Enholm B, Aase K, von Euler G, Eriksson U, Alitalo K & Joensuu H (1998) Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *Am J Pathol* 153: 103-108.

Salven P, Orpana A & Joensuu H (1999) Leukocytes and platelets of patients with cancer contain high levels of vascular endothelial growth factor. *Clin Cancer Res* 5: 487-491.

Saunders MP & Valle JW (2002) Why hasn't the National Institute been 'NICE' to patients with colorectal cancer? *Br J Cancer* 86: 1667-1669.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS & Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219: 983-985.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu X-F, Breitman ML & Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62-66.

Shen B-Q, Lee DY, Gerber H-P, Keyt BA, Ferrara N & Zioncheck TF (1998) Homologous up-regulation of KDR/Flk-1 receptor expression by vascular endothelial growth factor in vitro. *J Biol Chem* 273: 29979-29985.

Shi S-R, Key ME & Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 39: 741-748.

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R & Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci USA* 96: 5522-5527.

Siegfried G, Basak A, Cromlish JA, Benjannet S, Marcinkiewicz J, Chretien M, Seidah NG & Khatib A-M (2003) The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J Clin Invest* 111: 1723-1732.

Skobe M, Hamberg LM, Hawighorst T, Schimer M, Wolf GL, Alitalo K & Detmar M (2001a) Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 159: 893-903.

Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K & Detmar M (2001b) Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 7: 192-198.

Slack JK, Adams RB, Rovin JD, Bissonette EA, Stoker CE & Parsons JT (2001) Alterations in the focal adhesion kinase/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells. *Oncogene* 20: 1152-1163.

Sleeman JP (2000) The lymph node as a bridgehead in the metastatic dissemination of tumors. *Recent Results Cancer Res* 157: 55-81.

Sleeman JP, Krishnan J, Kirkin V & Baumann P (2001) Markers for the lymphatic endothelium: in search of the holy grail? *Microsc Res Tech* 55: 61-69.

Smith AJ, Stern HS, Penner M, Hay K, Mitri A, Bapat BV & Gallinger S (1994) Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. *Cancer Res* 54: 5527-5530.

Soker S, Fidder H, Neufeld G & Klagsbrun M (1996) Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. *J Biol Chem* 271: 5761-5767.

Soker S, Takashima S, Miao HQ, Neufeld G & Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 92: 735-745.

Soker S, Kaefer M, Johnson M, Klagsbrun M, Atala A & Freeman MR (2001) Vascular endothelial growth factor-mediated autocrine stimulation of prostate tumor cells coincides with progression to a malignant phenotype. *Am J Pathol* 159: 651-659.

Srivastava A, Laidler P, Davies RP, Horgan K & Hughes LE (1988) The prognostic significance of tumor vascularity in intermediate thickness (0.76-4.0mm thick) skin melanoma. A quantitative histologic study. *Am J Pathol* 133: 419-423.

Stacker SA, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, Roufail S, Simpson RJ, Moritz R, Karpanen T, Alitalo K & Achen MG (1999) Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J Biol Chem* 274: 32127-32136.

Stacker SA, Caesar C, Baldwin MA, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S-i, Kubo H & Achen MG (2001) VEGF-D promotes the metastatic spread of tumor cells *via* the lymphatics. *Nat Med* 7: 186-191.

Straume O, Jackson DG & Akslen LA (2003) Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. *Clin Cancer Res* 9: 250-256.

Stubbs RS (1983) The aetiology of colorectal cancer. *Br J Surg* 70: 313-316.

Szuba A, Skobe M, Karkkainen MJ, Shin WS, Beynet DP, Rockson NB, Dakhil N, Spilman S, Goris ML, Strauss HW, Quertermous T, Alitalo K & Rockson SG (2002) Therapeutic lymphangiogenesis with human recombinant VEGF-C. *FASEB J* 16: 1985-1987.

Takahashi A, Kono K, Itakura J, Amemiya H, Tang RF, Iizuka H, Fujii H & Matsumoto Y (2002) Correlation of vascular endothelial growth factor C expression with tumor-infiltrating dendritic cells in gastric cancer. *Oncology* 62: 121-127.

Takahashi Y, Kitadai Y, Bucana CD, Cleary KR & Ellis LM (1995) Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 55: 3964-3968.

Takahashi Y, Tucker SL, Kitadai Y, Koura AN, Bucana CD, Cleary KR & Ellis LM (1997) Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. *Arch Surg* 132: 541-546.

Takahashi Y, Bucana CD, Cleary KR & Ellis LM (1998) p53, vessel count, and vascular endothelial growth factor expression in human colon cancer. *Int J Cancer* 79: 34-38.

Takebayashi Y, Aklyama S-i, Yamada K, Akiba S & Aikou T (1996) Angiogenesis as an unfavorable prognostic factor in human colorectal carcinoma. *Cancer* 78: 226-231.

Takeda A, Shimada H, Imaseki H, Okazumi S, Natsume T, Suzuki T & Ochiai T (2000) Clinical significance of serum vascular endothelial growth factor in colorectal cancer patients: correlation with clinicopathological factors and tumor markers. *Oncol Rep* 7: 333-338.

Tamura M, Ohta Y, Kajita T, Kimura K, Go T, Oda M, Nakamura H & Watanabe G (2001) Plasma VEGF concentration can predict the tumor angiogenic capacity in non-small cell lung cancer. *Oncol Rep* 8: 1097-1102.

Tamura M & Ohta Y (2003) Serum vascular endothelial growth factor-C level in patients with primary nonsmall cell lung carcinoma: a possible diagnostic tool for lymph node metastasis. *Cancer* 98: 1217-1222.

Tanaka F, Otake Y, Yanagihara K, Kawano Y, Miyahara R, Li M, Yamada T, Hanaoka N, Inui K & Wada H (2001) Evaluation of angiogenesis in non-small cell lung cancer: comparison between anti-CD34 antibody and anti-CD105 antibody. *Clin Cancer Res* 7: 3410-3415.

Tang Y, Zhang D, Fallavollita L & Brodt P (2003) Vascular endothelial growth factor C expression and lymph node metastasis are regulated by the type I insulin-like growth factor receptor. *Cancer Res* 63: 1166-1171.

Tanigawa N, Amaya H, Matsumura M, Lu C, Kitaoka A, Matsuyama K & Muraoka R (1997) Tumor angiogenesis and mode of metastasis in patients with colorectal cancer. *Cancer Res* 57: 1043-1046.

Teng X, Li D & Johns RA (2002) Hypoxia up-regulates mouse vascular endothelial growth factor D promoter activity in rat pulmonary microvascular smooth-muscle cells. *Chest* 121: 82S-83S.

Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D & Bohlen P (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun* 187: 1579-1586.

Terry P, Giovannucci E, Michels KB, Bergkvist L, Hansen H, Holmberg L & Wolk A (2001) Fruit, vegetables, dietary fiber, and risk of colorectal cancer. *J Natl Cancer Inst* 93: 525-533.

The Association of Coloproctology of Great Britain and Ireland. (2001). Guidelines for the management of colorectal cancer. The Association of Coloproctology of Great Britain and Ireland at The Royal College of Surgeons of England: London.

Tille J-C, Wang X, Lipson KE, McMahon G, Ferrara N, Zhu Z, Hicklin DJ, Sleeman JP, Eriksson U, Alitalo K & Pepper MS (2003) Vascular endothelial growth factor (VEGF) receptor-2 signaling mediates VEGF-$C_{\Delta N \Delta C}$- and VEGF-A-induced angiogenesis in vitro. *Exp Cell Res* 285: 286-298.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC & Abraham JA (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 266: 11947-11954.

Tokunaga T, Oshika Y, Abe Y, Ozeki Y, Sadahiro S, Kijima H, Tsuchida T, Yamazaki H, Ueyama Y, Tamaoki N & Nakamura M (1998) Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. *Br J Cancer* 77: 998-1002.

Tomisaki S-i, Ohno S, Ichiyoshi Y, Kuwano H, Maehara Y & Sugimachi K (1996) Microvessel quantification and its possible relation with liver metastasis in colorectal cancer. *Cancer* 77: 1722-1728.

Tsuchiya N, Sato K, Akao T, Kakinuma H, Sasaki R, Shimoda N, Satoh S, Habuchi T, Ogawa O & Kato T (2001) Quantitative analysis of gene expressions of vascular endothelial growth factor-related factors and their receptors in renal cell carcinoma. *Tokohu J Exp Med* 195: 101-113.

Turnbull RB, Jr., Kyle K, Watson FR & Spratt J (1967) Cancer of the colon: the influence of the no-touch isolation technic on survival rates. *Ann Surg* 166: 420-427.

UICC. (2002). TNM classification of malignant tumours. Wiley-Liss: New York.

UK Flexible Sigmoidoscopy Screening Trial Investigators (2002) Single flexible sigmoidoscopy screening to prevent colorectal cancer: baseline findings of a UK multicentre randomised trial. *Lancet* 359: 1291-1300.

Valtola R, Salven P, Heikkila P, Taipale J, Joensuu H, Rehn M, Pihlajaniemi T, Weich H, deWaal R & Alitalo K (1999) VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 154: 1381-1390.

Van Trappen PO & Pepper MS (2002) Lymphatic dissemination of tumour cells and the formation of micrometastases. *Lancet Oncol* 3: 44-52.

Vasen HF, Mecklin JP, Khan PM & Lynch HT (1991) The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). *Dis Colon Rectum* 34: 424-425.

Vasen HFA, Watson P, Mecklin J-P & Lynch HT (1999) New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 116: 1453-1456.

Veikkola T & Alitalo K (1999) VEGFs, receptors and angiogenesis. *Semin Cancer Biol* 9: 211-220.

Veikkola T, Karkkainen M, Claesson-Welsh L & Alitalo K (2000) Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res* 60: 203-212.

Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA & Alitalo K (2001) Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 20: 1223-1231.

Verheul HMW, Hoekman K, Luykx-de Bakker S, Eekman CA, Folman CC, Broxterman HJ & Pinedo HM (1997) Platelet: transporter of vascular endothelial growth factor. *Clin Cancer Res* 3: 2187-2190.

Verheul HMW & Pinedo HM (2003) The importance of platelet counts and their contents in cancer. *Clin Cancer Res* 9: 3219-3221.

Vermeulen PB, Gasparini G, Fox SB, Toi M, Martin L, McCulloch P, Pezzella F, Viale G, Weidner N, Harris AL & Dirix LY (1996) Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. *Eur J Cancer* 32A: 2474-2484.

Vermeulen PB, Salven P, Benoy I, Gasparini G & Dirix LY (1999a) Blood platelets and serum VEGF in cancer patients. *Br J Cancer* 79: 370-373.

Vermeulen PB, Van den Eynden GG, Huget P, Goovaerts G, Weyler J, Lardon F, Van Marck E, Hubens G & Dirix LY (1999b) Prospective study of intratumoral microvessel density, p53 expression and survival in colorectal cancer. *Br J Cancer* 79: 316-322.

Vincenti V, Cassano C, Rocchi M & Persico G (1996) Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. *Circulation* 93: 1493-1495.

von Marschall Z, Cramer T, Hocker M, Burde R, Plath T, Schirner M, Heidenreich R, Breier G, Riecken E-O, Wiedenmann B & Rosewicz S (2000) De novo expression of vascular endothelial growth factor in human pancreatic cancer: evidence for an autocrine mitogenic loop. *Gastroenterology* 119: 1358-1372.

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M & Heldin C-H (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269: 26988-26995.

Wang JM, Kumar S, Pye D, Haboubi N & Al-Nakib L (1994a) Breast carcinoma: comparative study of tumor vasculature using two endothelial cell markers. *Journal of the National Cancer Institute* 86: 386-388.

Wang JM, Wilson PB, Kumar S, Pye D & Hunter RD (1994b) Quantitation of endothelial cell specific protein E-9 employing a single monoclonal antibody in an indirect sandwich ELISA. *J Immunol Meth* 171: 55-64.

Warren RS, Yuan H, Matli MR, Gillett N & Ferrara N (1995) Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 95: 1789-1797.

Wartiovaara U, Salven P, Mikkola H, Lassila R, Kaukonen J, Joukov V, Orpana A, Ristimaki A, Heikinheimo M, Joensuu H, Alitalo K & Palotie A (1998) Peripheral blood platelets express VEGF-C and VEGF which are released during platelet activation. *Thromb Haemost* 80: 171-175.

Webb NJA, Bottomley MJ, Watson CJ & Brenchley PE (1998) Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease. *Clin Sci* 94: 395-404.

Wei MH, Popescu NC, Lerman MI, Merrill MJ & Zimonjic DB (1996) Localization of the human vascular endothelial growth factor gene, VEGF, at chromosome 6p12. *Hum Genet* 97: 794-797.

Weidner N, Semple JP, Welch WR & Folkman J (1991) Tumor angiogenesis and metastasis - correlation in invasive breast carcinoma. *N Engl J Med* 324: 1-8.

Werther K, Christensen IJ, Brunner N & Nielsen HJ (2000) Soluble vascular endothelial growth factor levels in patients with primary colorectal carcinoma. The Danish RANX05 Colorectal Cancer Study Group. *Eur J Surg Oncol* 26: 657-662.

Werther K, Christensen IJ & Nielsen HJ (2001) The association between preoperative concentration of soluble vascular endothelial growth factor, perioperative blood transfusion, and survival in patients with primary colorectal cancer. *Eur J Surg* 167: 287-292.

Werther K, Christensen IJ & Nielsen HJ (2002) Prognostic impact of matched preoperative plasma and serum VEGF in patients with primary colorectal carcinoma. *Br J Cancer* 86: 417-423.

White JD, Hewett PW, Kosuge D, McCulloch T, Enholm BC, Carmichael J & Murray JC (2002) Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. *Cancer Res* 62: 1669-1675.

Wigle JT & Oliver G (1999) Prox1 function is required for the development of the murine lymphatic system. *Cell* 98: 769-778.

Wiley HE, Gonzalez EB, Maki W, Wu M-T & Hwang ST (2001) Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. *J Natl Cancer Inst* 93: 1638-1643.

Williams CS, Leek RD, Robson AM, Banerji S, Prevo R, Harris AL & Jackson DG (2003) Absence of lymphangiogenesis and intratumoural lymph vessels in human metastatic breast cancer. *J Pathol* 200: 195-206.

Wilting J, Papoutsi M, Christ B, Nicolaides KH, von Kaisenberg CS, Borges J, Stark GB, Alitalo K, Tomarev SI, Niemeyer C & Rossler J (2002) The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased human tissues. *FASEB J* 16: 1271-1273.

Witmer AN, van Blijswijk BC, Dai J, Hofman P, Partanen TA, Vrensen GF & Schlingemann RO (2001) VEGFR-3 in adult angiogenesis. *J Pathol* 195: 490-497.

Witte D, Thomas A, Ali N, Carlson N & Younes M (2002) Expression of the vascular endothelial growth factor receptor-3 (VEGFR-3) and its ligand VEGF-C in human colorectal adenocarcinoma. *Anticancer Res* 22: 1463-1466.

Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu J-S & Isner JM (1998) Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol* 153: 381-394.

Wong MP, Cheung N, Yuen ST, Leung SY & Chung LP (1999) Vascular endothelial growth factor is up-regulated in the early pre-malignant stage of colorectal tumour progression. *Int J Cancer* 81: 845-850.

Wu W, Shu X, Hovsepyan H, Mosteller RD & Broek D (2003) VEGF receptor expression and signaling in human bladder tumors. *Oncogene* 22: 3361-3370.

Wynter CVA, Simms LA, Buttenshaw RL, Biden KG, Young J, Leggett BA, Conrad RJ, Schoch EM, Jass JR & Praga Pillay S (1999) Angiogenic factor VEGF is decreased in human colorectal neoplasms showing DNA microsatellite instability. *J Pathol* 189: 319-325.

Yamada Y, Nezu J-i, Shimane M & Hirata Y (1997) Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics* 42: 483-488.

Yamamoto Y, Toi M, Kondo S, Matsumoto T, Suzuki H, Kitamura M, Tsuruta K, Taniguchi T, Okamoto A, Mori T, Yoshida M, Ikeda T & Tominaga T (1996) Concentrations of vascular endothelial growth factor in the sera of normal controls and cancer patients. *Clin Cancer Res* 2: 821-826.

Yanai Y, Furuhata T, Kimura Y, Yamaguchi K, Yasoshima T, Mitaka T, Mochizuki Y & Hirata K (2001) Vascular endothelial growth factor C promotes human gastric carcinoma lymph node metastasis in mice. *J Exp Clin Cancer Res* 20: 419-428.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ & Holash J (2000) Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242-248.

Yokoyama Y, Charnock-Jones DS, Licence D, Yanaihara A, Hastings JM, Holland CM, Emoto M, Sakamoto A, Sakamoto T, Maruyama H, Sato S, Mizunuma H & Smith SK (2003) Expression of vascular endothelial growth factor (VEGF)-D and its receptor, VEGF receptor 3, as a prognostic factor in endometrial carcinoma. *Clin Cancer Res* 9: 1361-1369.

Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K & Sasaki T (1999) Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 5: 1823-1829.

Yonemura Y, Fushida S, Bando E, Kinoshita K, Miwa K, Endo Y, Sugiyama K, Partanen T, Yamamoto H & Sasaki T (2001) Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)-3 in gastric cancer. *Eur J Cancer* 37: 918-923.

Yoon Y-s, Murayama T, Gravereaux E, Tkebuchava T, Silver M, Curry C, Wecker A, Kirchmair R, Hu CS, Kearney M, Ashare A, Jackson DG, Kubo H, Isner JM & Losordo DW (2003) VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. *J Clin Invest* 111: 717-725.

Ziche M, Morbidelli L, Choudhuri R, Zhang H-T, Donnini S, Granger HJ & Bicknell R (1997) Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 99: 2625-2634.

APPENDICES

APPENDIX I. British Journal of Cancer 2003;89:426-430

British Journal of Cancer (2003) 89, 426–430

www.bjcancer.com

Minireview

Vascular endothelial growth factors C and D and lymphangiogenesis in gastrointestinal tract malignancy

SE Duff[*,1,2], C Li[2], M Jeziorska[2], S Kumar[2], MP Saunders[3], D Sherlock[4], ST O'Dwyer[1] and GC Jayson[5]

[1]*Department of Surgery, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK;* [2]*Laboratory Medicine Academic Group, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK;* [3]*Department of Clinical Oncology, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK;* [4]*Department of Surgery, North Manchester General Hospital, Delauneys Road, Manchester M8 5RB, UK;* [5]*Department of Medical Oncology, Christie Hospital NHS Trust, Manchester M13 9PT, UK*

Vascular endothelial growth factor-C (VEGF-C) and VEGF-D are members of the VEGF family of cytokines and have angiogenic and lymphangiogenic actions. In gastric adenocarcinoma, VEGF-C mRNA and tissue protein expression correlate with lymphatic invasion, lymph node metastasis and in some reports, venous invasion and reduced 5-year survival. Patients with gastric adenocarcinomas containing high levels of VEGF-C expression have significantly reduced 5-year survival rates, and VEGF-C expression is an independent prognostic risk factor for death. The role of VEGF-C in oesophageal squamous and colorectal cancer and VEGF-D in colorectal cancer is not clear, with conflicting reports in the published literature. In order to exploit potential therapeutic applications, further research is necessary to define the precise roles of these cytokines in health and disease.
British Journal of Cancer (2003) **89**, 426–430. doi:10.1038/sj.bjc.6601145 www.bjcancer.com

Keywords: gastrointestinal neoplasms; lymphangiogenesis; lymphatic system; endothelial growth factors

Lymphangiogenesis, the development of new lymph vessels, is a relatively new area of clinical investigation. Increased interest in this field has been heightened by the discovery of new vascular endothelial growth factor (VEGF) family members, which possess lymphangiogenic roles.

Vascular endothelial growth factor-C (VEGF-C) and VEGF-D are secreted glycoproteins that are structurally similar, sharing areas of homology with one another and with the angiogenic growth factor VEGF-A (Joukov *et al*, 1996; Achen *et al*, 1998). They are specific ligands for the tyrosine kinase receptor, vascular endothelial growth factor receptor (VEGFR)-3 (flt-4) (Joukov *et al*, 1996; Achen *et al*, 1998). Both cytokines are subject to proteolytic processing, which also enables them to act as ligands for VEGFR2 (KDR/flk-1) (Joukov *et al*, 1997; Stacker *et al*, 1999). Vascular endothelial growth factor receptor 2 is expressed on vascular endothelial cells and is essential for the embryonic differentiation of endothelial and haematopoietic cells and formation of blood vessels (reviewed in Veikkola *et al*, 2000). Vascular endothelial growth factor receptor 3 is expressed on vascular endothelium early in development and on angiogenic endothelium, but is mainly restricted to the lymphatic endothelium in the adult (Kaipainen *et al*, 1995). Consequently, VEGF-C and D are implicated through their receptor affinities in angiogenic and lymphangiogenic pathways in health and disease (Stacker *et al*, 2002).

*Correspondence: Dr SE Duff; E-mail: sarah.duff@christie-tr.nwest.nhs.uk
Received 16 January 2003; revised 13 May 2003; accepted 21 May 2003

ROLES OF VEGF-C AND VEGF-D

Study of the lymphatic system and lymphatic endothelial cells has been limited by a lack of specific lymphatic vessel markers, lack of lymphatic endothelial cells for culture and limited animal models. These problems are currently being overcome with a variety of methods. The recent discovery of specific lymphatic vessel markers, such as the hyaluronan receptor LYVE-1, podoplanin and Prox-1, new antibodies to these markers and antibody combinations has aided the identification of lymphatic vessels in histological specimens (Stacker *et al*, 2002) (Table 1). The exploitation of the differential expression of these new specific cell surface markers by lymphatic and blood vascular endothelial cells has allowed the separation of stable lymphatic cell populations for study (Podgrabinska *et al*, 2002). Animal models have been adapted from angiogenesis research and specific tumour, transgenic and knock-out models developed.

Our current understanding of the roles of VEGF-C and VEGF-D is derived mainly from *in vitro* and *in vivo* studies. *In vitro* studies have shown that VEGF-C and VEGF-D exhibit mitogenic effects for vascular and lymphatic endothelial cells and survival-promoting abilities for lymphatic endothelial cells through VEGFR3 (Joukov *et al*, 1997; Achen *et al*, 1998; Marconcini *et al*, 1999; Veikkola *et al*, 2001). Both growth factors promote angiogenesis in *in vitro* assays (Joukov *et al*, 1996; Joukov *et al*, 1997; Marconcini *et al*, 1999). Vascular endothelial growth factor-C promotes the formation of capillary-tube structures by lymphatic endothelial cells, but not blood vascular endothelial cells, in a collagen sandwich assay (Podgrabinska *et al*, 2002).

In vivo studies, using models adapted from angiogenesis research, have confirmed the angiogenic abilities of VEGF-C and VEGF-D and the lymphangiogenic effect of VEGF-C (Oh *et al*,

Table 1 Immunohistochemical staining methods for the detection of lymphatic endothelial cells

	Sites of antigen expression	Advantages	Disadvantages
Single staining methods			
VEGFR3 antibody	LEC, tumour neovasculature	LEC specific in normal tissue	Lack of specificity in tumours, VEGFR3 upregulated in angiogenic endothelium
Podoplanin antibody	LEC, glomerular podocytes	LEC specific	Lack of commercial antibody availability
Prox-1 antibody	LEC	LEC specific	Lack of commercial antibody availability
LYVE-1 antibody	LEC, hepatic sinusoidal cells	LEC specific	Lack of commercial antibody availability
Enzyme histochemistry (5'NA activity)	All endothelial cells at different levels		Findings difficult to interpret Hampered by nonspecific staining
Double staining methods			
CD31/PAL-E antibodies	Pan-EC marker/BEC	Enhanced discrimination accuracy	Frozen tissue required
VEGFR3/PAL-E antibodies	LEC, angiogenic BEC/BEC	Enhanced discrimination accuracy	Frozen tissue required
LYVE-1/CD34 antibodies	LEC/Pan-EC marker	Enhanced discrimination accuracy	Lack of commercial antibody availability
Prox-1/CD31 antibodies	LEC/Pan-EC marker	Enhanced discrimination accuracy	Lack of commercial antibody availability

LEC = lymphatic endothelial cells; 5'NA = 5'nucleotidase; LYVE-1 = lymphatic vessel endothelial hyaluronan receptor-1; BEC = blood vascular endothelial marker; Pan-EC marker = pan-endothelial cell marker.

1997). Transgenic mouse models, which overexpress VEGF-C or VEGF-D in the epidermis, have shown cytokine-dependent, VEGFR3-mediated dermal lymphatic vessel enlargement and lymphatic endothelial cell proliferation without alteration in blood vasculature (Jeltsch *et al*, 1997; Makinen *et al*, 2001; Veikkola *et al*, 2001). Various tumour models have been constructed in which overexpression of VEGF-C or VEGF-D is demonstrated. These studies consistently show increased aggressiveness of the transfected cancer cell lines, intratumoural lymphangiogenesis, dilated and increased numbers of peritumoural lymphatics, enhanced rates of lymph node metastasis and increased tumour angiogenesis (Karpanen *et al*, 2001; Mandriota *et al*, 2001; Skobe *et al*, 2001).

Despite the implication of VEGF-C and VEGF-D in lymphangiogenic and angiogenic pathways in these studies, the role of the growth factors in the progression of human malignancy is unclear and the existence of functional lymphatics and lymphangiogenesis in human malignancy has been debated (Leu *et al*, 2000; Clarijs *et al*, 2001; Padera *et al*, 2002). Recent studies in head and neck cancer (Beasley *et al*, 2002; Maula *et al*, 2003) and melanoma (Straume *et al*, 2003) have demonstrated the existence of proliferating intratumoral lymphatic vessels. Further research is required to determine whether this is the case for all the different human malignancies that spread predominantly by the lymphatic route. The situation is likely to be clarified further by the use of antibodies and antibody combinations for the more specific lymphatic markers in conjunction with functional assays.

VASCULAR ENDOTHELIAL GROWTH FACTOR-C AND VEGF-D IN HUMAN MALIGNANCIES

The dissemination of malignant cells to the regional lymph nodes is an early step in the progression of many common solid tumours and is an important determinant of prognosis. Positive associations have been found between the expression of VEGF-C in human malignant tissue with adverse clinicopathological features including lymphatic invasion and lymph node metastasis. Expression of VEGF-C mRNA is increased in a variety of human malignancies (Salven *et al*, 1998). Tumour types investigated include breast, gastric, colorectal, oesophageal, prostate, pancreas, cervical, thyroid, non-small-cell lung cancers, lung adenocarcinoma and laryngeal cancers. Clinically important areas of interest are the association between VEGF-C and -D expression, intra- and peritumoral lymphatic density, lymphatic and venous invasion, lymph node metastasis and survival.

Methodological considerations

Many published reports conflict in their outcomes and conclusions. This may be partly explained by the use of different methodological tools and assumptions by their authors.

Immunohistochemical techniques and microvessel counting examine the tissue as near its condition *in vivo* as possible. Even so, results obtained examining malignant tissue at the invasive edge of tumours may not concur with results from central and superficial parts of the tumour (Furodoi *et al*, 2002). Scoring methods for both immunohistochemical staining and vessel counting vary between studies, with consequent difficulties in the extrapolation of results. Furthermore, the subjective nature of assessment of staining intensity and the frequent lack of positive or negative tissue controls in immunohistochemical analyses can confound analysis.

Studies examining mRNA levels provide an estimate of overall expression in the tissue fragment analysed, including tumour cells, stroma and normal mucosa, as RNA extraction necessarily entails tissue disruption. The nature of the interaction between expressed cytokines and the tumour microenvironment is at the cellular and paracrine level (Furodoi *et al*, 2002). Consequently, analysis of global tumour mRNA levels may miss subtleties of tissue expression that are crucial for tumour behaviour. The expression of mRNA in a tissue fragment may not necessarily equate with the expression of protein by the tumour.

Evidence for tumour-related lymphangiogenesis is derived from the presence of intratumoral lymphatics in xenograft studies. However, these vessels may be trapped in the tumour mass as a consequence of the methodology of model construction. Consequently, studies involving transgenic animals overexpressing VEGF-C, in which dilation of peritumoral lymphatics are seen (Mandriota *et al*, 2001) may reflect the situation in spontaneously arising human tumours more accurately (Karpanen and Alitalo, 2001).

Further discussion will focus on the current evidence for the role of VEGF-C and VEGF-D and their signalling receptors for the common sites of malignancy of the gastrointestinal tract (Table 2).

Gastric cancer

Gastric cancer is a leading cause of cancer death worldwide. Lymph node status is important in the prediction of prognosis. Potential molecular markers that predict lymphatic involvement would improve the clinical management of this disease. The role of VEGF-C in predicting lymphatic invasion and lymph node metastasis in gastric cancer has been investigated in several

Table 2 Immunohistochemical examination of VEGF-C expression in gastrointestinal malignancy

Tumour type	Number of cases	VEGF-C IHC positive (%)	Clinicopathological associations of increased VEGF-C expression with regard to: Lymphatic invasion	Venous invasion	Lymph node metastasis	Prognosis	Reference
Oesophageal SCC	48	40	**$P<0.01$**	**$P<0.01$**	**$P<0.01$**	NA	Kitadai *et al* (2001)
Oesophageal SCC	71	54	$P=0.51$	$P=0.092$	$P=0.085$	$P=0.80$	Noguchi *et al* (2002)
Gastric adenocarcinoma	117	26	**$P<0.05$**	**$P<0.01$**	**$P<0.05$**	**$P<0.001$**	Yonemura *et al* (1999)
Gastric adenocarcinoma	76	45	**$P=0.04$**	$P=0.07$	NS	NS trend	Ichikura *et al* (2001)
Early gastric adenocarcinoma	105	29	**$P=0.02$**	NS increase	NS increase	NA	Kabashima *et al* (2001)
Gastric adenocarcinoma	65	51	**$P<0.05$**	NS	**$P<0.05$**	**$P<0.01$**	Takahashi *et al* (2002)
Gastric adenocarcinoma	139	32	**$P<0.05$**	NS	**$P<0.05$**	NA	Amioka *et al* (2002)
Advanced colorectal adenocarcinoma	152	47	**$P<0.01$**	**$P<0.01$**	**$P<0.01$**	**$P<0.05$**	Furodoi *et al* (2002)
Colorectal adenocarcinoma	99	56	**$P<0.01$**	NS	**$P<0.01$**	NS trend	Akagi *et al* (2000)
Colorectal adenocarcinoma	59	35	NS	NA	NS	NA	George *et al* (2001)

SCC = squamous cell carcinoma; IHC = immunohistochemistry; NS = nonsignificant; NA = not assessed. Values in bold type indicate statistically significant results.

studies (Table 2). There are no studies that have examined the role of VEGF-D in gastric cancer.

Immunohistochemical analysis of tumour tissue has demonstrated that VEGF-C immunoreactivity is restricted to gastric cancer cells and is observed diffusely throughout the cytoplasm (Yonemura *et al*, 1999, 2001; Ichikura *et al*, 2001). The percentage of gastric tumours that are positive for VEGF-C protein expression varies from 26 to 51% (Table 2) (Yonemura *et al*, 1999; Ichikura *et al*, 2001; Kabashima *et al*, 2001; Takahashi *et al*, 2002), although this may be accounted for in part by the use of varying methodology as discussed.

Lymphatic invasion and lymph node status correlate positively with tissue expression of VEGF-C in gastric cancer (Yonemura *et al*, 1999; Ichikura *et al*, 2001; Kabashima *et al*, 2001; Amioka *et al*, 2002; Takahashi *et al*, 2002) (Table 2). In addition, positive VEGF-C tissue expression in early gastric cancer (confined to the mucosa or submucosa) was significantly associated with lymphatic invasion, potentially helping to predict those individuals who would benefit from more or less extensive surgical resections (Kabashima *et al*, 2001). Similar associations have been demonstrated concerning the expression of VEGF-C mRNA expression in gastric cancer tissue. Malignant tissue expressed increased VEGF-C mRNA compared with adjacent normal mucosa (47 *vs* 13% (Yonemura *et al*, 1999); 55 *vs* 13% (Yonemura *et al*, 2001)). Furthermore, positive lymph node status, lymphatic and venous invasion were also associated with expression of VEGF-C mRNA (Yonemura *et al*, 1999).

The clinical impact of the association between VEGF-C expression and prognosis is not fully understood (Table 2). Nonsignificant trends towards reduced survival in VEGF-C expressing gastric cancers have been found (Ichikura *et al*, 2001). However, in 117 patients with gastric cancer, Yonemura *et al* (1999) demonstrated that high levels of VEGF-C expression were associated with poorer prognosis and decreased survival. Further significant differences in survival associated with VEGF-C status have been reported by Takahashi *et al* (2002) in a group of 65 cancer patients. A potentially important clinical finding of this study was the negative correlation of dendritic cell density with VEGF-C expression in the tumour. The effect of VEGF-C on survival may be due, in part, to its regulatory function on dendritic cells with potential reduced immunosurveillance of the tumour (Kabashima *et al*, 2001).

In contrast to VEGF-C, VEGFR3 immunoreactivity in gastric tumours is restricted to endothelial cells of mucosal and submucosal vessels that are regarded primarily as lymphatic vessels but also to a very few small blood vessels. Consequently, the majority of VEGFR3-positive vessels in gastric cancer are considered as lymphatics (Yonemura *et al*, 1999, 2001). A positive correlation between VEGFR3 and VEGF-C mRNA expression was seen in gastric cancer tissue specimens (Yonemura *et al*, 1999, 2001). Microvessel counts for VEGFR3 positive vessels showed a significant increase in VEGF-C mRNA positive tumours compared to VEGF-C mRNA negative tumours (6.96 ± 6.05 *vs* 2.16 ± 2.00, $P<0.001$). However, there was no overall increase in the VEGFR3 positive vessel count in tumour stroma compared with normal gastric mucosa when both VEGF-C mRNA positive and negative tumours were considered together (4.62 ± 5.85 *vs* 2.48 ± 1.64, $P=0.067$) (Yonemura *et al*, 2001). Similar increases in VEGFR3 positive vessel counts are seen in gastric cancers that are lymph node positive, show lymphatic invasion or are poorly differentiated (Yonemura *et al*, 2001).

In summary, in gastric cancer, expression of VEGF-C mRNA is higher in tumour than in normal mucosa. Vascular endothelial growth factor-C mRNA and immunohistochemically detected tissue expression of the protein in gastric cancer correlate with lymphatic invasion and lymph node metastasis and in some studies, venous invasion with reduced survival (Table 2). Vascular endothelial growth factor receptor 3 expression is mainly found on lymphatic vessels in gastric tumours and VEGFR3 mRNA levels and tissue expression parallel that of VEGF-C. These results suggest that VEGF-C and VEGFR3 act together in a paracrine fashion in the microenvironment of the gastric tumour.

Oesophageal cancer

Oesophageal cancer has a poor prognosis, which is dependent on the presence of lymph node metastases. Limited and conflicting evidence exists for the role of VEGF-C in oesophageal cancer and no research is available concerning VEGF-D. Kitadai *et al* (2001) analysed the relationship between the expression of VEGF-C and clinicopathological characteristics in oesophageal squamous cell carcinoma. *In vitro* analysis demonstrated that four of the five oesophageal carcinoma cell lines studied expressed VEGF-C mRNA. *Ex vivo* analysis confirmed VEGF-C mRNA to be present in eight of the 12 oesophageal squamous carcinomas. In a further 48 archival specimens, 39.6% showed positive immunohistochemical staining for VEGF-C, which correlated with stage of disease, lymphatic invasion, venous invasion and lymph node metastasis ($P<0.01$) and depth of tumour invasion (Tumour *in situ* (Tis) *vs* T1, $P<0.05$; Tis *vs* T2, T3, $P<0.01$). Interestingly, the number of blood vessels detected by immunohistochemical staining for CD34 was significantly higher in the VEGF-C-positive tumours than the VEGF-C-negative tumours (Kitadai *et al*, 2001), suggesting that VEGF-C may be involved in both angiogenic and lymphangiogenic

processes in tumours. However, a similar study examined larger numbers of oesophageal squamous carcinomas for immunohistochemical expression of VEGF-C protein, but did not report a significant association between the expression of the cytokine and any clinicopathological factor other than histological grade (Noguchi *et al*, 2002) (Table 2).

Vascular endothelial growth factor-C expression is associated with neoplastic progression in the oesophageal mucosa. Using immunohistochemical detection, normal oesophageal mucosa does not express VEGF-C although there is an increase in expression in Barrett's epithelium as it progresses through dysplasia to adenocarcinoma, and this is paralleled by a similar increase in VEGFR3 expression on lymphatic vessels (Auvinen *et al*, 2002).

Colorectal cancer

Colorectal cancer is similar to oesophageal cancer, in that the role of VEGF-C is less well understood than in gastric carcinoma. Conflict also exists as to the role of VEGF-D. Recent publications illustrate conflicting results regarding protein and gene expression in relation to clinicopathological measures (Table 2).

With respect to VEGF-C expression, several authors have demonstrated associations between growth factor expression and poor clinicopathological outcome (Akagi *et al*, 2000; Furodoi *et al*, 2002). Immunohistochemical detection of VEGF-C expression at the deepest invasive site of colorectal carcinoma was found in 47% of 152 advanced tumours. Expression correlated with lymphatic and venous invasion, lymph node status, Dukes' stage, liver metastasis, depth of invasion, poorer histological grade and microvessel density (Furodoi *et al*, 2002). Vascular endothelial growth factor-C expression and lymph node metastasis were independent prognostic factors for 5-year survival on multivariate analysis (odds ratio (OR) 9.10, $P = 0.0272$ and OR 8.52, $P = 0.0322$, respectively). The study also emphasised the paracrine nature of the interaction between VEGF-C and the tumour microenvironment and the positive relationship between VEGF-C and tumour angiogenesis (Furodoi *et al*, 2002). Similar associations between tissue VEGF-C expression and clinicopathological factors have been described by Akagi *et al* (2000) with consistent patterns of VEGF-C expression in involved lymph nodes and primary tumours, although in this study only a nonsignificant trend towards decreased survival was identified in VEGF-C positive groups.

Contradictory evidence exists concerning the role of VEGF-C in lymphatic metastasis in colorectal cancer. Studies examining mRNA levels of various VEGF family members tend to show a lack of association with clinicopathological factors. George *et al* (2001) showed an increase in VEGF-A and VEGF-C mRNA in carcinomas ($P = 0.006$ and $P = 0.004$, respectively) but not in colonic polyps ($P = 0.22$ and 0.5, respectively). No association was found between the increased level of VEGF-C mRNA and lymph node status, although a positive relationship existed between positive lymph nodes and VEGF-A mRNA expression. Patterns of VEGF-C mRNA expression were similar in the primary tumour and lymphatic metastases. The mRNA findings of the study were confirmed by immunohistochemistry, which showed no correlation between positive staining for VEGF-A, VEGF-C or VEGF-D and lymphatic spread (George *et al*, 2001). Further analyses of VEGF family mRNA levels in the adenoma–carcinoma sequence showed that of VEGF-A, VEGF-B and VEGF-C, only VEGF-A mRNA levels were consistently raised in invasive malignancy and this became apparent early on in disease progression, as levels were elevated to a similar extent in tumours with and without lymph node metastases or distant spread (Andre *et al*, 2000).

A few studies have focussed on the role of VEGF-D in colorectal malignancy with conflicting results. Tumour expression, assessed by RT-PCR, of VEGF-D mRNA was less than in normal tissue (George *et al*, 2001), while White *et al* (2002) found higher levels of VEGF-D protein expression in cancers detected by immunohistochemistry. The increased VEGF-D protein levels detected were associated with lymph node involvement and reduced overall and disease-free survival (White *et al*, 2002).

The role of VEGF-D within tumours is not well understood, but it has been suggested that VEGF-D may act competitively as an antagonist to the other VEGF family members. George *et al* (2002) postulated that a reduction in VEGF-D levels in the adenoma–carcinoma sequence allowed the more potent angiogenic cytokines VEGF-A and VEGF-C to bind more readily to the signalling receptors VEGFR2 and VEGFR3. The balance between various members of the VEGF family, their relative levels within a tumour, the extent of proteolytic processing and receptor availability may be important in determining tumour behaviour. The importance of the balance between VEGF-C and VEGF-D is illustrated in lung adenocarcinoma, where a low ratio of VEGF-D:VEGF-C (i.e., low VEGF-D and high VEGF-C) is associated with lymph node metastasis and lymphatic invasion (Niki *et al*, 2000).

Upregulation of cytoplasmic VEGFR3 protein expression has been demonstrated immunohistochemically in colorectal cancer tissue specimens and increased expression was associated with poorer overall survival ($P < 0.05$) (Witte *et al*, 2002). This again demonstrates the potent paracrine nature of the interaction between the cytokines and their receptor in the microenvironment of the tumour.

In conclusion, conflicting reports exist for the precise involvement of VEGF-C and VEGF-D in lymphatic invasion, lymph node metastasis and prognosis in colorectal cancer. The importance of appropriate sampling and consistency in methodology of immunohistochemical staining and scoring are fundamental to interpretation and comparison between studies.

CONCLUSIONS

Lymphangiogenesis is an exciting area of research in cancer biology. The growth factors VEGF-C and D are involved in this process and possess angiogenic and lymphangiogenic properties. The expression of lymphangiogenic factors is increased in many human malignancies and this is illustrated with respect to malignancies of the gastrointestinal tract. In gastric adenocarcinoma, lymphatic metastasis and lymphatic invasion are enhanced by increased expression of VEGF-C. The precise role for VEGF-C in colorectal and oesophageal squamous malignancy and VEGF-D in other tumours is not clearly understood, but is clearly important at a paracrine level. Further studies using combinations of new lymphatic markers and functional assays will help clarify the influence of these and other cytokines in the future. However, an essential requirement to allow comparison between studies is the development of consistent experimental methodology. This must include the use of antibodies of defined specificity, consistent immunohistochemical protocols with appropriate use of controls and widespread consensus in scoring techniques. Further understanding of the function and actions of VEGF-C and VEGF-D is required to optimise therapeutic strategies, avoiding unwanted side effects, in the treatment of benign and malignant disease.

REFERENCES

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* **95**: 548–553

Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y, Sugimachi K (2000) Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* **83**: 887–891

Amioka T, Kitadai Y, Tanaka S, Haruma K, Yoshihara M, Yasui W, Chayama K (2002) Vascular endothelial growth factor-C expression predicts lymph node metastasis of human gastric carcinomas invading the submucosa. *Eur J Cancer* **38**: 1413–1419

Andre T, Kotelevets L, Vaillant J-C, Coudray AM, Weber L, Prevot S, Parc R, Gespach C, Chastre E (2000) VEGF, VEGF-B, VEGF-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. *Int J Cancer* **86**: 174–181

Auvinen MI, Sihvo EIT, Ruohtula T, Salminen JT, Koivistoinen A, Siivola P, Ronnholm R, Ramo JO, Bergman M, Salo JA (2002) Incipient angiogenesis in Barrett's epithelium and lymphangiogenesis in Barrett's adenocarcinoma. *J Clin Oncol* **20**: 2971–2979

Beasley NJ, Prevo R, Banerji S, Leek RD, Moore J, van Trappen P, Cox G, Harris AL, Jackson DG (2002) Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res* **62**: 1315–1320

Clarijs R, Ruiter DJ, de Waal RM (2001) Lymphangiogenesis in malignant tumours: does it occur? *J Pathol* **193**: 143–146

Purodoi A, Tanaka S, Haruma K, Kitadai Y, Yoshihara M, Chayama K, Shimamoto F (2002) Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. *Oncology* **62**: 157–166

George ML, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA, Swift RI (2001) VEGF-A, VEGF-C and VEGF-D in colorectal cancer progression. *Neoplasia* **3**: 420–427

Ichikura T, Tomimatsu S, Ohkura E, Mochizuki H (2001) Prognostic significance of the expression of vascular endothelial growth factor (VEGF) and VEGF-C in gastric carcinoma. *J Surg Oncol* **78**: 132–137

Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK, Alitalo K (1997) Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276**: 1423–1425

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* **15**: 290–298

Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K (1997) Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* **16**: 3898–3911

Kabashima A, Maehara Y, Kakeji Y, Sugimachi K (2001) Overexpression of vascular endothelial growth factor C is related to lymphogenous metastasis in early gastric carcinoma. *Oncology* **60**: 146–150

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* **92**: 3566–3570

Karpanen T, Alitalo K (2001) Lymphatic vessels as targets of tumor therapy? *J Exp Med* **194**: F37–F42

Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Ylä-Herttuala S, Jäättelä M, Alitalo K (2001) Vasular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* **61**: 1786–1790

Kitadai Y, Amioka T, Haruma K, Tanaka S, Yoshihara M, Sumii K, Matsutani N, Yasui W, Chayama K (2001) Clinicopathological significance of vascular endothelial growth factor (VEGF)-C in human esophageal squamous cell carcinomas. *Int J Cancer* **93**: 662–666

Leu AJ, Berk DA, Lymboussaki A, Alitalo K, Jain RK (2000) Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res* **60**: 4324–4327

Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, Kauppinen R, Jackson DG, Kubo H, Nishikawa S, Yla-Herttuala S, Alitalo K (2001) Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* **7**: 199–205

Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS (2001) Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* **20**: 672–682

Marconcini L, Marchio S, Morbidelli L, Cartocci E, Albini A, Ziche M, Bussolino F, Oliviero S (1999) *c-fos*-induced growth factor/vascular endothelial growth factor D induces angiogenesis *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* **96**: 9671–9676

Maula S-M, Luukkaa M, Grenman R, Jackson D, Jalkanen S, Ristamaki R (2003) Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res* **63**: 1920–1926

Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S (2000) Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res* **6**: 2431–2439

Noguchi T, Takeno S, Shibata T, Uchida Y, Yokoyama S, Muller W (2002) VEGF-C expression correlates with histological differentiation and metastasis in squamous cell carcinoma of the esophagus. *Oncol Rep* **9**: 995–999

Oh SJ, Jeltsch MM, Birkenhager R, McCarthy JE, Weich HA, Christ B, Alitalo K, Wilting J (1997) VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev Biol* **188**: 96–109

Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL, Jain RK (2002) Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* **296**: 1883–1886

Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, Jackson DG, Skobe M (2002) Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci USA* **99**: 16069–16074

Salven P, Lymboussaki A, Heikkila P, Jaaskela-Saari H, Enholm B, Aase K, von Euler G, Eriksson U, Alitalo K, Joensuu H (1998) Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *Am J Pathol* **153**: 103–108

Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M (2001) Induction of tumor lymphangiogenesis of VEGF-C promotes breast cancer metastasis. *Nat Med* **7**: 192–198

Stacker SA, Achen MG, Jussila L, Baldwin MA, Alitalo K (2002) Lymphangiogenesis and cancer metastasis. *Nat Cancer Rev* **2**: 573–583

Stacker SA, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, Roufail S, Simpson RJ, Moritz R, Karpanen T, Alitalo K, Achen MG (1999) Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J Biol Chem* **274**: 32127–32136

Straume O, Jackson DG, Akslen LA (2003) Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. *Clin Cancer Res* **9**: 250–256

Takahashi A, Kono K, Itakura J, Amemiya H, Tang RF, Iizuka H, Fujii H, Matsumoto Y (2002) Correlation of vascular endothelial growth factor C expression with tumor-infiltrating dendritic cells in gastric cancer. *Oncology* **62**: 121–127

Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA, Alitalo K (2001) Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* **20**: 1223–1231

Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K (2000) Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res* **60**: 203–212

White JD, Hewett PW, Kosuge D, McCulloch T, Enholm BC, Carmichael J, Murray JC (2002) Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. *Cancer Res* **62**: 1669–1675

Witte D, Thomas A, Ali N, Carlson N, Younes M (2002) Expression of the vascular endothelial growth factor receptor-3 (VEGFR-3) and its ligand VEGF-C in human colorectal adenocarcinoma. *Anticancer Res* **22**: 1463–1466

Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K, Sasaki T (1999) Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* **5**: 1823–1829

Yonemura Y, Fushida S, Bando E, Kinoshita K, Miwa K, Endo Y, Sugiyama K, Partanen T, Yamamoto H, Sasaki T (2001) Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)-3 in gastric cancer. *Eur J Cancer* **37**: 918–923

APPENDIX II. International Journal of Oncology 2003;22:399-343

INTERNATIONAL JOURNAL OF ONCOLOGY 22: 339-343, 2003

Immunodetection and molecular forms of plasma vascular endothelial growth factor-C

S.E. DUFF[1,2], C. LI[2], A. RENEHAN[1], S.T. O'DWYER[1] and S. KUMAR[2]

[1]Department of Surgery, Christie Hospital; [2]Department of Experimental Pathology, University of Manchester, Manchester, UK

Received September 12, 2002; Accepted October 30, 2002

Abstract. Vascular endothelial growth factor (VEGF)-C is a member of the VEGF family. VEGF-C is involved in developmental lymphangiogenesis and may be important in pathological lymphangiogenesis, lymphatic invasion and metastasis in carcinoma. We describe the development of an indirect enzyme-linked immunosorbent (ELISA) assay for the quantification of VEGF-C in plasma. Capture of VEGF-C was achieved using goat anti-human VEGF-C antibody, followed by detection with rabbit anti-human VEGF-C antibody. The sensitivity of the assay was amplified using the biotin-avidin and enhanced chemiluminescence (ECL) systems. The assay was highly sensitive and reproducible with a detection range of 0.4-100 U/ml and the intra- and inter-assay variations were less than 8%. Substitutional tests demonstrated that the assay was specific for VEGF-C with no cross-reaction with VEGF-A or VEGF-D. Practical application of the assay was evaluated in 41 colorectal cancer patients and 31 controls. Median plasma levels of VEGF-C were 35.0 U/ml (range: 17.4-75.9 U/ml) in colorectal cancer patients in contrast to 11.5 U/ml (range: 5.4-21.5 U/ml) in controls ($p<0.001$). Moreover, VEGF-C levels tended to be elevated in patients with advanced disease compared to early disease, but this was not statistically significant owing to a relatively small number of patients in each group. Immunoprecipitation and immunoblotting confirmed detection of VEGF-C in plasma and revealed that two forms of VEGF-C were present in the plasma corresponding to ~40 and ~80 kDa. The measurement of plasma VEGF-C offers opportunities to explore clinical applications in the management of malignancy, in particular in the prediction of lymphatic spread and in other lymphangiogenesis-related diseases.

Correspondence to: Dr S.E. Duff, Laboratory Medicine Academic Group, Department of Experimental Pathology, Stopford Building, University of Manchester, Manchester M13 9PT, UK
E-mail: sarah.duff@man.ac.uk

Abbreviations: VEGF, vascular endothelial growth factor; ELISA, enzyme linked-immunosorbent assay; ECL, enhanced chemiluminescence

Key words: endothelial growth factors, immunoassay, colorectal neoplasms

Introduction

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen and is crucial to the process of angiogenesis (reviewed in refs. 1,2). Levels of VEGF are elevated in the circulation of patients with cancer and predict tumour angiogenic potential and prognosis (3-5). VEGF-C is a recently discovered member of the VEGF family, sharing areas of homology with VEGF-A (6,7). VEGF-C is involved in the development of lymphatics in animal models (8), inducing lymphangiogenesis in the skin of transgenic mice overexpressing VEGF-C in the basal cell layer (9) and around tumours overexpressing VEGF-C (10-13). Over-expression of VEGF-C in tumour models has been associated with the development of lymph node metastases (12-14). Increased tumour expression of VEGF-C has been found in a variety of human tumours (15-17) and correlates with an increase in lymphatic density and metastasis (18-22). Lymph node metastasis is one of the earliest features of tumour dissemination in human solid tumours and is a crucial determinant of prognosis.

Detection of increased levels of factors regulating lymph-angiogenesis and lymph node metastasis could be useful in predicting advanced disease and individuals at risk of lymphatic dissemination. To investigate this process we have developed a quantitative enzyme-linked immunosorbent assay (ELISA) for the detection of VEGF-C in plasma and investigated levels of VEGF-C in normal individuals and in patients with colorectal cancer.

Materials and methods

Indirect ELISA for VEGF-C. Since VEGF-A and VEGF-D in the circulation can be detected using immunoassays, it is reasonable to speculate that VEGF-C in plasma can be quantified by an ELISA system.

VEGF-C present in the plasma was captured using goat polyclonal anti-human VEGF-C antibody (R&D Systems, Abingdon, UK). This antibody is produced in goats immunized with purified, *E. coli*-derived, recombinant human VEGF-C (rhVEGF-C) peptide, corresponding to amino acid residues

104-330. The detection antibody used was rabbit polyclonal anti-human VEGF-C antibody (Zymed Laboratories Inc., CA), raised against a synthetic peptide corresponding to the carboxyl terminal of human VEGF-C. A standard curve could not be created on the ELISA plate using the aforementioned rhVEGF-C (R&D Systems) as this peptide was not recognised by the detection antibody (data not shown and Fig. 4, lane 4), consequently human plasma with high levels of VEGF-C was used to form a standard curve as described below.

The procedure was as follows: 96-well white microtiter plates were coated with 100 µl/well goat anti-human VEGF-C antibody diluted to a final concentration of 1 µg/ml in 0.1 M carbonate buffer (pH 9.6). The coated plates were incubated overnight in a humidified chamber at 4°C then blocked with 100 µl/well of 1% BSA (w/v) in 0.1 M PBS and 0.1% Tween 20 (Sigma-Aldrich Ltd; PBS-Tween) for 3 h at room temperature. Test samples diluted 1 in 2 in PBS-Tween were added to the plates in duplicate. Plasma taken from a patient with a multisystem autoimmune disorder known to have a high level of VEGF-C by immunoprecipitation (see below) was serially diluted to generate a standard curve on each plate. The concentration of VEGF-C in plasma was defined as 100 units/ml in the standard plasma. After overnight incubation in a humidified chamber at 4°C, 100 µl/well of rabbit anti-human VEGF-C antibody was added at a final concentration of 0.5 µg/ml diluted in PBS-Tween and incubated at 4°C for 3 h. Amplification of signal was achieved by the addition of 100 µl/well of biotinylated goat-antirabbit IgG (Dako Ltd., Cambridgeshire, UK) at 1/1000 dilution in 1% BSA (w/v) and PBS-Tween, and incubated with shaking at room temperature for 1 h. 100 µl/well horseradish peroxidase (HRP)-streptavidin (Dako) was added and the plates incubated with shaking at room temperature for 30 min. Three washes with PBS-Tween were carried out between each of the procedures. Finally, 100 µl/well signal reagent (Orthoclinical Diagnostics, Bucks, UK) was added and the light emission immediately measured at 420 nm in a plate reader (Kodak Clinical Diagnostics, Aylesbury, UK).

Intra- and inter-assay variation. To evaluate the reproducibility of the assay, the intra-assay variation was measured using the same quantity of plasma in half the wells on a single plate and the inter-assay variation measured using plasma containing constant levels of VEGF-C across 8 separate experiments.

Assessment of the specificity of the ELISA system. The specificity of the system for detection of VEGF-C was determined by the substitution of standard plasma for known concentrations of recombinant VEGF-A and VEGF-D (both from R&D Systems) to assess for any evidence of cross-reactivity.

Immunoprecipitation and Western blotting of VEGF-C. To reveal the molecular forms of VEGF-C present in the plasma, immunoprecipitation and immunoblotting were performed. To eliminate the non-specific binding of plasma proteins, plasma samples were pre-cleared with Protein L-agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) by mixing 1/10 diluted standard plasma (2 µl plasma plus 18 µl PBS) with 10 µl Protein L-agarose at 4°C for 4 h. The beads were pelleted by centrifugation for 5 min at 1000 x g and the supernatant collected. VEGF-C was specifically precipitated from the pre-cleared supernatant with goat anti-human VEGF-C antibody coupled to Protein L-agarose. Goat anti-human immunoglobulins (Sigma-Aldrich Ltd.) coupled to Protein L-agarose were used as a negative control. The pre-cleared plasma samples were made up to 1 ml with PBS, 10 µl protease inhibitor cocktail (Calbiochem Biosciences UK, Nottingham, UK) was added followed by 10 µg goat anti-human VEGF-C antibody or negative control. Following 1 h of rotation at 4°C, 10 µl Protein L-agarose was added and incubation continued overnight. Immunoprecipitates were isolated by centrifugation, 500 µl NET buffer [50 mM Tris-HCl, 150 mM sodium chloride, 0.5 M EDTA (Sigma-Aldrich), 1% Igepal CA-630 (Sigma-Aldrich)] and 10 µl of protease inhibitor cocktail were added and the mixture centrifuged again. This process was repeated twice and finally, the bead pellet was washed with PBS, centrifuged, the supernatant discarded and the beads carefully dried. Reducing sample buffer was added to the tube and boiled for 10 min followed by cooling on ice, briefly centrifuged and loaded into a 4-10% SDS-PAGE gel and subjected to electrophoresis with electrophoretic transfer onto a PVDF membrane (Hybond-C Super, Amersham). Molecular weight markers (Bio-Rad, Hercules, CA) were run on the gel to aid detection of the molecular weights of the species observed. Filters were washed with PBS-Tween and blocked with 4% Marvel-PBS-Tween for 2 h at room temperature. To detect VEGF-C, the filters were divided and incubated overnight at 4°C with goat anti-human VEGF-C antibody at 1/1000 dilution or rabbit anti-human VEGF-C antibody at 1/500 dilution in blocking solution. Finally, the blots were incubated with rabbit anti-goat antibody (1/1000) conjugated with HRP or mouse anti-rabbit antibody (1/1000) conjugated with HRP (both from Dako) for 2 h at 4°C. The precipitated VEGF-C was detected using an enhanced chemiluminescence (ECL) system (Orthoclinial Diagnostics, Amersham, UK).

Evaluation of ELISA in colorectal cancer patients and controls. VEGF-C was measured in the plasma from 31 normal controls and 41 patients with colorectal cancer. Controls were healthy volunteers who were staff members in the University of Manchester. Patients with colorectal cancer were diagnosed histologically, blood samples, taken prior to resectional surgery were collected into an EDTA Vacutainer® (Becton Dickinson, Oxford, UK) bottle and plasma was harvested following centrifugation for 5 min at 1000 x g at 4°C. Plasma samples were aliquoted and stored at -80°C.

Statistical analysis. All statistical calculations were carried out using SPSS 10.1 statistical software (SPSS Inc, Chicago, IL). Unless specified, the data were expressed as median and range. The data were not parametrically distributed, consequently the significance of differences between groups was calculated by applying nonparametric tests. The Mann-Whitney U and Kruskal-Wallis tests were used to analyse the difference in plasma levels of VEGF-C between groups

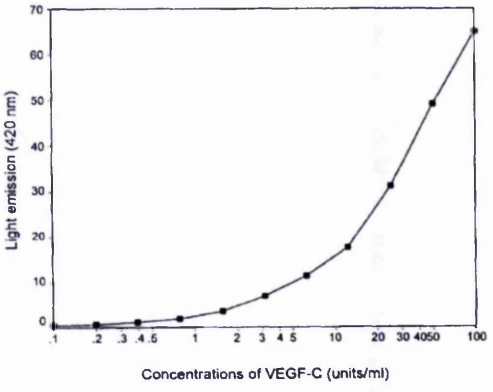


Figure 1. Standard curve of VEGF-C concentration in standard plasma. Standard plasma (100 units/ml) was serially diluted and the curve generated by logistic regression. The measured light emission of samples was converted into units/ml according to the standard curve on each plate.

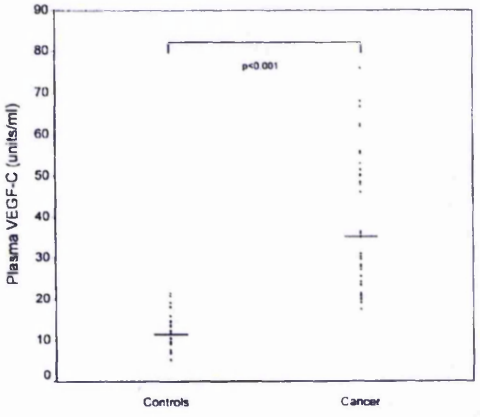


Figure 2. Plasma levels of VEGF-C in normal individuals (n=31) and colorectal cancer patients (n=41) determined by ELISA. Significantly elevated levels were found in cancer patients (p<0.001, Mann-Whitney U test). Horizontal lines indicate the medians.

of controls and patients and between controls and patients with early and advanced disease. A level of $p \leq 0.05$ was accepted as statistically significant.

Results

Detection of VEGF-C. The optimal signal was achieved with a coating antibody concentration of 1 µg/ml and a detection antibody concentration of 0.5 µg/ml. Use of the biotin-avidin system directed against the detection antibody doubled the signal detected. The approximate sensitivity of the assay was 0.4 units/ml, which represented twice the background level and the detection range for VEGF-C was up to 100 units/ml. Specific binding of the antibodies to VEGF-C in standard plasma was demonstrated by substitution tests. Substitution of standard plasma by recombinant VEGF-A or VEGF-D showed no detectable signal above background. The intra- and inter-assay variations were measured using plasma containing constant levels of VEGF-C and by repetition and were 5%, and <8%, respectively.

Standard curve for VEGF-C. To calculate the concentrations of VEGF-C in test plasma, a standard curve was included on each plate by serial dilution of the standard plasma (Fig. 1). In most of the assays, the curve fit was 100% and was never less than 99%. From this reference curve, the measured values of light emission of the test samples were automatically converted into concentrations in units per milliliter.

VEGF-C in plasma from cancer patients and normal controls. The optimised assay was employed to measure the levels of VEGF-C in plasma samples from 41 colorectal cancer patients and 31 healthy controls. The results shown in Fig. 2 demonstrate that the levels of VEGF-C were significantly elevated in plasma from colorectal cancer patients, 35.0 units/ml (17.4-75.9 units/ml) compared to normal controls 11.5 units/ml (5.4-21.5 units/ml) ($p<0.001$).

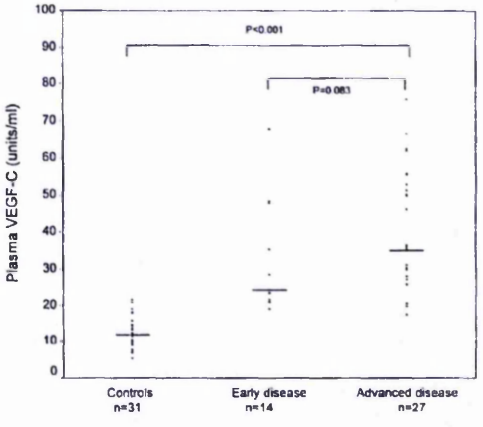


Figure 3. Plasma levels of VEGF-C in normal individuals and colorectal cancer patients divided into early disease (Dukes' A and B, n=14) and advanced disease (Dukes' C and D, n=27). Significant differences were found between the controls and either disease group, $p<0.001$, but not between early vs. advanced disease, $p=0.083$, NS.

Division of the colorectal cancer patients into groups of early [n=14, Dukes' A (n=5) and B (n=9)] and advanced disease [n=27, Dukes' C (n=5) and D (n=22)] showed a tendency towards increasing levels of VEGF-C in advanced disease but this was not statistically significant ($p=0.083$) (Fig. 3). Any potential significance may have been overshadowed by the relatively small number of patients in each group.

Molecular forms of VEGF-C in standard plasma. Standard plasma was subjected to immunoprecipitation and immuno-

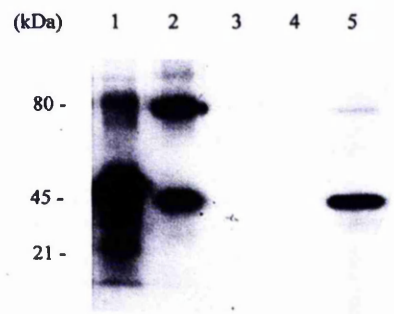


Figure 4. Immunoprecipitation and immunoblotting of VEGF-C in standard plasma. Samples were immunoprecipitated with goat anti-human VEGF-C antibody (lanes 1, 2, 4 and 5) or negative control antibody (goat anti-human IgG, lane 3). Lanes 1-3, VEGF-C was immunoprecipitated by goat anti-human VEGF-C antibody and revealed by rabbit anti-goat antibody conjugated to HRP. Lanes 4 and 5, VEGF-C was immunoprecipitated by rabbit anti-human VEGF-C antibody and revealed by goat anti-rabbit antibody conjugated to HRP. The blots were developed with ECL and the exposure time was 15 sec. Lane 1 contains recombinant VEGF-C loaded separately onto the gel, with a main band of ~45 kDa, and weaker bands of ~15, 23 and 80 kDa. Lane 2 is standard plasma, VEGF-C is seen as 2 bands of ~40 and ~80 kDa. Lane 3 is standard plasma immunoprecipitated with goat anti-human IgG as a negative control, no bands are seen. Lane 4 contains recombinant VEGF-C, no bands are seen (as the rabbit anti-human VEGF-C antibody does not recognise the recombinant protein). Lane 5 contains standard plasma, where a strong band of ~40 kDa and a weaker band of ~80 kDa can be seen.

blotting in an attempt to analyse the molecular forms of VEGF-C detected in plasma. Goat polyclonal anti-human VEGF-C antibody was used as the antibody for immunoprecipitation. Negative controls used goat anti-human IgG as the antibody for immunoprecipitation. Detection of the precipitated species on the gel was made by goat anti-human VEGF-C or rabbit anti-human VEGF-C antibodies. Recombinant human VEGF-C was also run on the gel to demonstrate reactivity with the goat but not the rabbit anti-human VEGF-C (further demonstrating the lack of effectiveness of recombinant human VEGF-C as a standard in the sandwich ELISA). The results of immunoprecipitation and immunoblotting with goat anti-human VEGF-C antibody are shown in Fig. 4. Recombinant VEGF-C is seen in lane 1, which has a major band of ~45 kDa, and weaker bands of approximately 15, 23 and 80 kDa. Standard plasma from the immunoprecipitation experiment with goat anti-human VEGF-C antibody is seen in lane 2. Two bands are of approximately 40 and 80 kDa, no bands are seen in lane 3 where the negative control goat anti-human IgG was used as the antibody for immunoprecipitation. Lanes 4 and 5 show the results of immunoblotting with rabbit anti-human VEGF-C antibody, recombinant VEGF-C is not detectable in lane 4 (as this does not react with the rabbit antibody used in detection) and standard plasma is seen in lane 5 as a band of approximately 40 kDa and a weaker band of ~80 kDa. These bands are identical in size to those detected in using goat anti-human VEGF-C for immunoprecipitation and detection (lane 2), demonstrating that the goat anti-human VEGF-C and the rabbit anti-human VEGF-C antibodies used in the indirect sandwich ELISA detect the same species of VEGF-C in the plasma.

Discussion

We have developed a novel indirect ELISA to quantify the levels of VEGF-C in plasma and have evaluated its potential use in patients with colorectal cancer. Sensitivity of the assay has been optimised by the use of an amplification step based on the avidin-biotin system and enhanced chemiluminescence has been substituted for conventional colorimetric ELISA. Intra- and inter-assay variations were minimal and compared well with other similar assays (23,24). Immunoprecipitation and immunoblotting confirmed the presence and detection of VEGF-C in standard plasma using the two antibodies employed in the ELISA system.

VEGF-C is secreted as a precursor protein and proteolytically processed to progressively smaller mature forms, which have higher affinity for the receptor flt-4 (VEGF-R3) and allow acquisition of VEGF-R2 (KDR) activating properties (25). The propeptide of ~58 kDa dimerises in the intracellular environment and is split into two near equal halves, resulting in polypeptides of 29 and 31 kDa bound to one another by disulfide and non-covalent bonds. This splitting occurs at the end of the secretory pathway or at the plasma membrane, although secretion is possible in the absence of proteolytic cleaveage. Further processing occurs in the extracellular environment, the N-terminal and C-terminal propeptides are split off to leave the mature 21 kDa form of VEGF-C (25). In this study, immunoprecipitation and immunoblotting of standard plasma detected two main bands of approximately 40 and 80 kDa. As the antibody combination used in the ELISA recognizes the central VEGF-homology and carboxyl terminal domains of VEGF-C, the forms detected in plasma may represent both the partially processed and the fully mature form of the protein.

VEGF-C has been detected in a variety of human tumours (15) and in colorectal cancer is associated with depth of tumour invasion, lymphatic invasion, lymph node metastasis and a trend towards a decrease in survival (22). More recently, Furodoi *et al* (26) have shown that expression of VEGF-C in the deepest invasive site of colorectal carcinoma is correlated with poorer histological grade, lymphatic and venous invasion, lymph node and liver metastasis. We found increased levels of VEGF-C in the plasma of colorectal cancer patients, although levels were not significantly different between groups of patients with early and advanced disease. Further evaluation of the detection of VEGF-C in plasma is currently underway in patients with early and advanced colorectal cancer.

References

1. Neufeld G, Cohen T, Gengrinovitch S and Poltorak Z.: Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13: 9-22, 1999.
2. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ and Holash J: Vascular-specific growth factors and blood vessel formation. Nature 407: 242-248, 2000.

3. Duque JL, Loughlin KR, Adam RM, Kantoff PW, Zurakowski D and Freeman MR: Plasma levels of vascular endothelial growth factor are increased in patients with metastatic prostate cancer. Urology 54: 523-527, 1999.
4. George ML, Eccles SA, Tutton MG, Abulafi AM and Swift RI: Correlation of plasma and serum vascular endothelial growth factor levels with platelet count in colorectal cancer: clinical evidence of platelet scavenging? Clin Cancer Res 6: 3147-3152, 2000.
5. Tamura M, Ohta Y, Kajita T, Kimura K, Go T, Oda M, Nakamura H and Watanabe G: Plasma VEGF concentration can predict the tumor angiogenic capacity in non-small cell lung cancer. Oncol Rep 8: 1097-1102, 2001.
6. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N and Alitalo K: A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15: 290-298, 1996.
7. Lee J, Gray A, Yuan J, Luoh SM, Avraham H and Wood WI: Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc Natl Acad Sci USA 93: 1988-1992, 1996.
8. Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V and Alitalo K: VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development 122: 3829-3837, 1996.
9. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK and Alitalo K: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 276: 1423-1425, 1997.
10. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Ylä-Herttuala S, Jäättelä M and Alitalo K: Vasular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res 61: 1786-1790, 2001.
11. Skobe M, Hamberg LM, Hawighorst T, Schimer M, Wolf GL, Alitalo K and Detmar M: Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am J Pathol 159: 893-903, 2001.
12. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K and Detmar M: Induction of tumor lymphangiogenesis of VEGF-C promotes breast cancer metastasis. Nat Med 7: 192-198, 2001.
13. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, *et al*: Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J 20: 672-682, 2001.
14. Mattila MM-T, Ruohola JK, Karpanen T, Jackson DG, Alitalo K and Harkonen PL: VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. Int J Cancer 98: 946-951, 2002.
15. Salven P, Lymboussaki A, Heikkila P, Jaaskela-Saari H, Enholm B, Aase K, von Euler G, Eriksson U, Alitalo K and Joensuu H: Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. Am J Pathol 153: 103-108, 1998.
16. Ohta Y, Shridhar V, Bright RK, Kalemkerian GP, Du W, Carbone M, Watanabe Y and Pass HI: VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. Br J Cancer 81: 54-61, 1999.
17. George ML, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA and Swift RI: VEGF-A, VEGF-C and VEGF-D in colorectal cancer progression. Neoplasia 3: 420-427, 2001.
18. Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K and Sasaki T: Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. Clin Cancer Res 5: 1823-1829, 1999.
19. Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K and Koji T: Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer 80: 309-313, 1999.
20. Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y and Hirohashi S: Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. Clin Cancer Res 6: 2431-2439, 2000.
21. Kajita T, Ohta Y, Kimura K, Tamura M, Tanaka Y, Tsunezuka Y, Oda M, Sasaki T and Watanabe G: The expression of vascular endothelial growth factor C and its receptors in non-small cell lung cancer. Br J Cancer 85: 255-260, 2001.
22. Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y and Sugimachi K: Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br J Cancer 83: 887-891, 2000.
23. Wang JM, Wilson PB, Kumar S, Pye D and Hunter RD: Quantitation of endothelial cell specific protein E-9 employing a single monoclonal antibody in an indirect sandwich ELISA. J Immunol Methods 171: 55-64, 1994.
24. Li CG, Wilson PB, Bernabéu C, Raab U, Wang JM and Kumar S: Immunodetection and characterisation of soluble CD105-TGFβ complexes. J Immunol Methods 218: 85-93, 1998.
25. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N and Alitalo K: Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 16: 3898-3911, 1997.
26. Furodoi A, Tanaka S, Haruma K, Kitadai Y, Yoshihara M, Chayama K and Shimamoto F: Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. Oncology 62: 157-166, 2002.

APPENDIX III. Poster presentation at British Microcirculation Society, 7-8th April 2003, Bristol, UK

Immunolocalisation of VEGF, VEGF-C and VEGF-D in colorectal carcinoma

SE Duff, M Jeziorska, S McGrath, N Haboubi, S Kumar, ST O'Dwyer, G Jayson

Laboratory Medicine Academic Group, University of Manchester and Departments of Surgery, Pathology and Medical Oncology, Christie Hospital NHS Trust, Manchester

THE UNIVERSITY of MANCHESTER

NHS

Introduction

The vascular endothelial growth factor (VEGF) family of cytokines is important for tumour angiogenesis. New members of the VEGF family, VEGF-C and VEGF-D, possess lymphangiogenic properties in addition. Elevated expression of these cytokines has been reported in various human tumours. Expression of VEGF-C has been correlated with lymph node metastasis, lymphatic invasion and poorer survival in both gastric and colorectal malignancy (1-3). Different authors have described tissue expression of VEGF-D in colorectal carcinoma to be either increased (4) or decreased (5).

Aims

The aim of this study was to assess the topographical distribution of VEGF, VEGF-C and VEGF-D in primary colorectal carcinoma and metastatic lymph nodes in cases with metastatic spread.

Materials and Methods

Immunohistochemical staining for VEGF, VEGF-C, and VEGF-D was performed in 21 colorectal carcinoma specimens and in 9 metastatic lymph nodes. The immunostaining protocols were optimized to achieve a similar range of staining intensities for each cytokine. Assessment of staining was made independently by four different researchers and discrepancies negotiated to reach consensus. Staining was examined at different sites of the primary tumour – normal mucosa (N), junctional mucosa (J), superficial tumour (TS), central tumour (TC), invasive edge (TI), and within the metastatic deposits in the involved lymph nodes (LN).

Results

Positive expression of cytokines was seen in increased numbers of cases at deeper tumour sites, being greatest at TI and in the metastatic lymph nodes (Table 1).

	N	J	TS	TC	TI	LN
VEGF-C	9(43)	13 (60)	18 (90)	20 (95)	20 (100)	9 (100)
VEGF-D	8(44)	12 (57)	11 (55)	19 (90)	20 (95)	9 (100)
VEGF	2 (11)	7 (39)	7(33)	14 (74)	18 (86)	9 (100)

Table 1. Number of cases (%) with any positive staining for the cytokines

The patterns of cytokine expression intensity were similar, with a significant increase throughout the tumour, to a maximum at TI and in the metastatic lymph nodes (Fig 1).

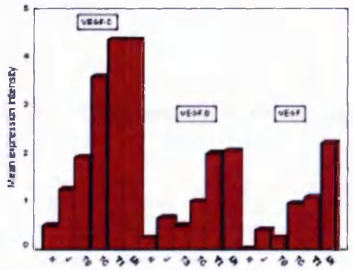
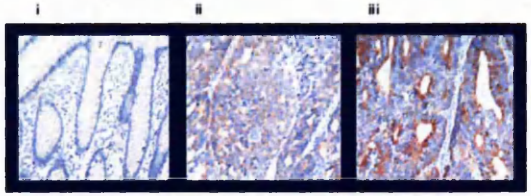


Fig 1. Mean cytokine expression intensity at varying tumour locations

The intensity of cytokine expression increased throughout the tumour for VEGF-C ($p< 0.001$), VEGF-D ($p= 0.027$) and VEGF ($p= 0.001$) (Fig 2).

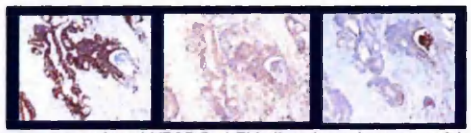
Fig 2. VEGF-C immunostaining within an individual case at i. normal mucosa, ii. superficial tumour, iii. invasive tumour



The expression of VEGF-C in metastatic lymph nodes was significantly greater than that of VEGF-D ($p= 0.04$) or VEGF ($p= 0.013$) at the corresponding site.

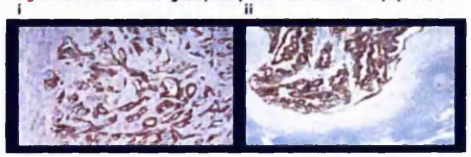
The expression of VEGF-C at TI was significantly greater than that of VEGF-D ($p< 0.001$) or VEGF ($p< 0.001$) at the corresponding site (Fig 3).

Fig 3. Colorectal carcinoma specimen immunostained at the same site for i. VEGF-C, ii. VEGF-D, iii. VEGF



The expression of VEGF-C at TI in the primary tumour correlated with that in the corresponding metastatic lymph node ($r= 0.79$; $p= 0.04$), this was not the case for VEGF-D ($p= 0.81$) or VEGF ($p= 0.76$) (Fig 4).

Fig 4. VEGF-C immunostaining in i. primary tumour and ii. metastatic lymph node.



Conclusions

The increased expression and distribution pattern of VEGF-C in colorectal carcinoma and metastatic lymph nodes is confirmed. VEGF-C is more highly expressed in colorectal carcinoma than VEGF-D and VEGF. Therefore, VEGF-C is likely to be of great importance in the progression and metastasis of colorectal carcinoma.

The distribution pattern of VEGF-D was similar to that of VEGF-C. There was no evidence of reduced VEGF-D expression in tumours.

References

1. Yonemura Y, Endo Y, Fujita H, Fushida S, Hiramoya I, Bandou E, et al. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. Clinical Cancer Research 1999;5:1823-1828.

2. Funahashi A, Tanaka S, Hiraumi K, Kikuta Y, Yoshihara M, Chayama K, et al. Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. Oncology 2002;62:15-21.

3. Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y, et al. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br J Cancer 2000;83(7):887-91.

4. White JD, Hewett PW, Kosuge D, McCulloch T, Eccleston DC, Carmichael J, et al. Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. Cancer Res 2002;62(6):1669-75.

5. George ML, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA, et al. VEGF-A, VEGF-C and VEGF-D in colorectal cancer progression. Neoplasia 2001;3(5):420-27.

E-mail: sarah.duff@christie.nwest.nhs.uk

APPENDIX IV. Poster presentation at British Association of Surgical Oncology, 24-25th November, 2003, Royal College of Surgeons of England, London, UK

Preoperative plasma levels of VEGF-A, VEGF-C and VEGF-D in the prediction of outcome in colorectal cancer

SE Duff, MP Saunders, V McCredie, S Kumar, E Levine, ST O'Dwyer, GC Jayson

Departments of Surgery and Oncology, Christie Hospital NHS Trust, Manchester and University of Manchester, UK

Christie Hospital NHS

Introduction

- Vascular endothelial growth factor (VEGF)-C and VEGF-D are members of the VEGF family and show increased expression in various cancers.
- VEGF-C and VEGF-D are involved in angiogenesis and lymphangiogenesis and may promote lymphatic invasion and metastasis in carcinoma.
- Increased VEGF-C expression in colorectal cancer correlates with lymphatic involvement, lymph node metastasis and a trend towards reduced survival (1,2).
- Circulating levels of VEGF-A are increased in cancer patients and may predict tumour angiogenic potential and prognosis (3).
- Detection of increased circulating levels of VEGF-C and VEGF-D could be useful as a prognostic marker, in particular in detection of those with or at risk of lymphatic dissemination.

The aim of this study was to measure and assess the use of plasma VEGF-A, VEGF-C and VEGF-D in colorectal cancer patients, in whom lymphatic spread is known to be a negative prognostic indicator.

Methods

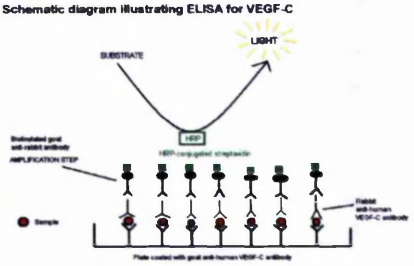
Patients

- **120 cancer patients** - rectal cancer patients undergoing preoperative radiotherapy at the Christie Cancer Centre and colon cancer patients undergoing surgery at South Manchester University Hospitals NHS Trust
- ***50* control patients** - patients undergoing endoscopic examination for bowel symptoms or polyp follow-up, with normal results
- All participants received information sheets and gave written informed consent to the study, which was approved by South Manchester Research Ethics Committee.

Sample processing

- Enzyme-linked immunosorbent assays (ELISAs) were performed for plasma VEGF-A, VEGF-D (R & D Quantikine ELISA Kits) and VEGF-C (4) (**Fig 1**). All samples were assayed in duplicate.

Fig 1. Schematic diagram illustrating ELISA for VEGF-C



Statistical analysis

- Two-sided non-parametric tests were used to compare median growth factor levels and a p value of <0.05 was taken as statistically significant.

Results

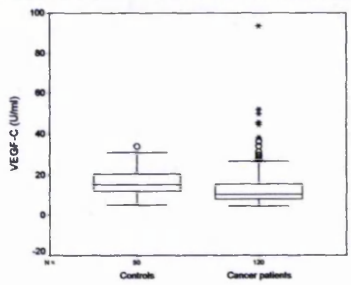
- Median plasma VEGF-C was increased in the control group (p=0.001) (**Table 1, Fig 2**).
- No differences in median plasma VEGF-C, VEGF-D or VEGF-A levels were seen in cancer patients of different T, N or M stages, different histological grades, different Dukes' stages, or in patients with and without lympho-vascular invasion or lymph node involvement.

Table 1. Comparison between cancer and control groups

	Cancer patients	Control patients	p-value
Number of cases	120	50	
Age (years)	66 (25-84)	62.5 (40-79)	0.103*
Gender	81 male 39 female	29 male 21 female	0.236**
Plasma VEGF-A (pg/ml)	39 (7-273)	36.5 (4-230)	0.99#
Plasma VEGF-D (pg/ml)	330 (0-1343)	370 (75-926)	0.09#
•LN positive	335 (113-1109)		} 0.68 #
•LN negative	317 (0-1343)		
Plasma VEGF-C (U/ml)	10.5 (4.4-93.4)	15.2 (5.2-34.0)	0.001#
•LN positive	11.2 (4.9-51.9)		} 0.90 #
•LN negative	9.9 (4.4-93.4)		

Numbers represent median (range). * independent samples t-test, ** chi-squared test, #Mann-Whitney U test.

Fig 2. Boxplot comparing plasma VEGF-C levels between cancer patients and controls. Medians are represented by the horizontal lines within the boxes. p<0.001.



- Plasma VEGF-A levels correlated with platelet count (Spearman's rho, 0.25, p=0.007). Plasma VEGF-C and VEGF-D levels did not correlate with platelet counts (Spearman's rho, 0.17 and –0.16; p values 0.06 and 0.08 respectively).

Conclusions

- Preoperative plasma levels of VEGF-A and VEGF-D could not discriminate between cancer patients and controls, whereas VEGF-C levels were elevated in controls.
- Circulating VEGF-C and VEGF-D levels could not predict lymph node involvement or lymphatic invasion.
- Any endocrine role for these growth factors is likely to be overshadowed by their paracrine and autocrine actions at tissue level in the tumour microenvironment.

REFERENCES

1. Akagi K, Ikeda Y, Miyazaki M, et al. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br J Cancer 2000;83:887-891
2. Furudoi A, Tanaka S, Haruma K et al. Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. Oncology 2002;62:157-166
3. Poon R, Fan S, Wong J. Clinical implications of circulating angiogenic factors in cancer patients. J Clin Oncol 2001;19:1207-1225
4. Duff SE, Li C, Renehan A, et al. Immunodetection and molecular forms of plasma vascular endothelial growth factor-C. Int J Oncol 2003;22:339-343

Printed by Department of Medical Illustration, Christie Hospital NHS Trust, Manchester. Tel 0161 446 3305/6. Email: illustration@physics.cr.man.ac.uk

APPENDIX V. Poster presentation at American Society of Clinical Oncology, GI Symposium, 22-24th January 2003, San Francisco, USA

Evidence for redundancy, cross-talk and autocrine circuits within the vascular endothelial growth factor signaling pathways in colorectal cancer

SE Duff, M Jeziorska, N Haboubi, S Kumar, ST O Dwyer, GC Jayson

THE UNIVERSITY of MANCHESTER

Christie Hospital NHS NHS Trust

Introduction

The first statistically significant survival advantage in favour of anti-angiogenic therapy was reported recently in colon cancer; bevacizumab, an anti-VEGF antibody, when administered with chemotherapy improved median survival (1). A detailed understanding of the VEGF system is critical to optimise this therapeutic advance.

VEGF-C and VEGF-D are members of the VEGF family with angiogenic and lymphangiogenic actions, with receptor specificity for VEGF receptors 2 (VEGFR2/KDR) and 3 (VEGFR3/flt4) and are expressed in colorectal cancer (2).

To understand the possible mechanisms underlying the response to anti-VEGF therapy, a detailed evaluation of expression of the VEGF family members and the VEGF receptor, VEGFR2 (KDR) was undertaken in primary colorectal cancer and associated metastases.

Materials and Methods

Specimens

- 30 primary colorectal cancers and their corresponding metastases (12 lymph node, 9 liver metastases)

Techniques

- Immunohistochemical staining for VEGF-A, VEGF-C, VEGF-D and VEGFR2 antigens, using the indirect immunoperoxidase technique.

Scoring

- Semi-quantitative score for staining intensity at different primary tumour areas including adjacent normal mucosa (N), junctional mucosa (J), superficial (TS), central (TC) and invasive tumour edge (TI).
- Scored by two independent observers blinded to clinical details.

Statistics

- Two-tailed non-parametric statistical tests, p-value <0.05 was considered significant.

Results

- VEGFR2 was expressed in colorectal cancer cells (**Fig 1**).
- Expression of VEGF-A, VEGF-C, VEGF-D and VEGFR2 increased throughout the tumour to a maximum at TI (p<0.001 for all antigens) (**Fig 1,2**).
- Expression of VEGF-C, VEGF-D and VEGF-A was not significantly different between the TI and either of the corresponding metastases (liver or lymph node).

Fig 1. VEGFR2 expression in a moderately differentiated colonic adenocarcinoma immunostained with mouse monoclonal anti-human VEGFR2 antibody: a, superficial tumour, TS; b, central tumour, TC; c, invasive edge of tumour, TI. Sections counterstained with haematoxylin, original magnification x100.

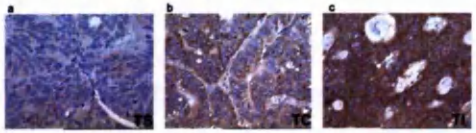
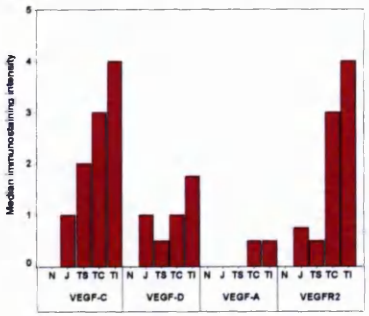
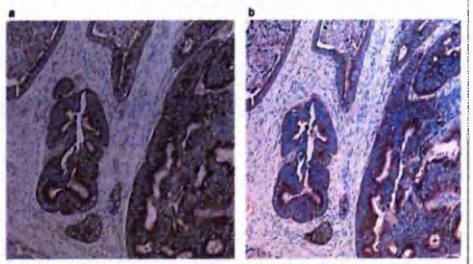


Fig 2. VEGF-A, VEGF-C, VEGF-D and VEGFR2 staining in primary tumours. N, normal mucosa, J, junctional mucosa, TS, superficial tumour, TC, central tumour, TI, invasive tumour edge



- No difference between VEGFR2 expression at TI and the corresponding lymph node metastasis was seen, but VEGFR2 expression was significantly reduced in associated liver metastases (p=0.009).
- VEGF-C and VEGFR2 were co-expressed in the same colorectal cancer cells (**Fig 3**) and expression correlated throughout the primary tumour (p-values: J, 0.03; TS, 0.028; TC, 0.001; TI, 0.019), lymph node metastases (p=0.009) but not in hepatic metastases.
- Expression of VEGF-C and VEGFR2 at the TI correlated with expression of the same antigens in the corresponding lymph node (p-values: VEGF-C, 0.009; VEGFR2, 0.02) but not liver metastases.

Fig 3. Serial sections of a moderately differentiated rectal adenocarcinoma immunostained for VEGF-C (a, chromogen DAB) and VEGFR2 (b, chromogen VIP) and counterstained with methyl green. Both antigens immunolocalise to the cytoplasm of the malignant colonic epithelial cells. Original magnification x40.



Conclusions

- Expression of VEGFR2 by colorectal cancer cells identifies potential autocrine circuits whereby tumour-produced VEGF-A, VEGF-C and VEGF-D can promote tumourigenesis.
- The correlation between VEGF-C and VEGFR2 expression throughout the primary tumour and in lymph node metastases suggests a functional pathway in colorectal cancer.
- Tumour VEGFR2 expression may account for the improved survival seen in response to bevacizumab in patients with metastatic colorectal cancer in the randomised study reported at ASCO 2003 (1).
- Anti-VEGF-A strategies may possess anti-tumour effects in addition to anti-angiogenic effects in VEGFR-expressing tumours. However, concurrent expression of other VEGFR2-activating VEGF family members may explain the limited duration of response to anti-VEGF treatment. Other VEGF family members and VEGFRs may provide additional therapeutic targets in conjunction with anti-VEGF-A treatments in colorectal cancer.
- The spatial co-expression of VEGF-C and VEGFRs in the same microenvironment should be taken into account by drug developers when developing inhibitors of VEGF.

REFERENCES

1. Hurwitz H, Fehrenbacher L, Cartwright T, Hainsworth J, Heim W, Berlin J et al. *ASCO Proceedings 2003*, Abstract 3646

2. Duff SE, Li C, Jeziorska M, Kumar S, Saunders PM, Sherlock D, O'Dwyer ST, Jayson G. Vascular endothelial growth factor C and D and lymphangiogenesis in gastrointestinal tract malignancy. *Br J of Cancer* 2003;89:426-430

Printed by Department of Medical Illustration, Christie Hospital NHS Trust, Manchester. Tel:0161 446 3305/6. Email: illustration@physics.cr.man.ac.uk