Angiogenesis in Resected Colorectal Liver Metastases

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By

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Abstract

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Aims

To assess the prognostic significance of angiogenesis using microvessel density (MVD) and angiogenesis-modulating cytokines, in a consecutive series of patients undergoing liver resection for colorectal metastases.

Methods

5μm sections from formalin-fixed, paraffin-embedded tissue blocks were immunohistochemically stained for microvessels, vascular endothelial growth factor (VEGF) thrombospondin-1 (TSP-1), thymidine phosphorylase (TP) and p53. MVD was measured using a computerised image analysis system. At the edge of the tumour, areas of highest vessel counts or hotspots, and the mean of contiguous x200 high power fields were counted. Within the tumour, hotspots and the random cumulative mean of vessel counts were analysed. The percentage expression of VEGF, TSP-1, TP and p53 was recorded. MVD and the cytokines were correlated using the χ^2 test. The Kaplan-Meier method, the log rank test, and the Cox proportional hazard model were used to correlate MVD, the cytokines and clinico-pathological variables with patient survival.

Results

182 patients, age range 25-81 (mean 61) were included.
On univariate analysis, bilobar disease ($p=0.003$), tumour edge hotspot ($p= 0.005$), lymphocytic TP ($p=0.02$), stromal TSP-1 ($p=0.01$) and perivascular TSP-1 ($p=0.03$) significantly correlated with poor prognosis.
High expression of TP correlated with high MVD ($p=0.04$ for hotspots, $p=0.001$ for contiguous vessels).
On multivariate analysis tumour edge hotspot, and bilobar disease were independent prognostic factors ($p= 0.038$, and $p= 0.04$ respectively).

Conclusion

Thymidine phosphorylase is an important cytokine associated with high tumour edge microvessel density.
Tumour edge hotspot and bilobar disease are independent prognostic markers of poor survival in patients who have undergone liver resection for colorectal liver metastases.

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I would like to thank Ken, Dave and Ashley for their support and enthusiasm from inception to completion of this project.

I particularly wish to thank Dr Louise Jones for her invaluable help with the interpretation and grading of my immunohistochemistry. Without her interest and enthusiasm this would not have been possible. Before meeting Louise I assumed immunohistochemistry merely involved looking at brown or purple dots, and now...

I would like to thank Professor Furness for his assistance with the computerised image analysis aspect of this project. One day I would like to be able to communicate on the same level as him!.

To my laboratory partner, Jonathan, thank you for saving my sanity!. I think we both learnt that there are indeed activities less interesting than watching paint dry!.

As for my experiences during counting and interpretation...I would like to ask that the controllers of Radio1 and Leicester Sound please change their play-list.

I would like to thank the staff of both Leicester and Liverpool Departments of Pathology for their teaching and assistance, especially Diana Cullen, Liza Wheatly and Wendy Prime. Further thanks extend to the Liverpool Tissue Bank for their kind tissue donation.

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Chapter 1

Introduction

1.1 Colorectal cancer

Commonly described as a disease of western society, colorectal cancer accounts for approximately 20,000 deaths per annum in the UK and 394,000 deaths per year worldwide (Scholefield, 2000). Demographically, 9.4% incidence occurs in men and 10.1% in women, with equal male to female distribution, peaking between the ages of 60 and 70 years (Boyle P and Langman J S, 2000).

The development of colorectal cancer has been associated largely with socio-economic and cultural practices, characteristic of the west. High dietary fat, low fibre and low physical activity have been shown in population studies to increase the risk of this disease (Boyle P and Langman J S, 2000; Giovannucci et al., 1996; Willett et al., 1990). Colorectal cancer is believed to arise from chromosomal and microsatellite instability through the adenoma-carcinoma cycle. Sequential tumour suppressor gene mutations (Adenomatous Polyposis Coli APC, the k-ras oncogenes, Deletion Colon Cancer DCC and p53) promote the progression of adenomas into carcinomas.

Colorectal cancer may present as rectal bleeding, altered bowel habit, and a palpable rectal or a right-sided abdominal mass associated with weight loss and iron deficiency anaemia (Hardy et al., 2000; Hobbs, 2000). Standard investigation includes sigmoidoscopy or colonoscopy and radiological imaging (for example barium enema, CT colography). Carcinomas are classified histologically by the TNM or modified Dukes' classification system (Cummings, 1994; Hardy et al., 2000). Management involves surgical and / or oncological care.

1.2 Current management of colorectal liver metastases

Colorectal cancer principally metastasises to regional lymph nodes and the liver. Up to 50% of all patients with colorectal cancer eventually develop liver involvement (Hughes et al., 1986). As many as 38% of patients that eventually die from the disease may have the liver as the only site of metastasis (Fong et al., 1997). Surgical resection of liver and lung secondaries can result in cure (Fong et al., 1997). Unfortunately, only 20-25% of patients with liver involvement are suitable for hepatic resection (Geoghegan and Scheele, 1999).

The largest published single centre series of resected colorectal liver metastases is from the Memorial Sloan-Kettering Cancer Centre (Fong et al., 1999), and the largest multicentre study is from France (Nordlinger B, 1996).These will therefore be used as the standards to compare other studies. They clearly show that there is a trend towards a more aggressive surgical approach with an increasing number of extensive resections being undertaken (Fong et al., 1999; Nordlinger B, 1996). Furthermore, repeat hepatic resections are being advocated, as recurrent colorectal cancer in the hepatic remnant can be treated effectively if all of the tumour is removed, with 5-year survival rates of between 26 and 32% for such patients being reported (Adam et al., 1997; Pinsen et al., 1996; Yamamoto et al., 1999).

The majority of patients treated with some form of liver resection eventually die due to recurrence of their disease (Geoghegan and Scheele, 1999). Therefore, there is a drive to identify those variables that predict which patients will benefit the most from liver resection. In their review of 1001 liver resections for colorectal metastases, Fong *et al* found the following factors to be of prognostic significance (Fong et al., 1999):

1.21 Operative factors

Extent of resection: those patients who had more than one lobe excised had a significantly reduced survival when compared to those that had a single liver lobe excised (5-year survival 33% and 40% respectively). This relationship remained significant even when it was adjusted for post-operative mortality (p=0.003 and 0.03 respectively) (Fong et al., 1999). Smaller, single centre studies have shown similar results (Doci R, 1991; Bolton and Fuhrman, 2000). However, Nordlinger and colleagues, in a multicenter analysis of 1568 patients, found that the extent of resection only had an impact on the post-operative mortality (p=0.04) and not on the 5- year survival (Nordlinger B, 1996).

1.22 Primary tumour characteristics

Fong *et al* found that the presence of lymph node metastases from the primary colorectal cancer an adverse predictor of outcome (Fong et al., 1999). This has been confirmed by a large number of other series including Nordlinger *et al* (Nordlinger B, 1996; Adson MA, 1984; Iwatsuki S, 1986; Butler J, 1986; Scheele J, 1990; Doci R, 1991; Minagawa et al., 2000). Only Nordlinger *et al* have demonstrated a relationship between the site of the primary tumour and survival (p=0.03), although the statistical significance was lost on multivariate analysis (Nordlinger B, 1996).

1.23 Characteristics of the liver metastases

Synchronous metastases are presumed to represent more aggressive tumour behaviour. Fong *et al,* in their univariate analysis of prognostic factors, found the period of twelve months between treatment of the primary colorectal cancer and discovery of metastases to have a direct correlation with survival. They analysed their data by categorising patients into two groups: those whose liver disease was detected < 12 months after colo-rectal resection and those whose liver disease was detected > 12 months post-resection. The former group had a significantly poorer outcome ($p=0.01$) (Fong et al., 1999). Nordlinger *et al* found that patients presenting within twenty four months with liver metastasis formed a worse prognostic group (Nordlinger B, 1996). Smaller studies, however, fail to show this association (Butler J, 1986; Foster JH., 1978; Iwatsuki S, 1986; Rees M, 1997).

Levels of carinoembryonic antigen (CEA) were found to be an important predictor of outcome by both Fong and Nordlinger, but the levels varied. Fong *et al* found that patients with a CEA level of >200 ng/ml had a significantly poorer prognosis than those who had lower levels (Fong et al., 1999), whereas in Nordlinger's series, a level above 30mg/ ml conferred a worse prognosis (Nordlinger B, 1996).

It is accepted that patients with solitary metastases have a favourable prognosis compared to those with multiple metastases (Fong et al., 1997; Fong et al., 1999; Foster JH., 1978; Minagawa et al., 2000; Nakamura S, 1999; Nordlinger B, 1996). There is agreement between Fong and Nordlinger that the size of the lesion(s)

influences prognosis, with those >5cm in diameter associated with a worse outcome. This relationship is significant under univariate and multivariate analysis (Fong et al., 1999; Nordlinger B, 1996). However, some smaller studies have failed to demonstrate this association (Butler J, 1986; Doci R, 1991; Rees M, 1997; Scheele J, 1990). There is some disagreement between Fong and Nordlinger about the prognostic significance of unilobar verses bilobar disease. Fong *et al* demonstrated a survival advantage for patients with unilobar disease but this relationship lost its significance when multivariate analysis was applied (Fong et al., 1999). Nordlinger *et al* found no such relationship (Nordlinger B, 1996).

Residual disease was the strongest predictor of recurrence in both Fong and Nordlinger's studies. This can be divided into lymph node spread and involvement at the resection margin (Fong et al., 1999; Nordlinger B, 1996). The presence of hepatic lymph node involvement is accepted as an extremely poor prognostic sign (Fong et al., 1999; Nordlinger B, 1996). In a recent review of fifteen studies that provided survival data on 145 node positive patients, only five patients were alive at five years, two of whom had recurrent disease (Rodgers M.S and McCall J.L, 2000). The majority of studies acknowledge that the resection margin is extremely important and the presence residual disease is associated with a dismal outcome (Fong et al., 1997; Fong et al., 1999; Foster JH., 1978; Nordlinger B, 1996; Rees M, 1997; Scheele J, 1990).

Much current research aims to elucidate the biological behaviour of tumours that may identify patients at risk of more aggressive disease, and may enable targeted therapy to be developed particularly for those patients not amenable for surgical resection.

The mechanisms by which tumours acquire and sustain their own blood supply, has been the focus of much research, which is reviewed below, and constitutes the main area of work in this thesis.

1.3 Definition of angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing venules, and may be a physiological or pathological process. Pathological angiogenesis occurs in diabetic retinopathy, psoriasis and rheumatoid arthritis, and also plays a major part in the growth and progression of cancer. Once a neoplasm has reached 2 to 3mm in diameter, it can no longer survive by diffusion of oxygen and waste products (Folkman, 1995). In order for a tumour to grow, invade locally, and metastasise, a blood supply must be recruited. The stages of tumour formation, angiogenesis and metastases to the liver from a primary colorectal malignancy, are summarised in Figures 1.1 and 1.2.

1.4 History of angiogenesis

One of the earliest descriptions of neovascularisation was made in the 19th century by Bilroth, who noted that in the youngest parts of tumours there were numerous fine vessels and vascular networks, whereas the older areas of tumours were associated with wider, thrombosed and atrophied vessels (Bilroth, 1871). This observation was confirmed by Goldmann and Bashford, who studied solid tumours using a combination of injection techniques and X-rays (Bashford et al., 1905; Goldman et al., 1993). Goldmann postulated that the trigger for neovascularisation originated from the tumour. This theory was supported by the work of Ide *et al.*, who inserted

fragments of epithelioma into a rabbit ear chamber, and demonstrated neovascularisation of the implanted tumours and not the controls (Ide et al., 1939).

Figure 1.1 Formation of primary colorectal tumour

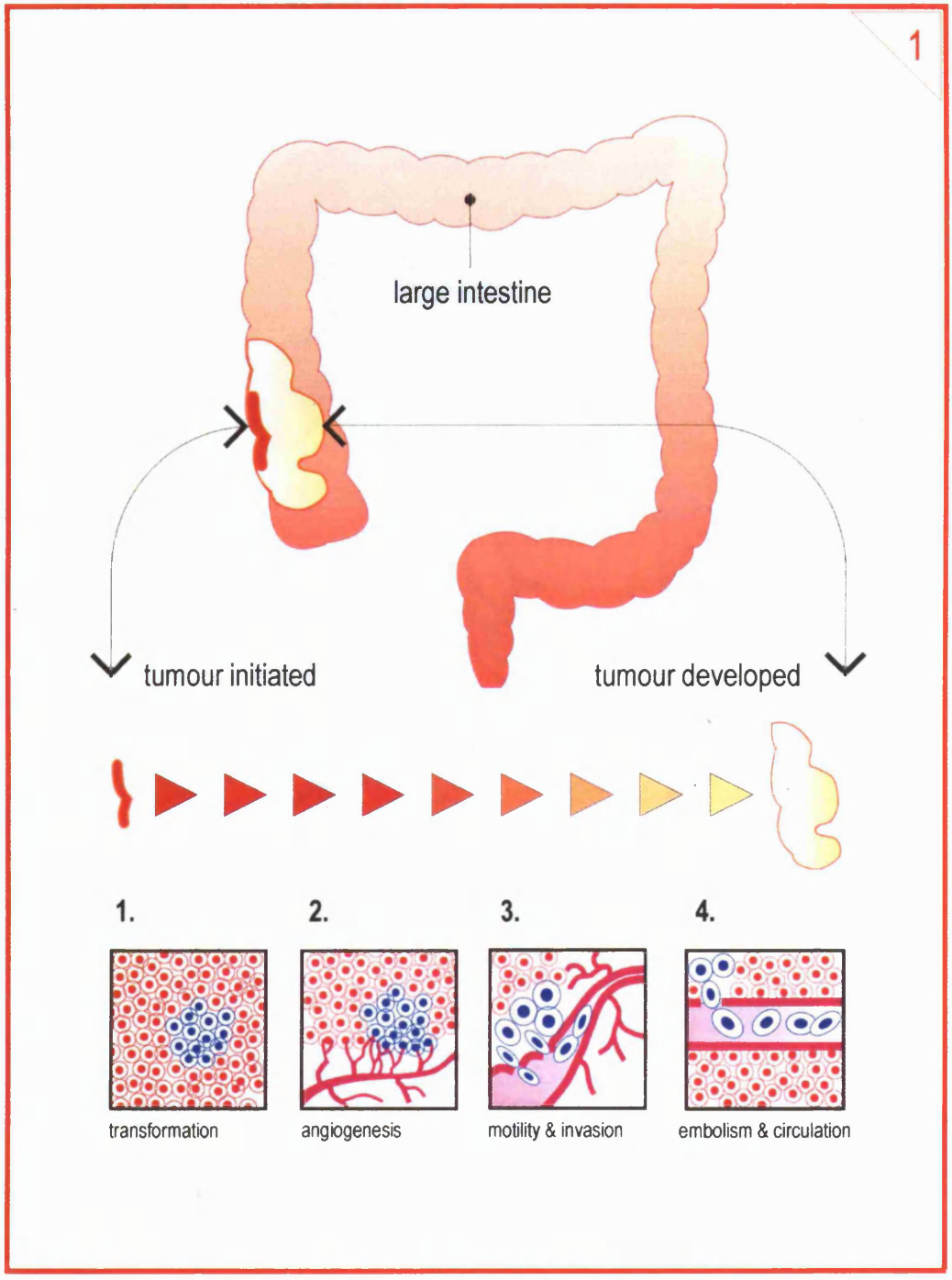
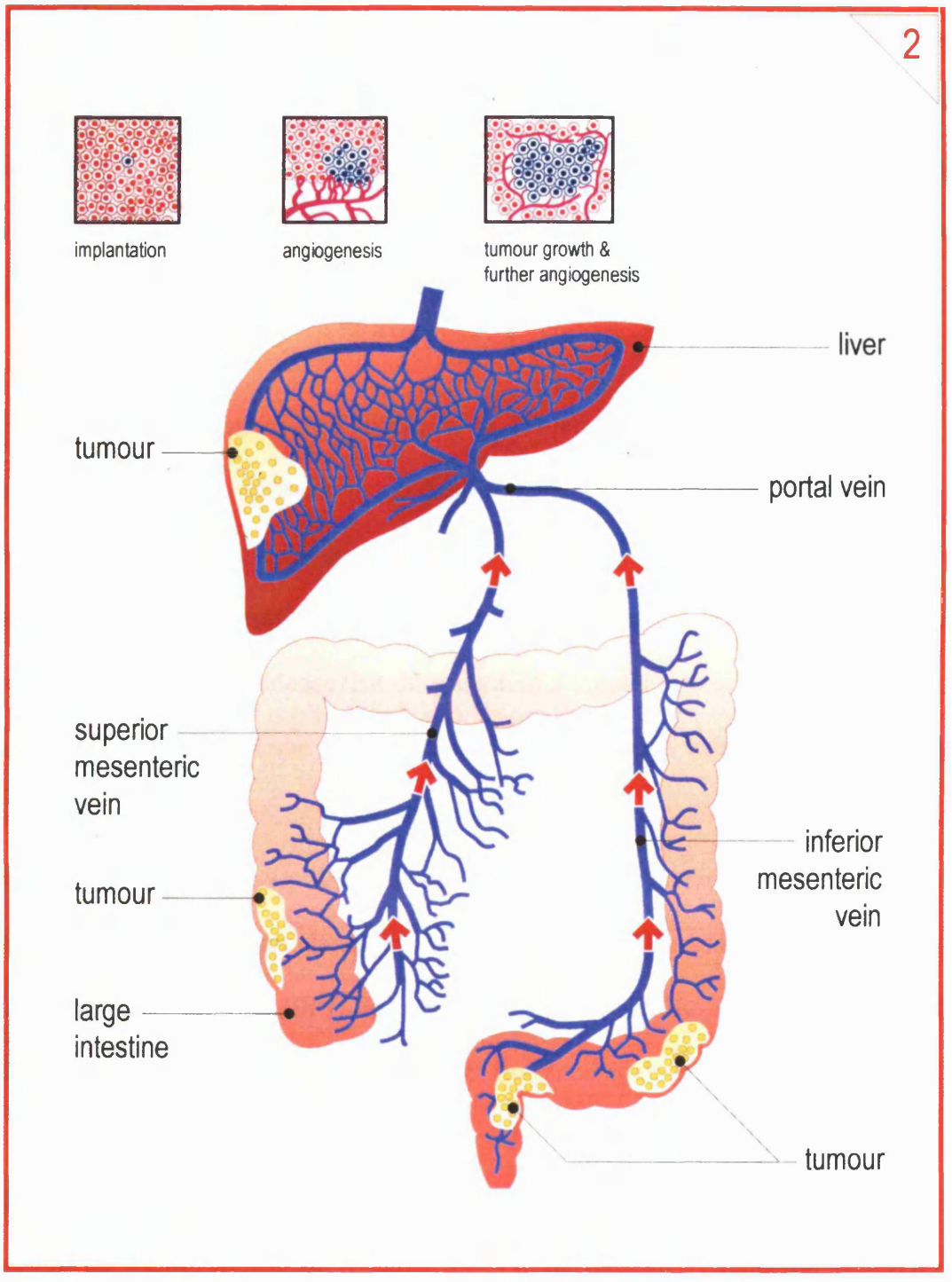


Figure 1.2 Implantation of a metastatis deposit, angiogenesis and tumour growth



In 1952, Green showed that human tumours became vascularised and grew following implantation into the anterior chamber of the guinea pig eye (Greene, 1952). Using the same anterior eye chamber model, he later demonstrated the failure of smaller fragments of tumour to gain a blood supply and grow, yet when these fragments were transplanted into a vessel-rich environment (muscle), they became vascularised and increased in size (Greene, 1952; Greene, 1961).

During the 1970s, studies by Folkman *et al*, (Folkman et al., 1966; Folkman, 1971) corroborated the above findings and further demonstrated that neovascularisation is essential in order for tumours to grow beyond 2mm in diameter, findings also described by Gimbrone (Gimbrone JR et al., 1972). Numerous other studies followed which provided clear evidence that the growth of a tumour is angiogenesis-dependent. In summary, these describe slow or absent tumour growth in avascular conditions, such as in the cornea, or the acqueous fluid of the anterior chamber of the eye, which upon implantation to a vascularised site, such as the iris undergo neovascularisation and increase in size in an almost exponential manner. (Conman and Sheldon, 1946; Folkman and Klagsbrun, 1987; Folkman, 1996; Gimbrone JR et al., 1973; Gimbrone JR et al., 1974; Conman and Sheldon, 1946; Knighton et al., 1976)

Having established its vital role in tumour growth, the process of angiogenesis became the subject of detailed research. Lysis of the basement membrane followed by endothelial migration and invasion of the extracellular matrix (ECM), culminating in the formation of new capillary networks, were carefully studied. Although Ide in 1939 (Ide et al., 1939) suggested that a "vessel growth-stimulating substance" derived from

tumours promoted the process of angiogenesis, it was not until the 1970s that research concentrated on identifying these factors, the first one identified being named "Tumour Angiogenic Factor" by Folkman and colleagues (Folkman, 1971). Since this neovascularisation was mainly detected alongside pre-existing vessels, it was believed that angiogenesis-promoting substances were capable of diffusing from tumours to host vessels, as demonstrated by the Millipore filter, the corneal pocket assay and the chorio-allantoic membrane of the embryonated chicken egg assay (Reviewed in (Folkman, 1971; Weiss, 2000))

Upon identifying factors promoting angiogenesis, Folkman postulated the existence of anti-angiogenic factors, thus proposing that angiogenesis could become the target for anti-cancer agents (Folkman, 1971). The first, naturally occurring anti-angiogenic agents were found in cartilage (cartilage-derived inhibitor, CDI) (Folkman, 1971; Moses et al., 1990). Numerous factors have since been identified. It was then suggested that the angiogenic process must depend on a balance between pro and anti-angiogenic factors (Folkman, 1971; Iruela-Arispe and Dvorak, 1997; Pepper et al., 1992).

The role of signalling pathways and intercellular interactions through a variety of surface molecules is at the forefront of current-day research, with the particular aim of identifying prognostic factors for the progression of neoplastic disease.

1.5 Stages and measurement of angiogenesis

Angiogenesis is a multistep process controlled by a balance of promoter and inhibitor substances. For neovascularisation to occur there must be increased expression of pro-angiogenic factors and concurrent down-regulation of anti-angiogenic factors that normally hold the vessels in a quiescent state (Bouck et al., 1996).

Neovessel buds arise from venules, the endothelial cells moving towards the angiogenic stimulus by proliferation, elongation and migration. The basement membrane must be broken down and the surrounding extracellular matrix altered by proteolytic digestion to allow this progression. The vessels sprout and develop a lumen, two of which form a capillary loop allowing blood to flow (Folkman and Klagsbrun, 1987). Angiogenic factors may be produced by the tumour cells, be present in the extracellular matrix or secreted by recruited cells such as macrophages (Polverini and Leibovich, 1984; Folkman, 1995). Alternatively, external stimuli, such as hypoxia, may cause inflammatory and tumour cells to generate these angiogenic factors (Plate et al., 1994; Shweiki et al., 1992).

Since then, angiogenesis has been quantified by two principal methods; firstly, by the direct identification and counting of microvessels or microvessel density (MVD), and secondly, by the measurement of pro-angiogenic and anti-angiogenic factors.

1.6 Microvessel Density

There are in excess of 500 published papers that document the use of MVD as a measure of angiogenesis in solid tumours. Many of these published reports have

related angiogenesis to tumour prognosis, such as in breast, (Fox et al., 1995) lung, (Cox et al., 2000; Fontanini et al., 1995), thyroid (Fontanini et al., 1996) prostate (Weidner et al., 1993) and bladder cancer (Dickinson et al., 1994).

Brem *et al* was the first to develop a method for measuring angiogenesis in tumours and relating the results to prognosis (Brem et al., 1972). The method was complicated, requiring measurement of several variables (one of which was the number of microvessels) to produce an angiogenesis score. Mlynek developed a laborious system in order to quantify the vascularity of colorectal cancer. Using photomicrographs of all stained tissue, he manually counted and measured all vessels (Mlynek et al., 1985). Weidner's method was simpler, estimating the density of microvessels in subjectively chosen areas of obvious high vessel density or hotspots (Weidner et al., 1991). This method has been widely used since then.

1.61 Microvessel density in colorectal cancer

Seventy papers up to April 2003, describe a method of determining MVD in human colorectal tumours, an additional four papers studied only colorectal liver metastases (Davies et al., 2000; Hillen et al., 1997; Nanashima et al., 2001; Terayama et al., 1996). All 74 papers are summarised in (Table 1.1).

Fifty-three papers stated the area of tissue that had been biopsied for subsequent analysis, the commonest area being the tumour edge (n=42). Five papers took biopsies from the main tumour mass, tru-cuts were used for two studies on hepatic metastases, and one study took central biopsies. Two papers analysed multiple biopsies from the tumour edge and the centre. All papers used immunohistochemistry to identify vessels. The most commonly used antibodies against pan-endothelial markers were

Anti-factor VIII (n=32), Anti-Cluster Determinant 34 [CD 34] (n=21) and Anti-Cluster Determinant 31 [CD 31] (n=20). One study used Fuschin B.

The 'hotspot' technique, namely, counts in the most vascular area of the tumour biopsy, was the commonest technique used (n=64). Sixty one of these papers counted the number of microvessels in a single high power field, and three papers used more than one contiguous field (3, 5 and 4 contiguous fields respectively) (Gallego MG et al., 2000; Kern et al., 2002; van Triest et al., 2000). The exact number of hotspots counted were not the same for all the papers: 1 hotspot n=10, 2 hotspots n=2, 3 hotspots n=18, 4 hotspots n=4, 5 hotspots n=14, 10 hotspots n=2, all hotspots n=4.

Eight papers counted more than 1 hotspot but failed to state how many in total, one paper counted more than 10, and one paper counted between 1 and 6 hotspots.

Two papers counted all the vessels in the study biopsy. Four papers used random sampling. Two papers counted vessels at the tumour edge, and a further two fail to say exactly where the counts were performed. Twelve papers used additional methods to assist counting: three used a Chalkley graticule, three used an optical grid, and six used computerised image analysis.

The magnification used to perform the microvessel counts ranged between x100 and x 500: x100 n=4, x250 n=5, x400 n=12, and x500 n=1. The most commonly used magnification was x200 (n=49), and four papers failed to state the magnification used.

Thirty-seven papers stated the field size used, ranging from $0.09mm^2$ to $0.9mm^2$. Half of these studies used $0.74mm^2$.

The final expression of MVD also varied between the research groups. 17 papers used the maximum count as MVD, 53 papers used the mean of several areas, and 4 papers did not state how they calculated MVD.

Table 1.1 Summary of studies measuring MVD in colorectal cancer

Paper	N°	Bx Site	Stain	Counting Method	*Mag*	Field (mm^2)	MVD Value
Abdalla (Abdalla et al., 1999)	111	Edge	CD 31	5 Hotspots (Chalkley)	400	ns	mean
Akagi (Akagi et al., 2002)	20	NS	CD 34	3 Hotspots	200	0.739	mean
Akagi (Akagi K et al., 2000)	40	NS	CD 34	3 Hotspots	200	0.739	mean
Amaya (Amaya et al., 1997)	136	NS	CD 34	5 Hotspots	200	ns	mean
Aotake (Aotake et al., 1999)	26	Edge	CD 34	5 Hotspots	200	0.74	mean
Banner (Banner et al., 1998)	22	Edge	VIII	>10 Hotspots	400	ns	mean
Bhatavdekar (Bhatavdekar et al., 1998)	98	NS	VIII	3 Hotspots	400	ns	mean
Bossi (Bossi et al., 1995)	178	Edge	CD 31	3 Hotspots	200	0.9	mean
Choi (Choi et al., 1998)	127	Edge	VIII	3 Hotspots	200	0.785	mean
Cianchi (Cianchi et al., 2002)	84	NS	CD 31	>1 Hotspots	250	ns	max
Davies (Davies et al., 2000)	12	Tru-cut	CD 31	40 Rnd areas (Chalkley)	200	0.42	mean
Engel (Engel et al., 1996)	35	Edge	CD 31	3 Hotspots	400	0.152	max
Etoh (Etoh T et al., 2000)	65	Edge	VIII	>1 Hotspots	200	0.785	ns
Fox (Fox et al., 1998)	36	Edge	VIII	4 Hotspots (0.5mm grid)	200	ns	mean
Frank (Frank et al., 1995)	105	NS	VIII	3 Hotspots (x100 grid)	100	ns	mean
Fukushima (Fukushima et al., 1998)	49	NS	VIII	5 undescribed areas	200	ns	mean
Gallego (Gallego MG et al., 2000)	126	NS	CD 34	1 Hotspot (3 fields)	200	0.74	max
Giatromanolaki (Giatromanolaki et al., 1999)	106	NS	CD 31	3 Hotspots	250	ns	mean
Harada (Harada Y et al., 2001)	259	Edge	VIII	3 Hotspots	Ns	ns	mean
Haraguchi (Haraguchi et al., 2002)	35	Edge	VIII	5 Hotspots	200	0.739	mean
Hasebe (Hasebe et al., 2001)	157	Main lesion	CD 31	>1	200	0.384	max
Hillen (Hillen et al., 1997)	18	Tru-cut	CD 34	18 Rnd areas	200	0.22	mean
Kaklamanis (Kaklamanis et al., 2000)	47	Edge	CD 31	3 Hotspots (Chalkley)	250	0.155	mean
Kang (Kang et al., 1997)	163	Edge	VIII	5 Hotspots	200	0.785	mean
Kawakami (Kawakami T et al., 2001)	53	Edge	CD 34	2 Hotspots (Automated)	200	0.739	mean
Kawasaki (Kawasaki et al., 2001)	60	Edge	CD 34	5 Rnd areas	200	0.74	mean
Kern (Kern et al., 2002)	11	NS	CD 34	2 Hotspots (5 fields) Automated	200	0.25	mean
Kim (Kim and Kim, 1999)	72	Edge	CD 34	>1 Hotspot	200	0.74	mean
Kondo (Kondo et al., 2000)	46	Edge	CD 31	3 Hotspots	200	0.739	mean
Konerding (Konerding et al., 2001)	46	Multiple	VIII	1-6 Hotspots (Automated)	ns	ns	ns
Konno (Konno et al., 2001)	71	Edge	VIII	4 undescribed areas	500	ns	mean
Losi (Losi et al., 1995)	116	NS	VIII	20 undescribed areas	400	ns	mean
Maeda (Maeda et al., 2000)	100	NS	VIII	5 Hotspots	200	0.785	mean
Maeda (Maeda K et al., 2001)	150	NS	VIII	5 Hotspots	200	0.785	mean
Masunaga (Masunaga et al., 2000)	100	Edge	CD 34	3 Hotspots	200	ns	mean
Matsumura (Matsumura et al., 1998)	148	NS	CD 34	5 Hotspots	200	ns	mean
Matsuura (Matsuura T et al., 2000)	32	Main lesion	CD 34	10 Hotspots	200	ns	mean
Matsuura (Matsuura et al., 1999)	64	Edge	CD 34	10 Hotspots	200	ns	mean
Mattern (Mattern et al., 1996)	15	NS	VIII	10 Rnd areas	400	0.152	mean
Mlynek (Mlynek et al., 1985)	10	Edge	Fuschin B	All vessels	100	0.09	mean
Mooteri (Mooteri et al., 1996)	32	Viable tumour	VIII	3 Hotspots (1cm grid) plus eyeball	ns	ns	mean

Nanashima (Nanashima et al., 1998)	68	Centre & Edge	CD 34	5 Hotspots	200	ns	mean
Nanashima (Nanashima et al., 2001)	62	Edge	CD 34	5 Hotspots	200	ns	mean
Ogura (Ogura et al., 2001)	46	Main lesion	VIII	4 Hotspots	400	ns	mean
Onodera (Onodera et al., 2000)	51	Edge	CD 31	3 Hotspots	200	ns	mean
Pavlopoulos (Pavlopoulos et al., 1998)	90	Main lesion	VIII	1 Hotspot (Automated)	200	ns	max
Saclarides (Saclarides et al., 1994)	48	Edge	VIII	3 areas at edge	200	ns	mean
Saeki (Saeki et al., 1997)	80	Edge	VIII	3 Hotspots	100	ns	mean
Saito (Saito et al., 2000)	86	Edge	CD 31	3 Hotspots	200	ns	NS
Shomori (Shomori et al., 1999)	97	Edge	CD 34	All Hotspots	100	ns	mean
Sternfeld (Sternfeld et al., 1999)	146	NS	CD 31	4 Hotspots	200	0.781	max
Tabara (Tabara et al., 2001)	60	Edge	CD 34	5 Hotspots	200	0.74	mean
Takahashi (Takahashi et al., 1997)	27	Edge	VIII	1 Hotspot	200	0.739	max
Takahashi (Takahashi et al., 1995)	52	Edge	VIII	1 Hotspot	200	0.739	max
Takahashi (Takahashi et al., 1998)	78	Edge	VIII	1 Hotspot	200	0.739	max
Takahashi (Takahashi et al., 1996)	96	Edge	VIII	1 Hotspot	200	0.739	max
Takebayashi (Takebayashi et al., 1995)	21	NS	VIII	>1 Hotspot	400	ns	ns
Takebayashi (Takebayashi et al., 1996)	163	Edge	VIII	>1 Hotspot	400	ns	ns
Takebayashi (Takebayashi et al., 1996)	166	NS	VIII	1 Hotspot	400	ns	max
Terayama (Terayama et al., 1996)	13	Edge	VIII	10 areas at edge	200	ns	mean
Tien (Tien et al., 2001)	58	Main lesion	CD 31	3 Hotspots	200	ns	mean
Tokunaga (Tokunaga et al., 1999)	61	Edge	CD 34	1 Hotspot (Automated)	200	0.739	max
Tomisaki (Tomisaki et al., 1996)	175	Edge	VIII	5 Hotspots	200	0.739	mean
Tsuchida (Tsuchida T et al., 1999)	65	Centre	CD 34	>1 Hotspot (Automated)	200	0.739	mean
van Halteren (van Halteren et al., 2001)	87	Edge	VIII	1 Hotspot	200	ns	max
van Triest (van Triest et al., 2000)	32	NS	CD 31	1 Hotspot (4 fields)	400	ns	max
Vermeulen (Vermeulen et al., 1997)	16	Edge	CD 31	All vessels	400	ns	mean
Vermeulen (Vermeulen et al., 1995)	21	Edge	CD 31	All Hotspots	250	0.4	max
Vermeulen (Vermeulen et al., 1995)	34	Edge	CD 31	All Hotspots	250	0.4	max
Vermeulen (Vermeulen et al., 1996)	42	Edge	CD 31	All Hotspots	200	ns	max
Vermeulen (Vermeulen et al., 1999)	145	Edge	CD 31	5 Hotspots	200	0.61	mean
Wynter (Wynter et al., 1999)	120	NS	CD 31	3 Hotspots	Ns	ns	mean
Yammamura (Yamamura et al., 2001)	171	NS	CD 34	>1 Hotspots	200	ns	mean
Yang (Yang Y et al., 2001)	30	Edge	VIII	4 Hotspots	200	0.739	mean
Yoshimura (Yoshimura et al., 2000)	60	NS	CD 34	5 Hotspots	200	0.298	mean

Ten papers have examined both adenomas and carcinomas. Nine of these have examined the relationship between MVD in adenomas and carcinomas. The results are summarised in Table 1.2.

As can be seen despite differing methodologies all studies found that carcinomas had significantly higher microvessel counts than adenomas. Four of these papers also examined the relationship of dysplasia and MVD. Three found that the more dysplastic the adenoma the higher the vessel counts whilst one failed to demonstrate such a relationship.

Microvessel density has been compared with a variety of clinicopathological factors in colorectal cancers. Twenty-eight papers have examined the relationship between MVD and survival and/or recurrence, shown in Table 1.3.

As can be seen, the results are not uniform. In terms of survival, 16 (of 27) studies found a significant relationship between MVD and survival whilst 11 studies did not. Eighteen papers studied recurrence, only half of which established a significant relationship between MVD and disease recurrence.

Table 1.2. MVD in adenomas and carcinomas

Paper	Adenomas	Carcinomas	Stain	Counting Method	Magnification	Field (mm^2)	MVD Value	Dysplasia	MVD and the adenoma carcinoma sequence
Akagi (Akagi et al., 2002)	54	20	CD 105	3 Hotspots	200	0.739	mean	Sig*	MVD from adenoma to carcinoma
Aotake (Aotake et al., 1999)	44	26	CD 34	5 Hotspots or all	200	0.74	mean	Sig	MVD from adenoma to carcinoma
Bossi (Bossi et al., 1995)	36	178	CD 31	3 Areas	200	0.9	mean	Not Sig	MVD from adenoma to carcinoma
Kaklamanis (Kaklamanis et al., 2000)	35	47	CD 31	3 Areas with Chalkley	250	0.155	mean	Nd**	MVD from adenoma to carcinoma
Kawasaki (Kawasaki et al., 2001)	213	60	CD 34	5 Rnd areas or All	200	0.74	mean	Sig	MVD from adenoma to carcinoma
Kondo (Kondo et al., 2000)	27	46	CD 31	3 Hotspots	200	0.739	mean	Nd	MVD from adenoma to carcinoma
Pavlopoulos (Pavlopoulos et al., 1998)	13	90	VIII	1 Hotspot Automated	200	ns	max	Nd	MVD from adenoma to carcinoma
Tabara (Tabara et al., 2001)	20	60	CD 34	5 Hotspots	200	0.74	mean	Nd	MVD from adenoma to carcinoma
Takahashi (Takahashi et al., 1995)	10	52	VIII	1 Hotspot	200	0.739	max	Nd	Not done
Takahashi (Takahashi et al., 1998)	15	78	VIII	1 Hotspot	200	0.739	max	Nd	MVD from adenoma to carcinoma

*Sig=significant

**Nd = not determined

Table 1.3. MVD, tumour recurrence and patient survival

Paper	N°	Site	Stain	Counting Method	Mag	Field (mm^2)	MVD	Treatment of Data	Survival	Recurrence
(Abdalla et al., 1999)	111	Edge	CD 31	5 Hotspots	400	ns	mean	Outcome groups quartiles and > or < 4.2 (median)	Sig Uni & Multi	Nd
(Amaya et al., 1997)	136	NS*	CD 34	5 Hotspots	200	ns	mean	Split into low & high MVD Around Median	Sig Uni & Multi	Sig Uni & Multi
(Banner et al., 1998)	22	Edge	VIII	>10 Hotspots	400	ns	mean	Outcome groups	Not sig	Not sig
(Bhatavdekar et al., 1998)	98	NS	VIII	3 Hotspots	400	ns	mean	Outcome groups plus Split Around Median	Sig Uni**	Nd
(Bossi et al., 1995)	178	Edge	CD 31	3 Hotspots	200	0.9	mean	Outcome groups	Not sig	Not sig
(Choi et al., 1998)	127	Edge	VIII	3 Hotspots	200	0.785	mean	Outcome groups plus Split Around Mean	Sig Uni & Multi	Sig uni
(Cianchi et al., 2002)	84	NS	CD 31	>1 Hotspots	250	ns	max	Split into low & high MVD Around Median	Not sig	Not sig
(Engel et al., 1996)	35	Edge	CD 31	3 Hotspots	400	0.152	max	Outcome groups Split > or < 65MVD (Split analysis)	Sig Uni	Sig Uni
(Fox et al., 1998)	36	Edge	VIII	4 Hotspots	200	ns	mean	Outcome groups	Not sig	Nd
(Frank et al., 1995)	105	NS	VIII	3 Hotspots	100	ns	mean	Outcome groups	Sig Uni	Sig Uni
(Gallego MG et al., 2000)	126	NS	CD 34	1 Hotspot (3 fields)	200	0.74	max	Outcome groups	Sig uni	Sig Uni
(Giatromanolaki et al., 1999)	106	NS	CD 31	3 Hotspots	250	ns	mean	3 groups around 33% and 66% percentiles	Sig Duke C	Not sig
(Hasebe et al., 2001)	157	Main lesion	CD 31	Multiple Hotspots	200	0.384	max	Outcome groups	Not sig	Not sig
(Masunaga et al., 2000)	100	Edge	CD 34	3 Hotspots	200	ns	mean	MVD> or <100 do not say why 100	Not sig	Not sig
(Matsumura et al., 1998)	148	NS	CD 34	5 Hotspots	200	ns	mean	MVD> or < 104 don't say why 104	Sig Multi***	Nd
(Mooteri et al., 1996)	32	Viable tumour	VIII	3 Hotspots & area	ns	ns	mean	Outcome groups	Sig Uni	Nd
(Nanashima et al., 1998)	68	Centre & Edge	CD 34	5 Hotspots	200	ns	mean	Outcome groups multiple plus Around Mean	Sig uni	Not sig
(Onodera et al., 2000)	51	Edge	CD 31	3 Hotspots	200	ns	mean	Outcome groups	Not sig	Nd
(Pavlopoulos et al., 1998)	90	Main lesion	VIII	1 Hotspot & area	200	ns	max	Outcome groups	Not sig	Nd
(Saclarides et al., 1994)	48	Edge	VIII	3 Areas At edge	200	ns	mean	Outcome groups	Sig uni	Nd
(Saito et al., 2000)	86	Edge	CD 31	3 Hotspots	200	ns	NS	MVD > or <37 do not say why 37	Not sig	Nd
(Sternfeld et al., 1999)	146	NS	CD 31	4 Hotspots	200	0.781	max	Split into low & high MVD Around Median	Sig Uni & Multi	Sig Uni & Multi
(Takahashi et al., 1997)	27	Edge	VIII	1 Hotspots	200	0.739	max	Outcome groups plus > or < 25 (Split pt analysis)	Nd	Sig Uni & Multi
(Takebayashi et al., 1996)	163	Edge	VIII	>1 Hotspots	400	ns	ns	Outcome groups	Sig Multi	Nd
(Takebayashi et al., 1996)	166	NS	VIII	1 Hotspots	400	ns	max	Outcome groups plus Split Around Median	Sig Uni & Multi	Sig Uni & Multi
(Tomisaki et al., 1996)	175	Edge	VIII	5 Hotspots	200	0.739	mean	Outcome groups plus Split Around Mean	Not sig	Not sig
(Vermeulen et al., 1999)	145	Edge	CD 31	5 Hotspots	200	0.61	mean	Outcome groups plus Split Around Median	Sig Uni & Multi	Sig uni
(Yoshimura et al., 2000)	60	NS	CD 34	5 Hotspots	200	0.298	mean	Split into low & high MVD do not say how	Not sig	Not sig

*NS=not significant **Sig uni=significant on univariate analysis ***Sig multi=significant on multivariate analysis

Twenty-three papers examined the relationship between MVD and Dukes' stage and/or differentiation, shown in Table 1.4.

Table 1.4. MVD and Dukes' stage

Paper	N°	Bx Site	Stain	Counting Method	Mag	Field (mm^2)	MVD Value	Dukes' Stage	Dysplasia
(Aotake et al., 1999)	26	Edge	CD 34	5 Hotspots	200	0.74	mean	NA	Sig uni
(Banner et al., 1998)	22	Edge	VIII	>10 Hotspots	400	ns	mean	Nd	Not sig
(Choi et al., 1998)	127	Edge	VIII	3 Hotspots	200	0.785	mean	Sig diff	Sig uni
(Cianchi et al., 2002)	84	NS	CD 31	>1 Hotspots	250	ns	max	Nd	Not sig
(Engel et al., 1996)	35	Edge	CD 31	3 Hotspots	400	0.152	max	Not sig	Not sig
(Gallego MG et al., 2000)	126	NS	CD 34	1 Hotspot (3 fields)	200	0.74	max	Not sig	Sig uni
(Giatromanolaki et al., 1999)	106	NS	CD 31	3 Hotspots	250	ns	mean	Not sig	Not sig
(Haraguchi et al., 2002)	35	Edge	VIII	5 Hotspots	200	0.739	mean	Sig diff	Nd
(Kawasaki et al., 2001)	60	Edge	CD 34	5 Rnd areas	200	0.74	mean	Nd	Sig uni
(Kim and Kim, 1999)	72	Edge	CD 34	>1 Hotspots	200	0.74	mean	Not sig	Sig uni
(Nanashima et al., 1998)	68	Centre & Edge	CD 34	5 Hotspots	200	ns	mean	Not sig	Not sig
(Pavlopoulos et al., 1998)	90	Main lesion	VIII	1 Hotspot & area	200	ns	max	sig diff	Nd
(Shomori et al., 1999)	97	Edge	CD 34	all Hotspots	100	ns	mean	Sig diff	Nd
(Sternfeld et al., 1999)	146	NS	CD 31	4 Hotspots	200	0.781	max	Not sig	Not sig
(Tabara et al., 2001)	60	Edge	CD 34	5 Hotspots	200	0.74	mean	Sig diff	Nd
(Takahashi et al., 1996)	96	Edge	VIII	1 Hotspot	200	0.739	max	Sig diff	Nd
(Takebayashi et al., 1996)	166	NS	VIII	1 Hotspot	400	ns	max	Sig diff	Not sig
(Tien et al., 2001)	58	Main lesion	CD 31	3 Hotspot	200	ns	mean	Sig diff	Not sig
(Tomisaki et al., 1996)	175	Edge	VIII	5 Hotspots	200	0.739	mean	Sig diff	Not sig
(van Triest et al., 2000)	32	NS	CD 31	1 Hotspot (4 fields)	400	ns	max	Not sig	Not sig
(Vermeulen et al., 1996)	42	Edge	CD 31	all Hotspots	200	ns	max	Not sig	Not sig
(Vermeulen et al., 1999)	145	Edge	CD 31	5 Hotspots	200	0.61	mean	Not sig	Not sig
(Yoshimura et al., 2000)	60	NS	CD 34	5 Hotspots	200	0.298	mean	NA	Not sig

Eighteen papers studied Dukes' stage. Half demonstrated a significant difference between MVD and stage. Eighteen papers examined the influence of differentiation on MVD: five showed a significant difference between MVD and differing tumour grades, whilst thirteen did not.

Twenty-three studies investigated the difference in MVD between metastatic and non-metastatic tumours (Table 1.5). Sixteen papers examined lymphatic metastases alone, a significant relationship being described in 7 studies, with the remaining nine not reporting any association. Fourteen papers looked at haematogenous metastases, with eight showing a significant relationship, and six failing to do so. Three studies analysed both lymphatic and haematogenous metastases as a single group; two found a significant relationship, and one did not.

Less work has been published on MVD and colorectal liver metastases (Table 1.6). However, only three out of the eight publications relate MVD to survival. Nanashima *et al* in two separate papers demonstrated that tumours with higher MVD had significantly lower survival rates (Nanashima et al., 2001; Nanashima et al., 1998). In contrast, Mooteri *et al* found that metastases with higher MVD had better prognosis (Mooteri et al., 1996).

The majority of published work has focused on colorectal carcinomas rather than adenomas and/or liver metastases, therefore those papers relating to clinicopathological variables and MVD in colorectal tumours will be discussed first, followed by an appraisal of the studies on MVD and adenomas and liver metastases.

Table 1.5. MVD in metastatic and non-metastatic colorectal cancer

Paper	N°	Site	Stain	Counting Method	Mag	Field (mm^2)	MVD Value	Treatment of Data (OG=outcome group)	Lymph Mets	Haem Mets
(Bossi et al., 1995)	178	Edge	CD 31	3 Hotspots	200	0.9	mean	OG	Not sig	Not Sig
(Choi et al., 1998)	127	Edge	VIII	3 Hotspots	200	0.785	mean	OG plus Split MVD Around Mean	Sig diff	Sig diff
(Engel et al., 1996)	35	Edge	CD 31	3 Hotspots	400	0.152	max	OG plus Split > or < 65 MVD	Not sig	Nd
(Gallego MG et al., 2000)	126	NS	CD 34	1 Hotspot	200	0.74	max	OG	Not sig	Nd
(Giatromanolaki et al., 1999)	106	NS	CD 31	3 Hotspots	250	ns	mean	3 groups around 33% and 66% percentiles	Nd	Not Sig
(Hasebe et al., 2001)	157	Main lesion	CD 31	Multiple Hotspots	200	0.384	max	OG	Sig diff	Not Sig
(Kim and Kim, 1999)	72	Edge	CD 34	>1 Hotspots	200	0.74	mean	OG	Sig diff grouped together	
(Konerding et al., 2001)	46	Multiple	VIII & Casts	1 to 6 Hotspots	Ns	ns	Ns	OG	Sig diff grouped together	
(Konno et al., 2001)	71	Edge	VIII	4 areas (?)	500	ns	mean	Split into 2 gps > and <35 do not say why	Nd	Not Sig
(Nanashima et al., 1998)	68	Centre & edge	CD 34	5 Hotspots	200	ns	mean	OG plus split Around Mean	Not sig	Sig diff
(Ogura et al., 2001)	46	Main lesion	VIII	4 Hotspots	400	ns	mean	OG	Sig diff	Not Sig
(Saclarides et al., 1994)	48	Edge	VIII	3 areas at edge	200	ns	mean	OG	Not sig grouped together	
(Saito et al., 2000)	86	Edge	CD 31	3 Hotspots	200	ns	NS	OG MVD > or <37 (mean in normal colon)	Not sig	Nd
(Sternfeld et al., 1999)	146	NS	CD 31	4 Hotspots	200	0.781	max	Split into low & high MVD Around Median	Not sig	Nd
(Takahashi et al., 1995)	52	Edge	VIII	1 Hotspots	200	0.739	max	OG	Sig diff	Nd
(Takahashi et al., 1997)	27	Edge	VIII	1 Hotspots	200	0.739	max	OG plus > or < 25 (Split pt analysis)	Sig diff	Sig diff
(Takebayashi et al., 1996)	166	NS	VIII	1 Hotspots	400	ns	max	OG plus Split Around Median	Sig diff	Sig diff
(Tien et al., 2001)	58	Main lesion	CD 31	3 Hotspots	200	ns	mean	OG	Sig diff	Sig diff
(Tomisaki et al., 1996)	175	Edge	VIII	5 Hotspots	200	0.739	mean	OG Split Around Mean	Not sig	Sig diff
(van Halteren et al., 2001)	87	Edge	VIII	1 Hotspots	200	ns	max	MVD <55, 55-68 & >68 and met +ve or -ve	Nd	Not Sig
(Vermeulen et al., 1999)	145	Edge	CD 31	5 Hotspots	200	0.61	mean	OG plus Split Around Median	Not sig	Sig diff
(Yamamura et al., 2001)	171	NS	CD 34	>1 Hotspots	200	ns	mean	OG	Nd	Sig diff
(Yoshimura et al., 2000)	60	NS	CD 34	5 Hotspots	200	0.298	mean	Split into low & high MVD gps unspecified	Not sig	Nd

Table 1.6. MVD in liver metastases

Paper	N°	Bx Site	Stain	Counting Method	Mag	Field (mm^2)	Expression	MVD
(Davies et al., 2000)	12	Tru-cuts	CD 31	40 Rnd areas using Chalkley Graticule	200	0.42	mm2	mean
(Hillen et al., 1997)	24	Tru-cuts	CD 34	18 Rnd areas or whole Bx	200	0.22	mm2	mean
(Kern et al., 2002)	11	NS	CD 34	2 hottest spots 5 areas from each using image analysis	200	0.25	mm^2	mean
(Mooteri et al., 1996)	53	Viable tumour	VIII	The 3 hottest spots plus subjectve 'eye-ball'	ns	ns	hpf	mean
(Nanashima et al., 1998)	44	Centre & Edge	CD 34	5 hottest spots in edge and centre respectively	200	ns	hpf	mean
(Nanashima et al., 2001)	62	Edge	CD 34	5 hottest spots in edge	200	ns	hpf	mean
(Terayama et al., 1996)	13	Edge	VIII	10 areas at tumour edge	200	ns	mm2	mean
(Yamamura et al., 2001)	9	NS	CD 34	>1 hotspot. Total not stated	200	ns	hpf	mean

In order to measure the microvessel density of a colorectal neoplasm, several methodological steps must be followed. A tumour area is selected for assessment, the microvessels are identified, a counting method is selected, the count performed, and the data expressed. As can be seen from Tables 1.3, 1.4 and 1.5, there is discordance between the studies in terms of the relationship between MVD and survival, recurrence, Dukes' stage, differentiation, and the development of metastases. The discrepancies can largely be explained on methodological grounds.

1.62.a Microcirculation of colorectal neoplasms and biopsy selection

Detailed studies of the microcirculation of both normal and cancerous large intestine have been described in work from Skinner *et al,* and Kondering *et al* (Konerding et al., 2001; Skinner et al., 1995), using similar techniques. Fresh, surgically resected specimens were flushed with saline and injected with a methacrylate-based acrylic resin. Following polymerisation and fixation, corrosion casts were prepared, scanned by electron microscopy and analysed. Skinner *et al* used manual measuring techniques (Skinner et al., 1995), whereas Kondering *et al* employed a computerised image analysis system (Konerding et al., 2001). Both papers describe a standard arrangement of the microvasculature of normal colon and rectum; that of a regular honeycomb pattern around the mucosal glands (Konerding et al., 2001; Skinner et al., 1995). There was agreement between the two papers that the vessel density was higher in carcinomas than in normal colon, and multiple abnormal branching vessels were observed in the tumours (Konerding et al., 2001; Skinner et al., 1995). Kodering et al observed heterogeneity of vascular density throughout the carcinomas, with significantly higher vascular densities at the tumour periphery.

The conventional view of MVD assessment is that areas within the tumour tissue that express high levels of neovascularisation should be evaluated. For most primary tumours, areas of intense angiogenesis may occur anywhere within the tumour substance but are most commonly located at the tumour periphery (Fanelli et al., 1999; Vermeulen et al., 1996; Weidner et al., 1991; Konerding et al., 2001). It would thus seem logical to choose biopsy specimens from the tumour edge. As can be seen from Tables 1.3, 1.4 and 1.5, the majority of studies did in fact sample the tumour edge, therefore, this alone does not explain the difference in outcomes detailed in the tables.

1.62.b Immunohistochemical detection of microvessels

Microvessel identification can be achieved by using immunohistochemical techniques to stain endothelial cells. Pan-endothelial cell markers are the most commonly used antibodies in formalin-fixed paraffin sections from human malignancy (Vermeulen et al., 1996). In colorectal carcinoma, four antibodies against such pan-endothelial markers have been used: Anti-factor VIII, Anti-Cluster Determinant 31 (CD 31), Anti -Cluster Determinant 34 (CD 34) and Anti-Ulex europaeus agglutinin 1 (UEA-1).

Factor VIII or von Willebrand's factor is involved in platelet adhesion and aggregation. Anti-factor VIII is a highly specific marker for endothelial cells, producing focal staining, although with some stromal interference and the detection of a proportion of lymphatics (Vermeulen et al., 1996).

CD 31 is associated with platelet adhesion in inflammation and wound healing (Martin et al., 1997). It is considered the most sensitive marker of endothelial cells presently available (Fanelli et al., 1999), however, it strongly cross-reacts with plasma cells (Fanelli et al., 1999; Martin et al., 1997).

CD 34 is believed to be involved with leucocyte adhesion and endothelial migration during angiogenesis (Martin et al., 1997). It is a sensitive endothelial marker, but some stromal and lymphatic staining has been reported (Fanelli et al., 1999; Vermeulen et al., 1996).

The final pan-endothelial marker, Anti UEA-1, has only been used in two studies of colorectal cancer patients (Terayama et al., 1996; Wynter et al., 1999). It recognises the sugar moieties present as part of the ABO blood group antigens on endothelial cells (Wynter et al., 1999). Although sensitive, it is not particularly specific, with obvious lymphatic and neoplastic staining (Vermeulen et al., 1996). It demonstrates up to 82% of microvessels in liver metastases, but the staining can be quite weak when compared to portal tracts (Terayama et al., 1996).

To date, there has not been a comparison between the three commonest pan-endothelial markers in colorectal cancer (factor VIII, CD 31 and CD 34). There has however, been such a study in breast cancer: Martin and colleagues used all three antibodies to identify microvessels in a series of 173 patients with breast cancer. They observed that anti-CD 34 staining was reproducible (98% consistency) and the most strongly expressed on microvessels, with no tumour or inflammatory cell staining and minimal background staining, enabling easy MVD assessment. Anti-factor VIII

staining was more reproducible (99% consistency), but it produced staining of tumour and inflammatory cells which impeded counting. In contrast, anti-CD 31 was the least reliable (87% consistency) as marked staining of inflammatory cells made accurate MVD assessment difficult. Therefore, anti-CD 34 was recommended as the antibody of choice for MVD assessment in breast carcinoma (Martin et al., 1997).

There have been three comparisons made between Anti -CD 34 and Anti-factor VIII in colorectal cancer (Hillen et al., 1997; Shomori et al., 1999; Tomisaki et al., 1996). Two studies favoured anti-CD 34, since anti- factor VIII produced non- specific background staining (Hillen et al., 1997; Shomori et al., 1999). The remaining study claimed that slides stained with anti-CD 34 had clearly recognisable lymphatics; therefore, anti- factor VIII was used (Tomisaki et al., 1996). Only one comparison has been drawn between anti-CD 31 and anti-factor VIII (Vermeulen et al., 1995). Anti-CD 31 was found to be more sensitive, but although inflammatory cell staining was observed, unlike Martin *et al*, MVD assessment was not impeded (Vermeulen et al., 1995).

Thus, the choice of antibody used for microvessel identification could explain some of the discrepancies between the studies. As there has not been a consensus of which pan- endothelial marker is the best for the assessment of microvessels in colorectal cancer, all three have been used with differing results, particularly in the studies of MVD and clinicopathological variables Tables 1.3, 1.4 and 1.5.

1.62.c Counting methods: hotspot and random

Mlynek *et al,* in 1985, were the first to describe a method of microvascular assessment in colorectal tumour tissue. Their method was complicated, involving vessel identification with Fuchsin B, followed by photomicrography of the section at x98 arranged into 'a map', with subsequent vessel counting. A vessel lumen was essential for its identification and subsequent numeration (Mlynek et al., 1985). This method is obviously time-consuming and impractical for clinical application; in addition, it is also likely to be inaccurate as normal colonic mucosa was found to have higher vessel counts than tumour tissue (Mlynek et al., 1985).

The most widely used method of assessing MVD in solid tumours is that described by Weidner and colleagues in 1991(Weidner et al., 1991). In brief, edge biopsies of invasive breast cancer were stained, and vessels were identified using anti- factor VIII. The sections were scanned at low power and the most vascular area or 'hotspot' selected. A vessel was defined as any separately stained endothelial cell or cluster, (lumens were not essential for vessel identification), and a count was performed in this hotspot at x200 (field size $0.74mm^2$). A significant association was found between the MVD and metastasis (Weidner et al., 1991). The majority of colorectal papers adhere to Weidner's definition of a countable microvessel, with only five exceptions wherein a vessel lumen was required for numeration (Gallego MG et al., 2000; Harada Y et al., 2001; Mlynek et al., 1985; Saclarides et al., 1994; Tabara et al., 2001).

The hotspot is thought to correlate best with clinical outcome because of its apparent relationship to haematogenous spread. McCulloch *et al* found a significant association

between the number of shed malignant cells and hotspot MVD in breast cancer patients (McCulloch et al., 1995). Tien *et al* found a similar relationship in colorectal cancer when they used reversed transcription polymerase chain reaction (rt-PCR) to identify circulating colorectal cancer epithelial cells in portal blood. Those patients with identifiable portal epithelial cells had significantly higher vessel counts than those who did not (Tien et al., 2001). Unlike Weidner, Tein *et al* counted three hotspots and expressed MVD as the mean value (Tien et al., 2001; Weidner et al., 1991).

Further justification for Weidner's technique comes from Martin *et al*, who compared MVD in ten hotspots with total tumour vascular density assessed using microangiography in breast cancer specimens, yielding a significant correlation (Martin et al., 1997). In their microangiographical study of colorectal cancer, Konerding *et al* found a significant relationship between vessel density calculated from multiple hotspots (exact number not stated) and metastasis, metastatic tumours having a higher vascular density than non-metastatic tumours. This result was cross-checked with a larger (n=46) immunohistochemical assessment of MVD in hotspots (anti-factor VIII), and the result remained significant (Konerding et al., 2001).
However, once again, more than one hot spot was used. Further evidence advocating the count of multiple hotspots in rectal cancer was described in a preoperative assessment of the angiogenesis of rectal carcinomas as investigated by Ogura *et al* (Ogura et al., 2001). In a prospective series of 46 patients with rectal cancer the tumours were examined with a combination of Colour Doppler Ultrasonography (n=46), microangiography (n=5), and immunohistochemistry (anti- factor VIII MVD assessment) (n=46). The total vascularity of the tumour was assessed by scanning the

lesion 1 cm at a time with the Doppler probe, and recording the number of highly vascular points (areas with maximum velocity and peak systolic velocity). The vascular point index (VPI) was defined as the number of vascular points divided by the area scanned, producing a value for the whole tumour (assuming that all of it was imaged). A comparison was made between the five lesions that underwent microangiography and the number of vascular points: in areas with a higher concentration of vessels, more vascular points were seen. A statistically significant correlation between the hotspot MVD of a tumour section (close to an area that had been examined with Doppler) and the VPI was observed ($p<0.0001$). The clinicopathological features of the study group were then examined in terms of prognostication of MVD and VPI. For patients with high MVD the only statistically significant relationship was the prediction of lymph node metastasis, whereas lymph node metastases, haematogenous metastases and venous invasion were significantly higher in patients with increased VPIs (Ogura et al., 2001). As single VPI measurements correlate with hotspot MVD, total tumour VPI represents multiple hotspot assessment.

As can be seen from Tables 1.1, 1.3,1.4 and 1.5, the majority of studies have used hotspot methodology, but the number of vascular areas assessed varies widely from 1 to all hotspots. The expression of the count also varies; some authors use the highest hotspots count (after Weidner (Weidner et al., 1991)), whilst others use the mean of several hotspots, which may be more representative (*vide supra).* In keeping with the original argument for using hotspots, fourteen papers have assessed the relationship between haematogenous metastases and hotspots. Eight studies found a significant relationship, whereas six did not (Table 1.5). The heterogeneity of number of areas

assessed and the expression of the counts are probably contributory factors to the differing results.

Only one paper has assessed MVD using random methodology in colorectal tumours (Kawasaki et al., 2001); however, the focus of this study was adenomas and T1 tumours, therefore it is not representative of colorectal cancer as a whole.
Two papers used random counting techniques to assess MVD in liver metastases (Table 1.6).

1.62.d Reproducibility of microvessel counts

The majority of researchers have used the hotspot method in assessing angiogenesis in colorectal cancer (Table 1.1). This assumes that the investigator(s) can reliably identify the area(s) of the tumour with the highest vascularity.

Martin *et al* investigated the accuracy with which investigators identified the areas of a tumour with the highest vascularity in a series of breast cancer specimens. The ten most vascular areas were assessed in descending order of vascularity; the 'hottest spot' was identified first in only 20% of cases, in the first five counts in 65% of cases and was always identified when ten areas were assessed (Martin et al., 1997).

Vermeulen *et al* noted that a period of training prior to embarking on a study of breast cancer angiogenesis significantly reduced the inter-observer variability (Vermeulen et al., 1997). Hansen *et al,* in a study of counting methodologies in breast cancer, concluded that computer-assisted, random sampling of the whole tumour was the

technique of choice to minimize inter and intra-investigator variability, followed by Chalkley counting (Hansen et al., 1998).

Although detailed studies of the effect of counting methodology and inter/intra observer variability in colorectal cancer are not currently available, at least 8 papers have assessed inter-observer variability in hotspot assessment. None of these demonstrated significant differences between the counters (Giatromanolaki et al., 1999; Takahashi et al., 1995; Takahashi et al., 1997; Vermeulen et al., 1995; Vermeulen et al., 1996; Abdalla et al., 1999; Bossi et al., 1995; Pavlopoulos et al., 1998). One paper assessed inter-observer variation in random counts and found no significant difference (Hillen et al., 1997). Four studies have assessed intra-observer variability with respect to hotspot counting, without demonstrating any significant differences (Giatromanolaki et al., 1999; Vermeulen et al., 1995; Vermeulen et al., 1996; Vermeulen et al., 1997).

In order to improve the accuracy of MVD assessment, some authors have adopted a simultaneous method wherein two investigators assess the sections for hotspots and count in unison (Amaya et al., 1997; Choi et al., 1998; Harada Y et al., 2001; Haraguchi et al., 2002; Ogura et al., 2001; Takebayashi et al., 1996; Takebayashi et al., 1996; Tien et al., 2001). Others advocate two independent counters, using a consensus when a significant difference is observed; unfortunately, the percentage of discrepancies was not reported (Hasebe et al., 2001; Maeda K et al., 2001; Maeda et al., 2000; Tabara et al., 2001).

It can therefore be proposed that some of the conflicting results regarding MVD and clinicopathological findings in colorectal cancer may be partly attributed to intra-observer variability, inadequate training in counting methods, lack of computer-assisted counting devices and inadequate sampling of hotspots. Counting less than 10 hotspots can fail to identify the hottest spot in 15% of cases (Martin et al., 1997).

1.62.e Expression of microvessel density

From Weidner's convention, the maximum value of the hottest spot from the most representative biopsy of invasive tumour should be quoted as the MVD for that cancer (Weidner et al., 1991). This has not been the case for most colorectal studies. Seventeen papers used the maximum count as MVD (after Weidner), but 52 papers used the mean of several areas. Clearly, these are two different measurements, which would make inter-study comparisons difficult, and may explain some of the conflicting results.

1.62.f Polyps

As can be seen from Table 1.2, despite obvious methodological differences between the papers, there is a general consensus that MVD increases from adenomas to carcinomas. There may also be a progressive relationship between increasing tumour differentiation and increasing MVD.

The largest study of adenomas was by Kawasaki *et al* who studied a series of 213 adenomas and 60 carcinomas in adenomas (all T stage 1). They found a significant stepwise increase in MVD (measured using anti-CD 34) between normal colonic

mucosa, mildly dysplastic, severely dysplastic adenomas and carcinomas (Kawasaki et al., 2001). There are some important methodological points from this study: Kawasaki *et al* chose to assess the background angiogenesis of the adenomas by counting 5 random areas, or if the specimen was too small, the whole lesion was assessed (Kawasaki et al., 2001). It may be that hot spots are not the primary driving force in the angiogenesis of early colonic neoplasms, but rather the total angiogenesis of the polyp (as measured by random sampling). Certainly Kaklamanis *et al* failed to find any hotspots in the adenomas they assessed (Kaklamanis et al., 2000).

There have been two separate papers describing corrosion casting of colorectal neoplasms (Konerding et al., 2001; Skinner et al., 1995), which differ in their description of the microvascular morphology of adenomas. Skinner *et al* found the overall morphology of adenomas was similar to that of normal colon; when the size of the adenoma increased, the density of the capillary network as well as the diameter and length of the vessels also increased, but there were no separate hotspots (Skinner et al., 1995). In contrast, Kondering *et al,* in a double-blind assessment, were unable to differentiate 40% of adenomas (4 out of 10) from carcinomas. Six of the ten lesions assessed had a similar vascular architecture to carcinomas. They do not comment on separate 'hot' areas, but rather on a change in the characteristics of the vascular morphomitary as a whole (Konerding et al., 2001).

The only study that failed to find an increase of MVD between adenomas and carcinomas using a pan-endothelial vessel marker was Akagi *et al* (Akagi et al., 2002), who not only used anti-CD 34 to assess a series of adenomas and carcinomas, but in addition used CD 105, a marker strongly expressed in the vascular endothelium

of tumours compared to normal tissues (Wang et al., 1993; Akagi et al., 2002). When they compared the MVD in hotspots of adenomas compared to carcinomas, no statistical difference was observed between those stained with anti-CD 34. However, when the sections stained with anti-CD 105 were counted, the adenomas had significantly lower MVD than the carcinomas (Akagi et al., 2002). The obvious discrepancy may be explained by either a sampling error, as the former study had a larger sample size (n=316) (Kawasaki et al., 2001; Wang et al., 1993), or by differences in MVD assessment: Kawasaki *et al* counted in random areas whilst Akagi counted hot-spots (Akagi et al., 2002).

1.62.g Liver metastases

Some of the initial work on the angiogenesis of liver metastases was performed in the 1980s. Strohmyer and colleagues performed a study employing resin injection to outline the microvasculature of liver metastases and noted that metastatic liver cancers in humans were initially supplied by sinusoidal blood up to 100-200μm in diameter. When the lesions increased beyond this size they grew new abnormal small blood vessels (Strohmeyer et al., 1987). This work was supported in one of the first studies to examine the MVD in liver metastases. Terayma *et al* studied 100 autopsy livers that contained metastases (colorectal n=13). They confirmed that for lesions below 200μm in diameter, no neovascularisation could be detected with anti-factor VIII or anti UEA-1. In tumours greater than 200μm diameter, MVD at the edge of the tumour increased with increasing tumour size, reaching a steady density when the tumour diameter was 3mm (Terayama et al., 1996).

Including the above work by Terayama, there are seven published studies investigating MVD in colorectal liver metastases, summarised in Table 1.6. (Davies et al., 2000; Hillen et al., 1997; Mooteri et al., 1996; Nanashima et al., 2001; Nanashima et al., 1998; Terayama et al., 1996; Yamamura et al., 2001). As can be seen from the table, all six research groups used differing counting methodologies. Three of the seven papers examined the relationship of metastasis size and MVD (Davies et al., 2000; Mooteri et al., 1996; Terayama et al., 1996). Davies *et al* found no relationship between MVD estimations in Tru-cut biopsies and tumour volume (Davies et al., 2000), whilst Mooteri *et al* could not establish a significant difference between the MVD of metastases measuring <4, 4-6, >6 cm respectively. Both of these studies provide some support to Strohmyer and Terayama's hypothesis of MVD stability in lesions >3mm diameter. However, Terayama used anti-factor VIII to identify microvessels and counted 10 areas at the tumour edge (Terayama et al., 1996). Although Mooteri *et al* employed the same antibody for microvessel identification the site of the biopsy was different and mean of the three hottest areas were used for MVD (Mooteri et al., 1996). Davies and co-workers used anti-CD 31 for vessel identification and assessed 40 random areas in Tru-cut biopsies; in addition, they used a Chalkley graticule (Davies et al., 2000). The methodological differences in these studies make comparisons therefore difficult.

Differences in MVD in colorectal liver metastases >3mm diameter may exist. Two groups have published three papers relating MVD to survival, but the results are contradictory. Nanashima *et al* in two separate publications demonstrated that tumours with higher MVD had significantly lower survival rates, although no statistical association with disease recurrence could be found (Nanashima et al., 2001;

Nanashima et al., 1998). In contrast, Mooteri *et al* found that metastases with higher MVD had a better prognosis (Mooteri et al., 1996). These differences may again be due to differing methodologies: Mooteri used factor VIII to identify microvessels in biopsies of liver metastases that contained viable tumour, and used the mean of 3 hotspots as the MVD (Mooteri et al., 1996); in contrast, Nanashima *et al* used anti-CD 34 and the mean of 5 hotspots in the edge of liver metastases (Nanashima et al., 2001; Nanashima et al., 1998).

1.7 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) was described independently in 1989 by Gospodarowicz *et al* (Gospodarowicz et al., 1989), Ferrara *et al* (Ferrara and Henzel, 1989) and Keck *et al* (Keck et al., 1989) as an angiogenic growth factor which induced endothelial cell mitogenesis. It was subsequently found to be identical to the previously described 'Vascular Permeability Factor' (VPF)(Dvorak et al., 1983).

The human VEGF gene consists of 8 exons separated by 7 introns, localised to chromosome 6p21.3 (Vincenti et al., 1996). Alternative splicing of the VEGF mRNA produces at least five distinct isoforms: 121, 145, 165, 189 and 206 (Tischer et al., 1991; Houck et al., 1991; Poltorak et al., 1997) producing disulfide-bonded dimeric glycoproteins of 34 to 45kD (Senger et al., 1993).

$VEGF_{165}$ is the most predominant iso-form and is produced by both normal and malignant cells. It is a basic, homodimeric glycoprotein demonstrating heparin binding capacity *in vitro* and heparan sulphate binding *in vivo,* hence its affinity for the ECM (Houck et al., 1992). In contrast, $VEGF_{121}$ is weakly acidic, has no heparin

binding characteristics and is therefore freely diffusible. $VEGF_{145}$ binds to heparin with an affinity similar to that of $VEGF_{165}$ (Poltorak et al., 1997) having an additional, independent heparin-binding site (Neufeld et al., 1994). $VEGF_{189}$ and $VEGF_{206}$ are more basic and have greater affinity for heparin than $VEGF_{165}$ and $VEGF_{145}$ (Houck et al., 1992). As a result, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequested by the ECM (Park et al., 1993). With the exception of $VEGF_{145}$, isoforms of VEGF may be liberated from the ECM by the action of heparinase (Houck et al., 1992; Poltorak et al., 1997).

1.71 VEGF Family

The VEGF family currently includes six known members: VEGF, Placental Growth Factor (PIGF), VEGF-B (VEGF Related Factor), VEGF-C (VEGF related protein), VEGF-D (c-fos-induced growth factor), and VEGF-E (Narko et al., 1999). They are all dimeric glycoproteins with homologous amino acid sequences shared with platelet derived growth factor (PDGF), and bind to similar tyrosine kinase growth factor receptors (Cox G et al., 2000).

PIGF was isolated from a term placenta cDNA library, hence the name, and is a 149-amino-acid-long protein with 53% homology to the platelet-derived growth factor-like region of human VEGF (Maglione et al., 1991). The PIGF gene is located on chromosome 14 (Maglione et al., 1993). There are two isomers, $PIGF_{129}$ and $PIGF_{152}$, differing only by the insertion of a highly basic, 21 amino acid stretch at the carboxyl end of $PIGF_{152}$, accounting for its heparin binding characteristics (Hauser and Weich, 1993). Both isomers are weakly mitogenic for endothelial cells (Ziche et al., 1997).

PIGF forms a heterodimer with VEGF and may modulate VEGF-induced angiogenesis (Cao et al., 1996).

The VEGF-B gene is located on chromosone 11q13 (Paavonen et al., 1996; Olofsson et al., 1996). Alternative splicing results in the production of two basic isoforms, $VEGFB_{167}$ and $VEGFB_{186}$. There is 44% homology between the two glycoproteins, however, only $VEGFB_{167}$ displays heparan binding characteristics (Olofsson et al., 1996; Olofsson et al., 1996) $VEGFB_{167}$ appears to be the most prevalent isomer. The exact function of VEGFB remains to be determined. It does not appear to undertake a major role in the development of the vascular system, as VEGFB knockout mice are viable and fertile (Olofsson et al., 1999). However, VEGFB forms heterodimers with VEGF and may therefore modulate its signalling (Olofsson et al., 1999).

The VEGFC gene is located on chromosone 4q34 (Paavonen et al., 1996; Olofsson et al., 1996; Olofsson et al., 1996). VEGFC stimulates the migration of endothelial cells, increases vascular permeability (Joukov et al., 1996) and stimulates the proliferation of lymphatic endothelial cells, despite being a weakly mitogenic to endothelial cells (Joukov et al., 1997).

VEGFD is structurally related to VEGFC. VEGFD mRNA is found most abundantly in heart, lung, skeletal muscle, colon, and small intestine (Achen et al., 1998).VEGFE isomers are viral homologues of VEGF encoded by different strains of the Orf virus (Ogawa et al., 1998).

1.72 VEGF Receptors

To date, two VEGF tyrosine-kinase receptors have been identified, VEGFR-1 (flt-1) (de Vries et al., 1992) and VEGFR-2 (KDR flk-1) (Terman et al., 1992), present both on vascular endothelium (Boocock et al., 1995) and non-vascular epithelium. The binding of VEGF to the receptors causes dimerisation followed by phosphorylation of their cytoplasmic kinase domains. However, information regarding the signalling cascades of VEGF and its receptors is not completely understood (Neufeld et al., 1999).

VEGF also binds to neuropillin-1 and neuropillin-2 receptors. Binding to these and to heparan-sulphate proteoglycans does not appear to induce a biological response in the absence of the tyrosine kinase receptors (Neufeld et al., 1999). An additional tyrosine kinase receptor has been identified, VEGFR-3 (Flt-4). This is not a receptor for native VEGF but binds VEGFC and VEGFD (Neufeld et al., 1999).

1.73 Functions of VEGF: role in tumour angiogenesis

1.73.a Increases Permeability

Studies have revealed that inhibition of VEGF signalling inhibits tumour angiogenesis, and consequently, tumour growth (Kim et al., 1993; Millauer et al., 1994; Millauer et al., 1994). VEGF is one of the most potent vasodilators known (Dvorak et al., 1995). It causes increased vascular permeability by the opening of vesiculo-vacuolar organelles, grape-like clusters of interconnecting smooth membrane bounded vesicles and vacuoles extending across the entire thickness of the venular

endothelial cytoplasm (Feng et al., 1996; Dvorak et al., 1992). This allows the extravasation of plasma proteins including fibrinogen (which clots forming a fibrin gel) into the extracellular matrix. This fibrin provides a medium that attracts and supports the growth of endothelial cells and fibroblasts, leading to angiogenesis (Dvorak et al., 1987). Microvascular hyperpermeability leads to a profound alteration in the extracellular matrix, transforming it from an anti-angiogenic to a pro-angiogenic phenotype. Chronic exposure to VEGF leads to a change in the vascular endothelial phenotype, from contiguous to fenestrated endothelium. (Kubitza et al., 1999).

1.73.b Induces serine proteases

VEGF induces the expression of urokinase-type and tissue-type plasminogen activators (PAs), PA inhibitor-1, and metalloproteinase interstitial collagenase (Pepper et al., 1992; Unemori et al., 1992), leading to further alteration of the ECM. The conversion of pro-thrombin to thrombin stimulates matrix metalloproteinase-2 (MMP-2), which in turn, further breaks down the ECM (Zucker et al., 1998).

1.73.c Mitogenic to endothelium

VEGF is a potent mitogen for endothelial cells (Ferrara and Henzel, 1989), inducing vessel sprouting from rat aorta (Nicosia et al., 1994). Activation of VEGFR2 leads to endothelial cell proliferation, while activation of both VEGFR1 and VEGF2 receptors leads to endothelial cell migration (Seetharam et al., 1995; Waltenberger et al., 1994). Transfection of VEGF cDNA leads to an increase in vascularity (Senger et al., 1994), which is inhibited by anti-VEGF antibodies (Kondo et al., 1993).

1.73.d Induces anti-apoptic proteins

VEGF inhibits endothelial cell apoptosis (Katoh et al., 1995), inducing the expression of anti-apoptitic factors such as bcl-2 (Alon et al., 1995; Gerber et al., 1998; Nor et al., 1999).

1.73.e Alters immune response

VEGF promotes both expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in endothelial cells, thereby allowing the adhesion of natural killer cells (NK) to the endothelium (Melder et al., 1996). VEGF has regulatory effects on blood cells: it is chemotactic to monocytes (Clauss et al., 1990), enhances the rolling and adhesion of leukocytes (Detmar et al., 1998) and induces colony formation of subsets of granulocyte-macrophage progenitor cells (Broxmeyer et al., 1995).

1.74 Regulation of VEGF

The VEGF gene can be induced by hypoxia in a number of cell types, including endothelial and smooth muscle cells (Namiki et al., 1995; Stavri et al., 1995). Endothelial cells respond to oxygen tensions below 70mmHg. Hypoxia in the cell leads to the redox state with the generation of free radicals. This activates the transcription factors Hypoxia Inducible Factor-1 (HIF-1) and NF-$_{\kappa}$B which in turn induce the expression of hypoxia-related genes (Huang et al., 1996; Koong et al., 1994). HIF-1 is a basic heterodimeric helix loop helix protein consisting of two subunits. It reacts with the specific DNA sequence, 5'-TACGTGCT-3'(Wang and Semenza, 1995). HIF-1 induces VEGF expression by binding to a cis element and

increasing VEGF mRNA transcription (Levy et al., 1995; Minchenko et al., 1994). Hypoxia-induced VEGF expression is biphasic, through transcriptional induction via HIF-1 and by increased stability of mRNA (Shih and Claffey, 1998). The rate of VEGF mRNA transcription equilibrates following hypoxia. VEGF mRNA stabilisation remains elevated throughout the hypoxic episode (Ikeda et al., 1995).

In parallel to the hypoxic upregulation of VEGF, the VEGF receptors VEGFR-1 and VEGFR-2 are also up-regulated *in vivo* (Li et al., 1996; Tuder et al., 1995). *In vitro* studies have shown that the VEGFR-1 gene is directly up-regulated by a hypoxia-response element however this has not been shown in VEGFR-2 (Gerber et al., 1997; Takagi et al., 1996). VEGF itself may directly up-regulate VEGFR-2 (Kremer et al., 1997; Shen et al., 1998).

Inactivation of tumour suppressor genes also leads to VEGF up-regulation. Wild type von Hippel Lindau gene (vHL) suppresses the production of hypoxia-regulated proteins including VEGF. Mutation of vHL results in increased VEGF expression by transcriptional and post transcritptional mechanisms (Mukhopadhyay et al., 1997; Siemeister et al., 1996; Stratmann et al., 1997). Mutant p53 promotes the expression of VEGF (Kieser et al., 1994), but evidence of wild-type p53 as an inhibitor of VEGF production is conflicting (Agani et al., 1997; Mukhopadhyay et al., 1995).

Expression of the mutant *ras* oncogene, one of the commonest genetic abnormalities in human cancer, is associated with marked up-regulation of VEGF (Okada et al., 1998). Disruption of the K-ras allele or mutant H-ras protein function in rat intestinal cells lead to VEGF suppression (Rak et al., 1995).

Several growth factors up-regulate VEGF expression, including fibroblast growth factor-4 (FGF-4)(Deroanne et al., 1997), tumour necrosis factor-alpha (TNF-alpha) (Ryuto et al., 1996), transforming growth factor beta (TGF-beta) (Pertovaara et al., 1994), keratinocyte growth factor (KGF)(Frank et al., 1995), epidermal growth factor (EGF) (Frank et al., 1995; Goldman et al., 1993), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) (Tsai et al., 1995). Insulin-like growth factor increases transcription of VEGF mRNA (Goad et al., 1996); however, evidence for a post transcriptional role is conflicting (Akagi et al., 1999; Warren et al., 1996; Akagi et al., 1999; Warren et al., 1996; Akagi et al., 1999).

Various interleukins play a part in the regulation of VEGF. Interleukin-6 (IL-6) up-regulates VEGF (Cohen et al., 1996; Warren et al., 1996); IL-1alpha and prostglandin E2 induce expression of VEGF in cultured synovial fibroblasts (Ben-Av et al., 1995; Warren et al., 1996); IL-1beta increases transcription (Li et al., 1995) but not stabilisation (Akagi et al., 1999); interleukin-10 (IL-10), interleukin-12 (IL-12) and interleukin-13 (IL-13) inhibit the release of VEGF (Matsumoto and Nakamura, 1997).

1.75 Vascular endothelial growth factor in colorectal cancer

The expression of vascular endothelial growth factor in colorectal cancer in relation to clinical and pathological parameters has been examined by numerous studies. Immunohistochemistry to determine protein expression, northern blotting and reverse transcription polymerase chain reaction (RT-PCR) to determine gene expression and enzyme-linked immunosorbant assays (ELISA) to determine serum, plasma and tissue levels of VEGF are techniques used to quantify VEGF.

1.75.a Immunohistochemical assessment of VEGF in colorectal cancer

Following antigen retrieval, either by protein digestion with casein (Berney C R et al., 1999; Berney et al., 1998; Berney C R et al., 1999), trypsin (Lee et al., 2000; Nanashima et al., 1998; Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1998; Takahashi et al., 1997; Takahashi et al., 1998; Nanashima et al., 1998; Lee et al., 2000), heat treatment with microwave (Cascinu et al., 2000; Cascinu et al., 2001; Kang et al., 1997) or a combination of these, (Amaya et al., 1997) studies that examined tumour expression of VEGF using immunohistochemistry typically used the rabbit polyclonal anti-VEGF antibody clone number #sc-152 (Santa Cruz Biotechnology, Santa Cruz, USA) (Berney C R et al., 1999; Berney et al., 1998; Kang et al., 1997; Kondo et al., 2000; Nanashima et al., 1998; Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1998; Takahashi et al., 1997; Takahashi et al., 1998; Kang et al., 1997; Nanashima et al., 1998; Berney et al., 1998; Berney C R et al., 1999), or Ab-2 (Oncogene Science Cambridge, MA, USA and Biogenex CA USA respectively) (Amaya et al., 1997; Cascinu et al., 2000; Cascinu et al., 2001) which recognises the 121, 165 and 189 isoforms of VEGF, and polyclonal anti-VEGF antibodies generated by the immunisation of rabbits with N terminal region of VEGF 165 (Calbiochem USA) (Lee et al., 2000).

There is no clear consensus, however, on how to evaluate tissue VEGF staining and it has therefore been performed in a variety of ways. Intensity of staining using categories 0, 1+, 2+ and 3+ is the most basic, semi-quantitative method used by some researchers (Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1998).

Percentage of positive stained cells has been more widely used (Berney et al., 1998; Cascinu et al., 2000; Cascinu et al., 2001; Kang et al., 1997; Lee et al., 2000; Nanashima et al., 1998), followed by dividing the data into different groups:

- Positive versus negative expression: those with negative expression (<10% positive stained tumour cells) and positive expression of VEGF (>10% stained positive tumour cells (Cascinu et al., 2000; Kang et al., 1997). Greater than 30% positive stained tumour cells has also been used as a cut-off point (Ono and Miki, 2000).
- Categories of staining:

1) 0, 1= focal/weak staining, 2= staining of 25% of cells, 3= staining of 25-50% of cells and 4=staining of >50% of cells (Berney C R et al., 1999; Berney et al., 1998; Berney C R et al., 1999).

2) 0, 1+ (<50% tumour cells stained), 2+ (>50% tumour cells stained)(Nanashima et al., 1998).

3) 0: no VEGF positive tumour cells, 1+: < 20% positive cells, 2+: 20-50% positive cells and 3+: > 50% positive cells (Kondo et al., 2000).

4) 0, 1+ <5% stained cells, 2+ 5-25% stained cells and 3+ >25% positive cells (Lee et al., 2000).

5) VEGF positive, if tumour cells stained more intensely than surrounding normal colonic epithelium and VEGF negative, if the reverse was true (Cascinu et al., 2000).

Any results arising from such studies must be interpreted with some degree of caution due to the heterogenicity between the methods used (*vide supra*). However, despite the absence of a consistent grading system for VEGF staining, there is a general consensus regarding most of the clinical and pathological factors examined. There

appears to be a correlation between microvessel density in colorectal primary tumours and VEGF staining. The majority of studies that examined these two parameters produced a statistically significant result (Amaya et al., 1997; Kang et al., 1997; Kondo et al., 2000; Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1998; Kang et al., 1997; Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1998). Kondo *et al* examined the relationship between VEGF expression and MVD in non-invasive colorectal cancer, by studying adenomas, lesions confined to the submucosa and those that had breached the submucosa. They found a significant difference in the vessel counts of mucosal lesions expressing VEGF (higher MVD) compared to those that did not. Amongst the submucosal lesions, those with higher numbers of VEGF positive cells had significantly higher vessel counts (Kondo et al., 2000). This is in contrast with work by Lee *et al* who failed to demonstrate any VEGF expression in adenomas (n=30), regardless of their histological differentiation. This may have been due to the type of antibody used in this study, as it only recognised the 165 isoform of VEGF; indeed, the grading system employed was set 50% lower when compared to the scales employed by other groups (*vide supra)* (Lee et al., 2000). Fox *et al* also failed to show a significant correlation (Fox et al., 1998), but they only examined 10 patients.

VEGF staining has been used to predict survival, disease-free episodes and recurrence following a colorectal resection. Statistically significant results with uncensored survival data have been demonstrated (Amaya et al., 1997; Kang et al., 1997; Lee et al., 2000). VEGF negative tumours have been correlated with disease-free survival (Kang et al., 1997), whereas a correlation between VEGF positive tumours and metastatic disease has been shown as significant by some studies (Takahashi et al.,

1995; Takahashi et al., 1997; Takahashi et al., 1998; Lee et al., 2000; Takahashi et al., 1997; Takahashi et al., 1998) but not others (Berney C R et al., 1999; Berney et al., 1998). Similarly, VEGF status and recurrence does not appear to be straightforward. In one study, Cascinu *et al* concentrated their research on stage two tumours ($pT3N_0M_0$), demonstrating a significant difference in the recurrence rate of tumours that stained positive for VEGF compared to those that did not (Cascinu et al., 2000; Cascinu et al., 2001). Berney *et al* failed to associate VEGF status with recurrence (Berney et al., 1998).

VEGF status in association with other markers of angiogenesis has also been evaluated, with VEGF positive tumours correlating with p53 staining and higher MVD (Kang et al., 1997; Takahashi et al., 1997; Takahashi et al., 1998) also in association with the VEGF receptor VEGFR1 (Takahashi et al., 1995). Once again, others failed to reproduce these results (Amaya et al., 1997).

Conflicting results have been yielded regarding the expression of VEGF in colorectal liver metastases compared with the primary tumour. Berney *et al* demonstrated a significant reduction in VEGF in liver secondaries compared with matched primary tumours (Berney et al., 1998), whereas Nanashima *et al* showed no such reduction (Nanashima et al., 1998). Nanashima *et al* confirmed a statistically significant relationship between MVD and VEGF expression in colorectal liver metastases. Both authors examined the relationship between VEGF expression and outcome in patients who underwent liver resection for colorectal hepatic metastases, and neither found a predictive relationship with disease or recurrence. Whilst Nanashima *et al* examined hepatic resection patients alone (Nanashima et al., 1998), Berney *et al* evaluated a

heterogeneous group consisting of liver resection patients and those who underwent colorectal resection alone (Berney et al., 1998).

VEGF immunostaining has also been compared between metastatic sites. Cascinu *et al* analysed VEGF expression in liver and abdominal metastases, and although the sample population was small (19 liver metastases and 22 abdominal metastases) they demonstrated statistically higher levels of VEGF in the abdominal deposits as compared with the liver deposits, the importance of which is claimed to be in developing targeted chemotherapy (Cascinu et al., 2001).

1.75.b Assessment of VEGF in colorectal cancer by molecular biology

Studies differ in the exact methodology chosen to investigate VEGF gene expression. Northern blot analysis (Ishigami et al., 1998; Nakata et al., 1998; Nakata et al., 1998), semi-quantitative PCR (Cheung et al., 1998; Wong et al., 1999; Wong et al., 1999), non-quantitative PCR (Kondo et al., 2000), and a combination of these techniques have been used (Andre et al., 2000; Tokunaga et al., 1998; Tokunaga et al., 1999). The majority of studies examined the isoforms 121, 165, 189 and 206 (Andre et al., 2000; Cheung et al., 1998; Kondo et al., 2000; Tokunaga et al., 1998; Tokunaga et al., 1999; Wong et al., 1999; Cheung et al., 1998; Wong et al., 1999). No study attempted to quantify the expression of $VEGF_{145}$ in colorectal carcinoma.

There is a consensus amongst the studies that the VEGF gene is up-regulated in colonic carcinoma compared to normal colonic mucosa (Andre et al., 2000; Cheung et al., 1998; Kondo et al., 2000; Tokunaga et al., 1998; Tokunaga et al., 1999; Wong et

al., 1999; Wong et al., 1999), the 121 and 165 isoforms are the predominant types expressed, with no expression of the 206 isoform in either normal or malignant colonic tissue (Andre et al., 2000; Cheung et al., 1998; Tokunaga et al., 1998; Tokunaga et al., 1999; Wong et al., 1999; Wong et al., 1999). These studies differ however in the precise timing of VEGF up-regulation and whether it continues throughout the progression of colonic cancer.

Analysis of colonic adenomas yielded differing results (Andre et al., 2000; Cheung et al., 1998; Wong et al., 1999; Andre et al., 2000). Kondo *et al* examined 20 adenomas with RT-PCR and failed to demonstrate any positive cases. Wong *et al* demonstrated significant expression of the 165 and 121 isoforms of VEGF in adenomas and carcinomas compared to control samples. Although up-regulation of VEGF was also observed between adenoma and carcinoma stages, the increase did not reach statistical significance. This may mean that either VEGF is up-regulated to a set level very early in the malignant process, or a sampling error has occurred as only 12 adenomas and 11 carcinomas (of differing Dukes' stage) were used (Wong et al., 1999). Andre *et al* described isoforms 121, 165 and 189 in two adenomas examined, but failed to show up-regulation of VEGF compared to control samples. They only examined isoform 121, however, and not the 165 and 189 splice variants, therefore up-regulation of $VEGF_{165}$ or $VEGF_{189}$ may have occurred and was missed (Andre et al., 2000).

Similarly, analysis of VEGF mRNA according to Dukes' stage has produced controversial results. Nakata *et al* showed a significant increase in VEGF expression in Dukes' B tumours compared with Dukes' A tumours, but failed to demonstrate any further up-regulation between C and D staged lesions; however, the D group consisted

of only 5 patients (Nakata et al., 1998). In contrast, Ishigami *et al* demonstrated that the expression of VEGF mRNA was highest in Dukes' D, but no difference between the other stages was shown (Ishigami et al., 1998). Andre *et al* did not find a statistical difference between any of the Dukes' stages (Andre et al., 2000). Tokunaga *et al* used a different approach, and divided the cancers into three groups depending on isoform expression: 121 only, 121 and 165, and finally those that expressed 121, 165 and 189. Patients with tumours that expressed all three isoforms were statistically more likely to develop liver metastases; however, there was no correlation with T stages (Tokunaga et al., 1998; Tokunaga et al., 1999).

Few data are available regarding VEGF gene expression and survival. Once again, the results are opposing. High VEGF expression has been shown to correlate with poor prognosis for uncensored survival by some authors (Ishigami et al., 1998), but not others (Andre et al., 2000). However, this may be due to the fact that different VEGF isoforms were considered: for example, in Ishigami *et al's* study, VEGF positively correlated with survival using isoforms 121,165 and 189, (Ishigami et al., 1998); whereas in Andre *et al's* study, which failed to show such a relationship, only isoform 121 was measured , and therefore, a correlation could have been missed (Andre et al., 2000).

In summary, there appears to be early up-regulation of VEGF expression in colonic carcinomas. The soluble VEGF isoforms 121 and 165 are the most predominant but tumours also expressing the 189 isoform have an increased tendency to metastasise to the liver.

1.75.c ELISA assessment of VEGF in colorectal cancer

Studies detailing VEGF levels in patients with colorectal carcinoma as measured by ELISA have mainly concentrated on peripheral serum levels of VEGF (Chin KF et al., 1999; Dirix et al., 1996; Dirix et al., 1997; Fujisaki et al., 1998; Kumar et al., 1998; Landriscina et al., 1998; Werther et al., 2000; Werther et al., 2001; Werther et al., 2000; Werther et al., 2001) (Karayiannakis et al., 2002). Tissue levels of VEGF (Kondo et al., 1993; Kondo et al., 2000)(Ono and Miki, 2000) plasma VEGF in patients with colorectal liver metastases (Davies et al., 2000) both serum and tissue levels of VEGF in patients with colorectal cancer (Fujisaki et al., 1998; Landriscina et al., 1998; Landriscina et al., 1998) mesenteric serum (Landriscina et al., 1998), and both serum and plasma levels (Werther et al., 2002) have also been examined. The soluble isoforms of VEGF, 121 and 165 were the most widely measured, although individual isoforms have been studied (Chin KF et al., 1999)
(Dirix et al., 1996; Dirix et al., 1997; Fujisaki et al., 1998; Kumar et al., 1998; Landriscina et al., 1998; Werther et al., 2000; Werther et al., 2001)

Dirix *et al* investigated the relevance of serum VEGF in the tumour kinetics of colorectal cancer patients. They claim that patients with progressive cancer have higher levels of VEGF when compared with those in whom disease is static. However, this study consisted of a heterogeneous group of malignancies including colorectal, breast, ovarian and renal carcinomas, dictating that any inferences to colorectal cancer alone cannot be made (Dirix et al., 1997). In a separate study, Dirix

et al established that for a group of patients with recurrent and/or metastatic colorectal tumours, those whose disease had more rapid growth (doubling in tumour size in less than 6 months) had significantly higher serum VEGF levels than those that displayed slower growth (doubling in tumour size in more than 6 months)(Dirix et al., 1996).

There is some agreement between the relevant studies that serum VEGF levels (Fujisaki et al., 1998; Kumar et al., 1998; Landriscina et al., 1998; Landriscina et al., 1998; Karayiannakis et al., 2002) and plasma VEGF (Werther et al., 2002) statistically correlate with Dukes' stage. However, Werther *et al* only demonstrated a difference between Dukes' stage D and A to C inclusive; they did not show differences between groups A to C (Werther et al., 2000). Moreover, patients with liver metastases had significantly higher serum VEGF levels compared with those without liver involvement (Fujisaki et al., 1998; Kumar et al., 1998; Landriscina et al., 1998; Werther et al., 2000; Karayiannakis et al., 2002) This also applied to patients with haematogenous metastases, also classified as Dukes' D staging (Fujisaki et al., 1998; Kumar et al., 1998; Karayiannakis et al., 2002). In a separate investigation, Chin *et al* reported that pre-operative serum levels of VEGF accurately predicted those patients who would develop distant metastases regardless of Dukes' stage or lymph node status (Chin KF et al., 1999). With the exception of Landriscina *et al*, significant differences were shown between serum VEGF levels and control samples (Fujisaki et al., 1998; Kumar et al., 1998; Landriscina et al., 1998; Werther et al., 2000; Werther et al., 2001; Werther et al., 2001). This may be due to a sampling error, as only 35 cases and 10 controls were used in Landriscina's study (Landriscina et al., 1998).

In a study of 524 patients with colorectal cancer, Werther *et al* showed that high serum vascular endothelial growth factor was an independent predictor of poor survival, contrary to plasma vascular endothelial growth factor.(Werther et al., 2002) Karayiannakis *et al* also found that elevated serum VEGF correlated with poor outcome (Karayiannakis et al., 2002).

There is a consensus between studies examining tissue expression of VEGF that the amount of the glycoprotein is higher in the tumour than in healthy colonic mucosa (Fujisaki et al., 1998; Ono and Miki, 2000; Kondo et al., 2000; Konno et al., 1998; Landriscina et al., 1998; Landriscina et al., 1998; Fujisaki et al., 1998). Konno *et al* attempted to correlate tissue expression of VEGF with clinico-pathological factors; the only significant finding was that tissue levels of VEGF correlated with tumour bulk (Konno et al., 1998). Ono and Miki also found increased levels of VEGF protein with increasing tumour bulk (Ono and Miki, 2000), and further demonstrated significant differences in the concentration of VEGF with increasing histological grade and vascular invasion. They also showed that increasing amounts of tissue VEGF significantly correlated with systemic hypoxia (Ono and Miki, 2000).

Davies *et al* examined plasma for the VEGF isoforms 121 and 165 in a series of patients with colorectal liver metastases and controls. Although, as with serum there was a degree of overlap between cases and controls, significantly higher plasma levels were seen in patients with metastases as compared with controls. In addition, plasma levels of VEGF were significantly correlated to metastatic volume and tumour vessel counts (Davies et al., 2000).

1.76 VEGF and the coagulation pathway

It is well established that most patients with cancer have some form of abnormality with their coagulation pathway, including increased platelet turnover and increased blood concentrations of fibrinogen breakdown products (Harker and Slichter, 1972; Sun et al., 1979; Sun et al., 1979). Thrombocytosis has been identified as an independent prognostic indicator in patients with both lung (Cox et al., 2000; Pedersen and Milman, 1996) and colorectal malignancy (Monreal et al., 1998).

In 1997 Möhle and co-workers demonstrated that megakaryocytes produce VEGF (Mohle et al., 1997), which is incorporated in the cytoplasm of platelets. Following activation, VEGF is released in combination with β-thromboglobulin suggesting that it is carried within the α-granules (Wartiovaara et al., 1998). Platelets were therefore identified as major transporters of VEGF in the circulation (Verheul et al., 1997), in addition to B and T lymphocytes, previously identified as carriers of VEGF (Harker and Slichter, 1972).

Platelet counts and serum VEGF have been correlated in both cancer patients and controls (Salgado et al., 1999; Salven et al., 1999; Salven et al., 1999; O'Byrne et al., 1999); this may suggest that the prognostic relevance of serum VEGF in colorectal cancer patients is simply a reflection of thrombocytosis that is known to occur with the disease. However, when a group of patients with advanced malignancy were compared with controls, the cancer patients had higher lysed whole blood VEGF concentrations than healthy individuals who had been matched for leukocyte and platelet counts (Salven et al., 1999). These results suggest that the high whole blood

and serum VEGF concentrations found in cancer patients cannot be explained by the presence of leukocytosis or thrombocytosis alone. Platelets are known to take-up several hormones and proteins (Bridges and Baldini, 1966; Heilmann et al., 1994; Heilmann et al., 1994). Therefore they may also scavenge tumour-released angiogenic cytokines. Wynendaele *et al* demonstrated that for a group of patients with heterogeneous malignancy, VEGF measurements derived from platelet-poor plasma as opposed to EDTA-plasma or serum, allowed the most accurate identification of cases rather than controls (Wynendaele et al., 1999). This has yet to be repeated for a group of patients with colorectal malignancy.

1.77 VEGF as a potential anti-angiogenic target

VEGF and its receptors are extremely attractive therapeutic targets for anti-angiogenic therapy. This is because, of all the pro-angiogenic cytokines, VEGF appears to be the most important for the development of the vascular system, as the loss of a single VEGF allele results in embryonic lethality (Carmeliet et al., 1996; Carmeliet et al., 1996). Antibody treatment against VEGF has been reported in numerous experimental models (Asano et al., 1995; Kim et al., 1993). These studies found that it was not possible to obtain complete angiosupression and total dissolution of experimental tumours using this strategy alone. Tumours may therefore be relying on concurrent production of other angiogenic cytokines, and so multiple blockades would be necessary. Two other strategies have been used. Firstly, mouse monoclonal antibodies raised against VEGF have been 'humanised'; they have a long half-life, high degree of specificity and little immunogenicity. One such monoclonal antibody is rhuMab VEGF (Presta et al., 1997) (Ferrara and Alitalo, 1999). A phase III study using

RhuMab VEGF for the treatment of colorectal carcinoma has shown a significant improvement in patient survival (unpublished data presented at the American Society of Clinical Oncology, 2003).

The second strategy involves inhibition of the VEGF receptor-2. This can be achieved either by the use of a 'humanised' monoclonal antibody against the receptor or by inhibition of signal transduction. Both these techniques are being assessed in clinical trials (Ferrara and Alitalo, 1999). Antibody inhibition of VEGFR-2, in combination with low dose vinblastine, has been shown to produce complete regression of experimental tumours in nude mice, without signs of toxicity (Klement et al., 2000). At the same time, a selective kinase inhibitor (PTK787) which blocks phosphorylation by VEGF and PDGF receptors, was shown to completely prevent retinal neovascularisation in a murine model (Ozaki et al., 2000). In a recently performed phase I/II study, PTK787 has been demonstrated to inhibit the growth of colorectal liver metastases (Morgan et al, *in press JCO*). The inhibition of VEGF receptors appears therefore to hold more promise as an effective anti-angiogenic therapy. The results of clinical trials are eagerly awaited.

1.8 Thymidine Phosphorylase

Another potent, pro-angiogenic factor is thymidine phosphorylase (TP). It was originally described as platelet-derived endothelial growth factor (PD-ECGF) since, following its purification from human platelets, TP was found to be an identical dimmer of 45kDa subunits (Miyazono et al., 1987), observed to be chemotactic for endothelial cells *in vitro* and angiogenic for endothelial cells *in vivo* (Ishikawa et al., 1989). These initial reports were disputed with claims that PD-ECGF did not induce

endothelial cell proliferation (Moghaddam and Bicknell, 1992). The apparently contradictory findings were resolved when it was discovered that PD-ECGF and TP were identical (Usuki et al., 1992; Furukawa et al., 1992).

TP is a member of the pyrimidine nucleoside phosphorylase family of enzymes that are essential for DNA synthesis. TP has been shown to stimulate angiogenesis and endothelial cell chemotaxis in animal models (Moghaddam et al., 1995). Possibly through one of its metabolites, 2-deoxy-D-ribose. (Haraguchi et al., 1994).

TP expression is elevated in several tumour types including carcinoma of the breast (Moghaddam et al., 1995; Toi et al., 1995), bladder (O'Brien et al., 1995), ovary(Reynolds and Redmer, 1998), stomach (Maeda et al., 1996) oesophagus, lung(Giatromanolaki et al., 1997) and pancreas (Takebayashi et al., 1996). TP appears to be involved in the development of colorectal cancer from an early stage (Enomoto et al., 2000; Takebayashi et al., 1995).

1.81 Polyps

The largest study of colorectal polyps was by Enomoto *et al*, who described the expression of TP in 120 polyps categorised into five groups: controls (20 hyperplastic polyps and 20 inflammatory polyps), 20 polyps containing a focus of carcinoma and 60 adenomas, subdivided into mild, moderate and severely dysplastic lesions (n=20 for each division). Although TP was detected in stromal macrophages and fibroblasts in all specimens, no association was found between any clinicopathological factors.

Significant differences were observed in the expression of TP in the polyps themselves: 20% of the severely dysplastic adenomas and 25% of the carcinomas expressed the enzyme, whereas no expression was detected in the control group or the polyps with lower dysplasia. Larger polyps were found to have significantly more TP than smaller ones. In addition, increased cellular proliferation as measured by Ki-67 expression, was associated with higher levels of TP expression. No association was observed with p53 expression (Enomoto et al., 2000). These findings are similar to other studies. Shomori *et al* described nuclear staining in 24% of colonic adenomas with increased staining in those cells with increasing nuclear atypia (Shomori et al., 1999), whereas Takebayashi *et al* observed cellular staining in 23% of adenomatous polyps (Takebayashi et al., 1995). A more recent study has shown a lower expression of TP in adenomas (9%); this may be due to a sampling error, as the degree of dysplasia in the study group was not documented (Kaklamanis et al., 2000).

1.82 Colorectal carcinomas

It is accepted that the expression of TP is higher in colorectal carcinomas than in normal colon or rectal mucosa (Enomoto et al., 2000; Ohi and Miki, 2000; Tabara et al., 2001; Takebayashi et al., 1995). It is also accepted that TP is found not only in the tumour cells but also in the stroma, the latter usually being in macrophages and lymphocytes (Folkman, 1996). The literature may be divided into the ways by which TP in colorectal tumours has been analysed. The majority of studies have evaluated the amount of TP present in tumour cells only. In two separate studies, Takebayashi and co-workers demonstrated a significant relationship between MVD and TP expression (Takebayashi et al., 1995; Takebayashi et al., 1996) also, significant relationships with tumour, lymphatic and venous invasion, Dukes' stage, and lymph

node metastases were demonstrated (Takebayashi et al., 1996). In addition, they demonstrated, using both a univariate and multivariate model, that high TP expression is a poor prognostic factor for survival (Takebayashi et al., 1996). Similar relationships with MVD have been shown by Matsuura *et al*, Shomori *et al* and van Halteren *et al*. Shomori *et al* confirmed the relationship between increasing Dukes' stage and increasing TP expression, and described a relationship between p53 and TP expression, the latter being lower in p53 negative cells (Shomori et al., 1999).

Matsuura *et al* found a similar relationship between p53 and TP expression (Matsuura T et al., 2000). van Halteren *et al* found a significant difference between Astler Coller B1 and B2 tumours, the former having significantly lower TP expression. They also confirmed TP as an independent prognostic risk of haematogenous metastases (van Halteren et al., 2001). Although Matsuura *et al* failed to show a relationship between Dukes' stage, p53 and TP expression, they did report a significantly lower apoptitic index for those tumours expressing TP compared with those that did not (Matsuura et al., 1999). By grading the total amount of TP present in the tumour and stroma, Saeki *et al* found a significant correlation between depth of invasion, increased TP expression and microvessel counts (Saeki et al., 1997).

In an attempt to clarify whether TP in colorectal tumour cells or stromal cells act independently or synergistically, Matsumura and co-workers assessed TP expression, the MVD and macrophage infiltration (using anti-CD68 a macrophage marker) in a series of 148 patients. The patients were divided into three groups: those that did not express TP (grade I), those that expressed TP either in the stroma or the tumour, (grade II), and those that expressed TP in both the stroma and the tumour cells (grade

III). The incidence of advanced disease or haematogenous metastases was higher in the grade II and grade III groups compared with grade I; there was no difference in the clinicopathological variables between grade II and grade III tumours. A significant difference was demonstrated between each of the three groups when analysed in terms of MVD: the higher the grade, the higher the mean MVD. In univariate analysis of survival, patients with grade I tumours survived significantly longer than those with grade II or grade III tumours; however, this relationship was not independent of MVD when analysed using a Cox proportional hazard model (Matsumura et al., 1998). These results suggest that both stromal and tumour TP are important in angiogenesis, with higher MVD being found in those tumours that expressed both.

In contradiction to all the above studies, Saito and colleages could not demonstrate a relationship between MVD and TP expression. In addition, they found an inverse correlation between TP expression and both lymph node and haematogenous metastases. This may be explained in part by the grading system employed, as they only graded stromal expression of TP (Saito et al., 2000). TP is expressed in approximately 80% of colorectal liver metastases (Collie-Duguid et al., 2001). There is little, if any work available on the prognostic significance of TP expression in colorectal liver metastases.

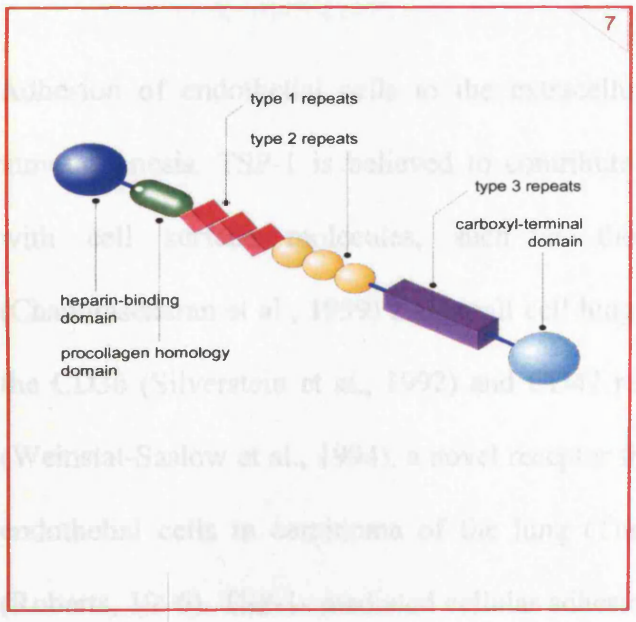
1.10 Thrombospondin

1.11 Structure

Thrombospondin (TSP-1) is a large glycoprotein, which is stored in the alpha granules of thrombin-stimulated platelets, secreted in response to platelet-derived growth factor and incorporated into the extracellular matrix. It is also a major secretory product of several vessel wall cells including smooth muscle cells, endothelial cells and fibroblasts (Baenziger et al., 1971; Bornstein, 1992; Sargiannidou et al., 2001; Majack et al., 1986).

Five subclasses of thrombospondin exist, TSP 1-5, of which TSP-1 and TSP-2 are the most closely related structurally. TSP-1 consists of a heparin domain, a pro-collagen-like domain, type 1,2 and 3 repeats and a cell-binding domain (Sheibani and Frazier, 1999) as illustrated in Figure 1.3.

Figure 1.3 Structure of TSP-1



The pro-collagen domain and the type 1 repeats, shared by TSP-1 and 2 are believed to confer anti-angiogenic properties and impair migration of endothelial cells in vitro(Weinstat-Saslow et al., 1994). It is the multi-domain structure of these molecules that enable it to interact with a variety of molecules.

1.12 Functions of TSP-1

Thrombospondin-1 modulates platelet aggregation, wound healing, protease activity and cellular functions such as adhesion, motility and growth (Lawler, 1986). However, the precise function of TSP-1 in angiogenesis and tumour growth is not straightforward as controversy surrounds the actual role of TSP-1 in neovascularisation. Evidence suggests that TSP-1 has both pro-angiogenic and anti-angiogenic properties, as described below, thus displaying a regulatory role in tumour neovascularisation.

1.12.a Cellular adhesion

Adhesion of endothelial cells to the extracellular matrix is an important step in tumourigenesis. TSP-1 is believed to contribute to cellular adhesion by interacting with cell surface molecules, such as the integrins in breast carcinoma (Chandrasekaran et al., 1999) and small cell lung cancer cell lines (Guo et al., 2000), the CD36 (Silverstein et al., 1992) and CD47 receptors in certain breast neoplasms (Weinstat-Saslow et al., 1994), a novel receptor found in invasive malignant cells and endothelial cells in carcinoma of the lung (Tuszynski et al., 1993), and heparins (Roberts, 1996). TSP-1- mediated cellular adhesion can prevent cellular proliferation,

and it has been shown in small cell lung cancer cell lines that it promotes neuroendocrine rather than carcinogenic differentiation. (Guo et al., 2000)

1.12.b Cellular motility

Cellular motility is promoted by chemotaxis in response to TSP-1 exposure. Receptors such as CD47 promote cellular motility by signalling pathways that modulate integrin-mediated cellular migration (Wang et al., 1999). It is also possible that different fragments of the TSP-1 molecule can be responsible for different angiogenesis- modulating properties which may often be opposing: a 25kD heparin-binding fragment induced a notably stronger angiogenic response in the rabbit cornea assay for neovascularisation than the TSP-1 molecule as a whole (Tarabolleti et al., 2000), whereas a 140kD fragment, completely inhibited the angiogenic response (Tarabolleti et al., 2000). TSP-1 has also been shown to promote migration at high concentrations yet inhibit migration at low concentrations if the whole TSP molecule is considered, thus emphasizing that different domains within the TSP-1 molecule may account for different tumour-modulating properties (Weinstat-Saslow et al., 1994). This is further supported by studies in breast cancer cell lines in which the deletion of a pentapeptide sequence containing a cysteine residue near the carboxyl terminus of TSP-1 in proximity to the calcium binding domain, resulted in increased tumour growth and metastatic potential (Weinstat-Saslow et al., 1994).

1.12.c Tumour invasion

TSP-1 may potentiate tumour invasion by:

- up-regulating protease components of the plaminogen/plasmin system, such as thrombin, plasmin and urokinase plasminogen activator (Sargiannidou et al., 2001)
- modulating the action of matrix metalloproteinases upon the extracellular matrix in a concentration-dependent manner
- activating other growth factors such as TGF-beta-1 (Sargiannidou et al., 2001)
- acting synergistically with other growth factors such as epidermal growth factor, which stimulates mitogenesis in smooth muscle cells *in vitro* via an autocrine pathway (Majack et al., 1986)

These processes ultimately result in the digestion of the extracellular matrix, thereby promoting tumour invasion (Sargiannidou et al., 2001).

It has also been suggested, however, that TSP-1 may inhibit tumour growth and invasion by binding to growth factors and proteases, thus preventing the digestion of he extracellular matrix (Weinstat-Saslow et al., 1994). Thrombospondin has been shown to inhibit cellular proliferation *in vitro*, as demonstrated in carcinoma cell lines (Yamashita et al., 1998).

1.12.d Vascularity

Historically, TSP-1 has been regarded as a potent inhibitor of angiogenesis. TSP-1 is found in abundance in association with mature quiescent vessels, but is absent from actively growing vessels (O'Shea and Dixit, 1988; Tolsma et al., 1997). It has been

found to suppress capillary formation *in vitro*,(Iruela-Arispe et al., 1991; Tolsma et al., 1997) possibly by inhibiting chemotaxis, proliferation, (Bagavandoss and Wilks, 1990; Weinstat-Saslow et al., 1994) and the formation of focal adhesions in endothelial cells (Murphy-Ullrich and Höök, 1989). It may be TGF beta coupled to TSP-1 that is responsible for this inhibitory effect on endothelial cell proliferation (Murphy-Ullrich et al., 1992), but not all studies support this theory (Weinstat-Saslow et al., 1994). TSP-1 also inhibits basic fibroblastic growth Factor (bFGF)- driven angiogenesis *in vivo* in the rodent eye model (Rastinejad et al., 1989).

Few clinically-based studies have examined the role of TSP-1 in tumourigenesis. Of particular interest are those that studied TSP-1 in colorectal cancer. The overall findings attribute an anti-angiogenic role to TSP-1, with improved patient survival in tumours expressing this glycoprotein, although direct comparisons cannot be made since different parameters were examined by each study.

Maeda *et al* studied TSP-1 levels by immunohistochemical methods in 150 patients with primary colon cancer, 125 having undergone curative surgery and 30, non-curative surgery and found 59% of tumours to express TSP-1. Any degree of either cytoplasmic or membranous tumour cell staining was considered positive. TSP-1 positive tumours had lower microvessel counts compared with TSP-1 negative tumours. TSP-1 expression did not correlate with the development of liver metastases, indeed, liver metastases were more frequent in TSP-1 negative primary tumours. TSP-1 expression was associated with increased disease-free survival rates (84% 5 year survival as compared with 55% in TSP-1 negative tumours). Disease recurrence was also less frequent in TSP-1 positive patients: 8% of patients developed recurrence as

compared with 81% of patients with TSP-1 negative tumours. TSP-1 was not studied in the liver metastases.. This study therefore supports the role of TSP-1 as an inhibitor of angiogenesis by decreasing tumour vascularity, thus reducing the risk of recurrence and prolonging patient survival (Maeda K et al., 2001).

In another study by Maeda and colleagues the relationship between TSP-1, VEGF and microvessel counts using 100 resected primary colon cancers was examined. An inverse relationship between TSP-1 and microvessel counts was demonstrated. Tumours with high VEGF and low TSP-1 had higher microvessel counts, and by multivariate analysis worse prognosis, with a 73% recurrence rate. Similarly, in hepatocellular carcinoma, high levels of TSP-1 and low levels of VEGF equated with low microvessel density (Kawahara et al., 1998).

Maeda *et al* failed to demonstrate a direct relationship between TSP-1 and VEGF expression, but in endometrial cancer, a strong relationship of TSP expression to weak VEGF expression has also been found, wherein extracellular intratumoural or peritumoural thrombospondin was graded as weak, moderate or strong (Salvesen and Akslen, 1999). TSP expression did not correlate with pathological features of the primary colorectal tumour, as shown by other researchers (Maeda K et al., 2001; Maeda et al., 2000; Tokunaga et al., 1999; Bertin et al., 1997).

Maeda *et al's* work also showed that TSP-1 expression independently correlated to better 5-year survival (91% versus 44% in TSP-1 negative tumours) (Maeda et al., 2000). Studies in breast cancer have described reduced TSP-1 levels in association

with more aggressive tumours (Weinstat-Saslow et al., 1994). No anti-angiogenic role was found for TSP-1 in endometrial cancer (Salvesen and Akslen, 1999).

Thrombospondin-1 may be up-regulated by other cytokines. Kawakami *et al*, in a study of 53 primary colon cancers demonstrated higher expression of TSP-1 and TSP-2 mRNA (TSP-2 also being described as an anti-angiogenic agent) in tumours expressing interleukin-10 mRNA. Vessel counts were also lower in tumours with increased interleukin-10 expression, but no relationship was found when compared to other angiogenesis markers (Kawakami T et al., 2001).

Since up-regulation of thrombospondin-1 may be a cytokine-dependent process, thrombospondin may equally need to interact with other cytokines in order to account for the anti-angiogenic effects seen in some tumours. In a study of gene expression of TSP-1 and its receptor CD36 in 65 colon cancers, Tsuchida *et al* showed that TSP-1 did not correlate with tumour vascularity, whereas CD36 correlated not only with lower vessel counts, but with improved patient survival. Activation of CD36 is believed to activate signalling pathways that inhibit angiogenesis through ill-understood mechanisms, possibly interacting in conjunction with protein kinases (Dawson et al., 1997). The authors therefore concluded that the inhibition of angiogenesis described, might not be due to TSP-1 *per se,* but to the presence of its receptor CD36, which may explain the discrepancies between studies regarding the role of TSP-1 in angiogenesis (Tsuchida T et al., 1999).

To analyse the roles of both TSP-1 and TSP-2 in the risk of the development of liver metastases, Tokunaga *et al*, in a study of 61 patients with colorectal cancer (Tokunaga et al., 1999) found TSP-1 gene expression in 6.5% of primary tumours, TSP-2 gene

expression in 28% of tumours and gene expression of both markers in 34% of tumours. Both TSP genes were more significantly expressed in the primary tumour than in the surrounding tissue. Interestingly, TSP-1 expression did not correlate with the development of liver metastases as documented by Maeda *et al* (Maeda K et al., 2001) whereas, TSP-2 expression was associated with a lower incidence of metastatic disease, 15.8% as compared to 43.5% incidence in tumours not expressing TSP-2. In addition, 71.1% of patients with hepatic disease did not express TSP-2 compared with 37.5% who did express this marker. The relationship between TSP-2 and VEGF was examined, which statistically showed better prognosis in patients with TSP-2 expression and no VEGF 189 expression. Conversely, VEGF189 expression was associated with higher venous invasion. TSP-2 has been shown in endothelial migration assays to inhibit endothelial cell migration, but in higher concentrations than TSP-1 (Volpert et al., 1995).

Plasma TSP-1 levels have also been examined. Yamashita *et al*, in a study of 115 patients with colorectal cancer, measured the concentration of plasma TSP-1 levels by enzyme-linked immunoadsorbent assay (ELISA) and correlated the results with Dukes' stage and venous invasion. Plasma levels were statistically increased in Dukes' B to D as compared with controls, in Dukes' A-C tumours with no venous invasion, and in Dukes' A, C and D tumours with venous invasion. Plasma levels were linearly higher in accordance to degree of tumour venous invasion. Venous invasion may result in increased thrombospondin levels, most likely from platelet activation and/or from the endothelium, however, the authors argued that another source must exist to account for the increased levels in patients with no venous invasion. They further argued that high plasma thrombospondin levels might promote

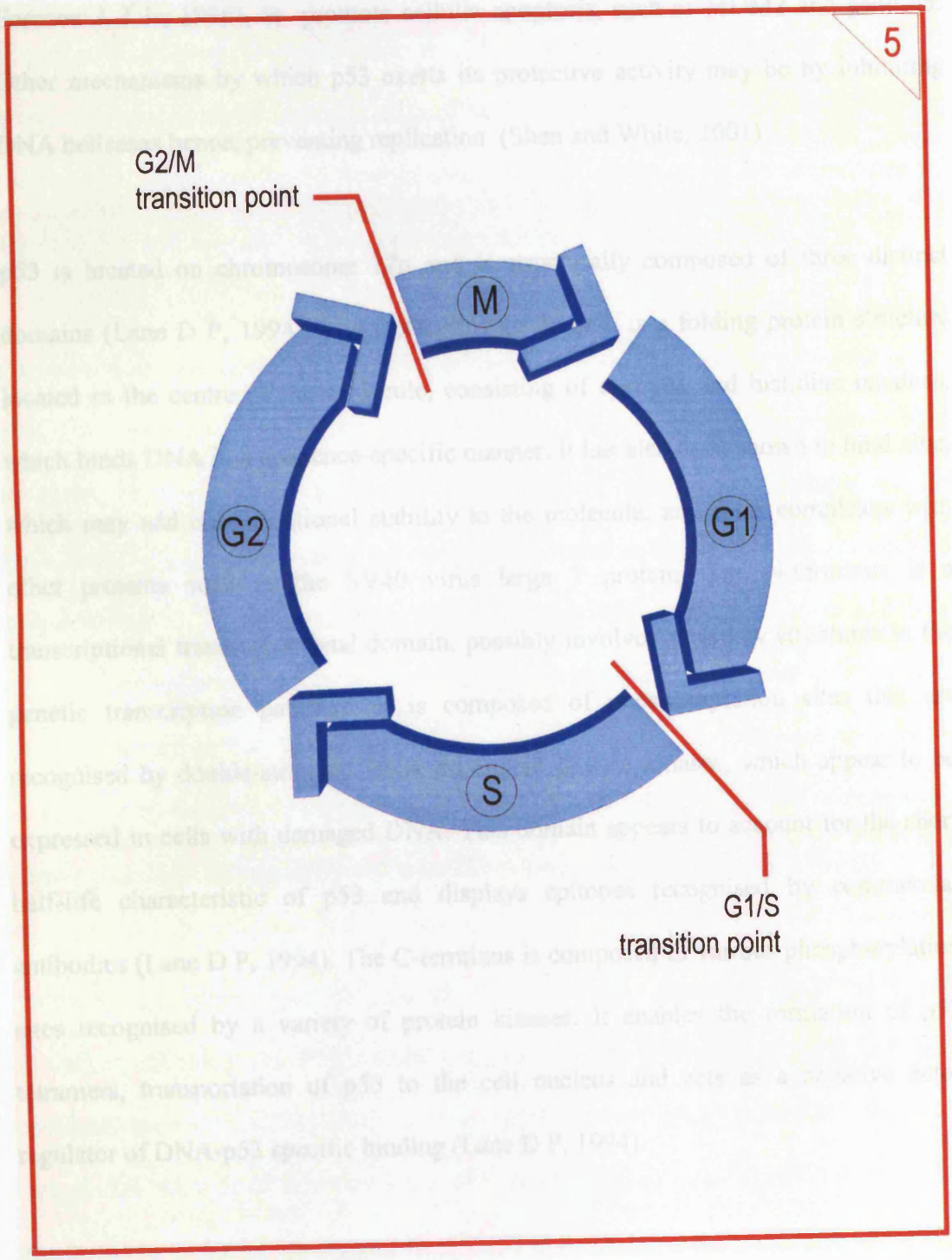
metastatic disease, as high thrombospondin stimulated cell invasion in experimental models, and because patients with venous invasion have been shown to be at greater risk of developing hepatic metastases (Yamashita et al., 1998). Therefore TSP-1 has been proposed as a marker of possible hepatic dissemination, contrary to the results obtained in immunohistochemical studies (*vide supra*) (Maeda K et al., 2001).

It can therefore be surmised that although the main role of TSP-1 appears to be anti-angiogenic, the exact mechanisms by which it exerts its effects are open to speculation. Some studies have not demonstrated a correlation between TSP-1 and markers of angiogenesis, and others have further postulated that it may be the action either of separate TSP-1 domains or indeed of its receptor(s) rather that TSP-1 itself that accounts for the modulation of tumour vascularity. It has also been suggested that the actions of TSP-1 may result from coupling to other growth cytokines such as TGF-beta. The presence of TSP-1 appears to confer a survival advantage, but more data are required.

1.11 p53

Cell propagation is achieved via a tightly regulated pathway known as the cell cycle (Figure 1.4). Throughout the cycle, a number of distinct transition zones or checkpoints exist to prevent cells with genetic damage from propagating, thus protecting against the development of neoplasia. Specific genes control each checkpoint: the G1 phase by the tumour suppressor genes and the G2 phase by proto-oncogenes.

Figure 1.4 The cell cycle



p53 is one such tumour suppressor gene, which acts to prevent genetic damage by detecting damaged DNA and up-regulating genes that either arrest the cell cycle to allow for DNA repair, most notably p21 (WAF-1) (Lane D P, 1994; Smith M L and Fornace A J Jr, 1996), or promote cellular apoptosis, such as MDM2 and gadd 45. Other mechanisms by which p53 exerts its protective activity may be by inhibiting DNA helicases hence, preventing replication (Shen and White, 2001)

p53 is located on chromosome 17p and is structurally composed of three distinct domains (Lane D P, 1994). The DNA binding domain is a folding protein structure located in the centre of the molecule, consisting of cysteine and histidine residues, which binds DNA in a sequence-specific manner. It has also been shown to bind zinc, which may add conformational stability to the molecule, and form complexes with other proteins such as the SV40 virus large T protein. The N-terminus is a transcriptional transactivational domain, possibly involved with key structures in the genetic transcription pathway. It is composed of phosphorylation sites that are recognised by double-stranded DNA dependent protein kinases, which appear to be expressed in cells with damaged DNA. This domain appears to account for the short half-life characteristic of p53 and displays epitopes recognised by commercial antibodies (Lane D P, 1994). The C-terminus is composed of various phosphorylation sites recognised by a variety of protein kinases. It enables the formation of p53 tetramers, transportation of p53 to the cell nucleus and acts as a negative auto-regulator of DNA-p53 specific binding (Lane D P, 1994).

1.11.1 Regulation of p53

The p53 response is initiated by damage to DNA. Although phosphorylation in a protein-C dependent manner is required to activate p53 (Chiarugi V et al., 1998), the overall mechanism of DNA detection remains unclear; however, coupling to a multi-protein structure may be involved. Control of p53 activity is achieved by proteins that bind to P53, which prevent it from binding to DNA, and target it for degradation by the ubiquitin pathway and by mechanisms of self-regulation (Lane D P, 1994).

The most common genetic change in solid tumours is mutation of the p53 gene (Lane D P, 1994; Smith M L and Fornace A J Jr, 1996). The mutated gene is unable to bind to DNA, -either due to molecular conformational changes or changes in the constituent residues-, decreasing the rate of DNA repair and apoptosis, thus promoting tumourigenesis. In addition, it has been suggested that early mutation in p53 may confer increased susceptibility to cancer through increased genomic instability, which predisposes to further cancer-generating mutations. The Li Fraumeni syndrome, wherein germ-line mutations predispose individuals to the development of neoplastic disease, exemplifies this (Smith M L and Fornace A J Jr, 1996).

Although numerous types of mutations have been described, point missense mutations, affecting the DNA binding domain of the p53 gene remains the most common (Lane D P, 1994; Tullo A et al., 1999). In the heterozygous form, this may increase the capacity of the mutant allelle to inactivate the remaining wild -type allele, which would promote tumorigenesis in the first instance, and secondly, result in the expression of proteins that are more metabolically stable, thus confering a survival advantage for cancer cells (Lane D P, 1994; Smith M L and Fornace A J Jr, 1996).

Mutations to p53 therefore result in genomic instability which itself predisposes to the development of neoplasia, and promotes tumorigenesis by impairing the protective mechanisms usually triggered by damage to DNA.

Point mutations of the p53 gene and the expression of p53 protein has been determined by a variety of methods in solid tumours, and correlated with other factors promoting tumourigenesis and patient survival. Genomic analysis (PCR amplification, for example) has been used to isolate p53 mRNA and determine its sequencing (Heide I et al., 1997), and immunohistochemistry has been used to detect the expression of mutant p53 protein in tissues (Heide I et al., 1997; Kondo et al., 2000). Greater than 20% of cells demonstrating nuclear staining of p53 are considered positive as reports have correlated this percentage with p53 gene mutations by PCR (Ishida et al., 1997). In tumours where wild-type p53 is conserved, malignancy may result from loss of p53 function secondary to depleted protein C levels in response to oncogene-stimulated glycolysis (Chiarugi V et al., 1998).

1.11.2 p53 in colorectal cancer

The most common genetic alteration in colorectal cancer is loss of the p53 tumour suppressor gene (Fearon and Vogelstein, 1990). More than 50% of sporadic colorectal cancers arise due to p53 missense mutations (Tullo A et al., 1999). Point mutations mainly involving exons 5 to 9 (Kahlenberg M S et al., 2000; Yao J et al., 1996) have been associated with metastatic disease and poor outcome (Yao J et al., 1996).

The mutation rate of p53 in colorectal cancer has been reported as averaging 50-70%,

(Heide I et al., 1997; Kahlenberg M S et al., 2000; Yao J et al., 1996) independent of tumour stage, size or patient gender, although study populations only included 19 primary colon tumours in Heide's study (Heide I et al., 1997) and 56 consecutive, sporadic colorectal tumours in Kahlenberg's study (Kahlenberg M S et al., 2000).

Point mutations of p53 have also been identified in liver metastases, once again in small study populations: 70% expression rate in 33 liver metastases in Heide's study (Heide I et al., 1997), 45% expression in 40 metastases in Tullo's study (Tullo A et al., 1999), and in 7 out of 8 metastases in Yao's study (Yao J et al., 1996). A high concordance between the expression of mutations in primary and secondary colorectal tumours has been observed (Heide I et al., 1997; Tullo A et al., 1999) which may reflect positive selection for a small cohort of cells that promote disease dissemination (Heide I et al., 1997; Yao J et al., 1996). However, some studies have yielded a higher incidence of mutations in the liver metastases as compared with the primary tumour including 77% in the liver compared with 44% in the primary tumour in a study by Kastrinaki *et al* (Kastrinakis W V et al., 1995). This study also identified mutations in the liver metastases not detected in the primary tumour, which may suggest that the metastases arose from a minority cohort of cells within the primary tumour (Kastrinakis W V et al., 1995; Yao J et al., 1996).

Interestingly, in mice, it was shown by one group that the effectiveness of surgical resection of the primary colorectal tumour on the subsequent development of metastases might be potentially influenced by genetic alterations to p53 (Pocard et al., 2001). All mice transfected with colorectal cells containing mutated p53 developed

metastases. Although this was a very simplistic model, it could have significant implications (Pocard et al., 2001).

p53 status has also been correlated to the number of liver metastases and the temporal distribution of metastatic disease. Tullo's work showed that patients with mutated p53 developed up to eight metastases, 70% having synchronous lesions, whereas patients with wild-type tumours developed up to four metastases and only 29% had synchronous lesions. Once again, tumour size did not correlate with p53 status.

Mutant p53 status has been correlated with overall decreased 4-year survival, 54% as compared with 71% in patients with wild-type p53 tumours, and decreased 4-year disease-free survival, 83% in patients with wild-type tumours and 62% in patients with mutant p53 tumours. Tumour stage was found to be the only predictor of disease-free survival (Tullo A et al., 1999). Correlation with recurrence failed to achieve statistical significance in Kahlenberg's study (Kahlenberg M S et al., 2000); however in Tullo's study, 73% of patients with mutant p53 developed recurrence compared with 33% of patients with wild-type p53 (Tullo A et al., 1999).

Using immunohistochemistry, p53 expression in primary colorectal cancers has been demonstrated in 50-70% of cases (Kakolyris et al., 2000). Belluco *et al* demonstrated 72% p53 expression in primary colorectal tumours in 50 consecutive patients with inoperable synchronous liver metastases, with a corresponding percentage staining in the case-matched liver metastases. De Jong found 83% p53 expression in a series of 33 primary colorectal tumours, with 71% expression in 45 corresponding resected liver metastases. In both studies, p53 expression was independent to tumour stage and

histological grade (Belluco C et al., 1996; De Jong K P et al., 1998; Kakolyris et al., 2000). Kimura *et al* also found higher p53 expression in 25 liver metastases as compared with the corresponding primary tumours by flow cytometry (Kimura O et al., 1996). The differences in detection of p53 between the primary tumours in different studies may be related to the fact that those citing higher percentage expression were found in more advanced tumours that had metastasised. Okano *et al* showed that p53 expression in the primary tumour was higher (71%) in patients with synchronous liver metastases than patients without liver metastases (55%) (Okano K et al., 1999). In de Jong's study of 38 patients who underwent liver resection for colorectal metastases, the expression of p53 in the metastases did not correlate to the temporal distribution of liver metastases or resectability of the liver lesions (De Jong K P et al., 1998).

In a study by Berney *et al* (Berney C R et al., 1999), p53 was only weakly associated with the risk of developing metastases as an independent factor, yet in combination with other markers, c-erbB-2, nm23, urokinase-like plaminogen activator, and vascular endothelial growth factor, the risk was increased.

2-year survival rates in Belluco's work (Belluco C et al., 1996) indicated a 41.7% survival in patients with p53 positive primary tumours, and 78.6% in p53 negative primary tumours; 2- year survival for p53 positive liver metastases was 50% as compared with a 95% survival in p53 negative liver metastases in de Jong's work (De Jong K P et al., 1998). On multivariate analysis, p53 status in the primary tumour was found to be the most important predictor of overall survival (Belluco C et al., 1996)

and p53 status in the liver metastases was predictive of disease-free survival after partial hepatectomy (De Jong K P et al., 1998).

Nitti *et al* (Nitti et al., 1998) examined the risk of tumour recurrence following potentially curative hepatectomies in 69 patients with a primary diagnosis of colorectal cancer. 63.8% of these metastases were p53 positive. 51 patients developed recurrence (30 to the liver only, 6 to the lungs only, and 9 to the liver plus another site, 5 to the pelvis and one to the brain). The 5-year estimated survival with p53 positive metastases was 21% and 53.1% for p53 negative metastases. Three-year survival was 31.5% and 71.8% respectively, and p53 was an independent factor associated with poor survival. Cascinu *et al* (Cascinu et al., 2002) also demonstrated over-expression of p53 and VEGF in 79 rectal tumours having received chemoradiation. Both were found to be independent prognostic factors of local recurrence (p53 overexpression), distant metastases (VEGF overexpression) and poor disease-free, and overall survival on multivariate analysis.

Paradoxically, however, Yang *et al*, in a molecular and immunohistochemical study of 39 primary colorectal cancer and 37 liver metastases (Yang Y et al., 2001), showed statistically significant better survival in patients with mutant p53 as compared with wild-type p53 in the liver metastases following hepatectomy, independent of other clinicopathological variables; the risk of recurrence was reduced after 30-35 months (Yang Y et al., 2001). Similarly, Rosty *et al* (Rosty et al., 2001) found in a series of 56 liver metastases of primary colorectal tumour origin that patients with p53 mutations in the liver metastases showed better overall survival in those patients who did not undergo a liver resection.

These, and other studies have evaluated response to chemotherapy and chemoradiation. It has been accepted that chemotherapy requires the apoptotic system to work effectively, which is mediated by p53 and members of the bcl-2 family. Therefore, any defects in the DNA repair mechanisms would be associated with chemoresistance (Cascinu et al., 2002). One study examining the association between p53 and BAX failed to demonstrate a relationship of statistical significance between BAX and p53 and BAX and survival (Cascinu et al., 2002).

However, *in vitro* work suggests that p53 negative cells respond better to chemotherapy, a finding replicated by Rosty who found better response to 5-FU in patients with mutated p53 in liver metastases compared with those with wild-type p53 (Rosty et al., 2001). Yang however, did not corroborate these results, showing no correlation between response to chemotherapy and p53 status (Yang Y et al., 2001). The reason for this discrepancy is unclear.

1.11.3 p53 and angiogenesis

It may be postulated that mutant p53 adversely affects patient survival, as demonstrated by the majority of studies, due to its role in promoting tumour angiogenesis, as evidenced by various studies examining the correlation between p53 and cytokines involved in angiogenesis. However, one study showed improved survival in patients with colorectal liver metastases displaying mutated p53.

Wild-type p53 is involved in the secretion of thrombospondin-1 (TSP-1) (Dameron et al., 1994; Dameron et al., 1994). Mutations of the p53 gene cause down- regulation of

TSP-1 mRNA in cell lines (Vermeulen et al., 1996) and up-regulation of VEGF in human embryonic kidney cells (Kieser et al., 1994); transduction of colon cancer cells with wild-type p53 decreases VEGF mRNA and protein levels (Bouvet et al., 1998). There appears to be a p53-VEGF pathway regulating tumour angiogenesis in human colorectal cancer (Kang et al., 1997; Takahashi et al., 1998). Kondo *et al* demonstrated increasing expression of p53 and VEGF in accordance with histological tumour progression (mild-to-moderate dysplasia did not localise VEGF whereas severe dysplasia and carcinoma did express VEGF), which also correlated to MVD. This may support the role of mutant p53 as an up-regulator of VEGF thereby promoting angiogenesis (Kondo et al., 2000).

It appears clear, therefore, that more research is needed, in a larger study population, to determine the relationship of p53 expression to angiogenesis and angiogenic regulatory pathways and to overall and disease-free survival.

1.12 Summary

Angiogenesis as measured by microvessel density is an important prognostic factor in colorectal primary tumours (Banner et al., 1998; Bhatavdekar et al., 1998; Choi et al., 1998; Engel et al., 1996; Frank et al., 1995; Saclarides et al., 1994; Saeki et al., 1997; Takahashi et al., 1996; Takahashi et al., 1997; Takahashi et al., 1998; Tomisaki et al., 1996; Tsuchida T et al., 1999; Vermeulen et al., 1999). Some of the most important mediators of angiogenesis in colorectal primary tumours are tissue vascular endothelial growth factor (VEGF) (Takahashi et al., 1995) (Nakata et al., 1998; Tokunaga et al., 1998) thymidine phosphorylase (TP)(Amaya et al., 1997; Nakata et al., 1998; Tokunaga et al., 1998) and thrombospondin 1 (TSP-1) (Tsuchida T et al., 1999) and p53(Kakolyris et al., 2000).

1.13 Aims and objectives of this Thesis

1.13.1 Aims

The aim of this study is to clarify the prognostic significance of angiogenesis as measured by microvessel density (MVD), and to evaluate the significance of vascular endothelial growth factor, p53, thrombospondin and thymidine phosphorylase in resected colorectal metastases.

1.13.2 Objectives

To determine:

- The relationship between microvessel density in colorectal liver metastases and outcome following a potentially curative liver resection, using the following counting techniques:
  1) Weidner's intra-tumour and tumour edge hotspot method.
  2) Random intra-tumour and tumour edge vessel counts.
- Protein expression of pro-angiogenic markers (VEGF, TP) and anti-angiogenic markers (TSP-1 and p53), as determined by immunohistochemical techniques.
- The prognostic significance of microvessel density and the aforementioned angiogenesis-modulating factors on patient survival by univariate and multivariate analysis.
- To construct a prognostic angiogenesis biological model for colorectal cancer with a view to developing targeted chemotherapy.

Chapter 2

Patients and Methods

2.1 Introduction (Miller, 2002)

Immunohistochemistry is a series of techniques used to detect specific cell components (proteins, carbohydrates or lipids) and render them visible by light microscopy. These cell constituents are termed antigens, which can be detected using antibodies that specifically bind to antigens by a "lock and key" mechanism: their complementary structures interlock and are held together by electrostatic forces, van der Waals' forces, and hydrogen bonds.

Antibodies consists of a basic protein core structure formed by two kappa or lambda light chains and two heavy chains, each composed of constant and variable domains linked by inter and intra-chain disulphide bonds. The variable domains sub-classify these immunoglobulins into five distinct categories: IgG, IgM, IgA, IgE and IgD, of which IgG and IgM are the two principal antibodies used in immunohistochemistry. In addition to the core heavy and light chains, the latter being further composed of two monovalent antigen-binding fragments (Fab), and one bivalent antigen binding fragment F(ab')2 and one or two crystalline fragments (Fc), IgG achieves further structural specificity by displaying hypervariable domains on the light and heavy chains, and in the hinge region on the heavy chain. Two heavy chains, two light chains and a J chain arranged as a pentamer characterise IgM.

Antibodies can either be derived from identically cloned plasma cells harvested from antigen-inoculated mice (monoclonal antibodies) that can then bind to a specific antigen site, or from a variety of cells harvested from antigen inoculated rabbits (polyclonal antibodies) that bind to various epitopes on a specified antigen.

For immunohistochemical purposes, optimal antibody activity is achieved by using appropriate dilutions (the ratio between antibody concentrate to volume of diluent). Although the manufacturer usually recommends optimal dilutions, the researcher confirms this figure experimentally.

Once an antibody-antigen complex is formed, it needs to be labelled to allow visualisation by light microscopy. The principal labelling molecules are most notably horseradish peroxidase and alkaline phosphatase enzymes, which, upon incubation with a chromogen, most notably 3,3'-diaminobenzidine tetrahydrochloride (DAB), generate a coloured precipitate.

Non-specific staining using these visualisation methods, recognised as a homogenous trace of colour, may occur due to the endogenous tissue presence of peroxidases and alkaline phosphatases, and background staining, caused by ionic protein-antibody interactions away from the specific antigen site. To ensure that the final precipitate has been generated by specific, antibody-antigen binding only, endogenous substances and background staining must be blocked. Endogenous peroxidases are blocked using hydrogen peroxide, and levamisole blocks endogenous alkaline phosphatase. Incubating tissue with serum that will not bind to the antigen-antibody complex reduces or eliminates background staining.

2.2 The study population

All patients who underwent a liver resection for colorectal liver metastases at the University Hospitals of Leicester NHS Trust and the Royal Liverpool Infirmary from 1993-1999 were identified. Data derived from Liverpool was prospective, and all data from Leicester was retrospective from 1993-99, and prospective from 1999 onwards. The patients' medical notes were reviewed and a database compiled detailing patient demographics, and a series of clinico-pathological factors including:

- Site of disease (colon or rectum)
- Dukes' stage of primary tumour
- Number of metastases (one or greater than one metastasis) (Nordlinger B, 1996), less than three or greater than three metastases (Fong et al., 1999))
- Unilobar or bilobar disease
- Synchronous or metachronous lesions (metachronous being defined as occurring greater than 12 months from detection of the primary) (Fong et al., 1999)
- Lesion size (less than, and greater than 5 cm diameter) (Fong et al., 1999), and median diameter
- Clearance at the resection margin
- Tumour recurrence
- Survival data.

In Leicester, paraffin-embedded tissue blocks pertaining to each resected liver metastasis were obtained from the Department of Pathology. Tumour edge sections (abutting normal hepatocyte tissue) were selected with a Consultant Histopathologist.

Paraffin-embedded tissue sections were similarly obtained from Liverpool. The Liverpool Tissue Bank also comprised an anonymised patient database detailing the same information as the Leicester database.

2.3 Immunohistochemical techniques

Direct and indirect antibody-antigen links and visualisation are most commonly employed in immunohistochemical detection methods. Direct techniques apply enzyme- linked primary antibodies directly onto the antigen generating a complex that is then visualised with a chromogen. Indirect techniques were used in this thesis and involve the use of a chromogen-detected, labelled secondary antibody directed against the unconjugated primary antibody that targets the antigen.

2.31 The Dextran Polymer Conjugate Two-step Visualisation system (Envision, DAKO™, Ely, UK)

This is a two- stage visualisation system that uses dextran polymers conjugated to the secondary antibody followed by chromogen.

1) The primary mouse antibody binds to the antigen.
2) The secondary goat anti-mouse immunoglobulin conjugated to a peroxidase-labelled polymer binds to the primary antibody.
3) Substrate-chromogen solution reacts with the peroxidase, generating a brown precipitate detectable by light microscopy.

2.32 The avidin- biotin horseradish peroxidase technique

This method uses the affinity of streptavidin (avidin) for biotin. Streptavidin is preferable to avidin as it has a neutral isoelectric point and lacks oligosaccharide residues, thus reducing the possibility of interactions with charged cell constituents and hence non-specific background staining.

The primary mouse antibody is applied to the tissue section to bind to the antigen.

A secondary anti-mouse, biotin-bound antibody binds to the primary antibody.

The pre-formed Avidin-Biotin-enzyme Complex (ABC) containing biotinylated alkaline phosphatase binds to the biotin on the secondary antibody.

Chromogen detection is undertaken using DAB (see Table 2.2)

All methods used in this thesis are summarised in Table 2.1.

2.33 Preparation of tissue slides

Formalin-fixed, paraffin-embedded dehydrated tissue blocks were cut into 5μm sections using a microtome and mounted on silane-coated (Surgipath, UK), glass slide.

Racked slides were placed in a 60^{o} oven for 5 minutes to melt the paraffin wax, except for the detection of p53, as dry heat is believed to denature the protein.

Sections were de-waxed in xylene (Genta Medical) and re-hydrated through graded alcohols (99%, 99%, 95% Industrial Methylated Spirit, Genta Medical) by periodic agitation for approximately two minutes.

2.34 Antigen retrieval

Formalin fixation and paraffin wax cross-linking may mask antigen epitopes. Antigen retrieval techniques therefore become necessary to unmask these antigens and render them detectable by immunohistochemistry.

The following techniques were the used in the study:

2.34.a Pressure cook

Three litres of 0.01M citrate buffer (see section 2.10.1) were boiled in a stainless steel pressure cooker (Duromatic, Kuhn Rikon, Switzerland), on an electrical heated plate. Dewaxed and re-hydrated, racked slides were placed in the boiling citrate buffer and the lid sealed to allow maximum pressure to be generated. On attainment of full pressure, identified by a change in whistle pitch, the slides were left in the pressure cooker for two minutes. The pressure cooker was then placed in the sink and cooled with running cold tap water; the steam let out via the valve on the lid to reduce the pressure inside. The lid was removed and the slides were washed firstly in running water, and then in Tris-buffered saline (see Table 2.3 and section 2.10.2) for 5 minutes.

This technique was used for p53 antigen retrieval.

2.34.b Enzyme proteolytic digestion

The proteolytic enzymes trypsin and pronase were used:

A solution of 0.1% trypsin (Becton Dickinson &Co, France) in 0.1% calcium chloride (Fisher Scientific, UK) in 300mls distilled water, was placed in a Hallendahl jar and placed in a 37^0C water bath. The pH was adjusted with 0.1 M NaOH (Fisher Scientific, UK) to reach 7.8. The dewaxed and rehydrated racked slides were immersed in the solution for 10 minutes, rinsed in running water and bathed in TBS

buffer (see section 2.10.2) for 5 minutes. 1μg/ml pronase (Sigma UK) at 37^0C was utilised in a similar fashion for thrombospondin antigen retrieval.

2.34.c Microwave

Dewaxed and re-hydrated, racked slides were placed in 300mls citrate buffer and microwaved for 15 minutes. The slides were then washed in running water, and placed in TBS buffer for 5 minutes. This technique was used for VEGF antigen retrieval .

2.4 Microvessel Density

2.41 Immunohistochemistry and Image Analysis System

After immunohistochemical detection of vessels, an accurate and reproducible image analysis system was created (detailed below), that measured MVD in solid tumour types, which can be used, at minimal cost, in any department with an image capture facility.

2.42 Immunohistochemical identification of microvessels

A monoclonal antibody against CD34, a surface molecule on endothelial cells, was used to identify the vessels using the ABC streptavidin biotin immunoperoxidase development technique (DAKO^{TM}, Ely, UK).

Following de-paraffinisation and re-hydration, (*vide supra*), the liver metastases tissue sections were washed in 6% hydrogen peroxide solution (Sigma Chemicals, Germany) for 10 minutes to block endogenous peroxide and then washed in buffer.

The slides were transferred into a humid chamber and 100 µl normal goat serum pipetted onto each slide, using a Gilson's pipette. 100 µl of CD34 primary antibody was then applied and incubated overnight at 4°C. After washing in phosphate buffered saline, two drops of secondary linking antibody (reagent C, see Table 2.2) were applied for 30 minutes, followed by 100 µl of streptavidin peroxidase complex (reagents A+B, see Table 2.2) prepared 30 minutes before use, using:

1000µl TBS

1µl Streptavidin (Reagent A)

1µl Biotinylated alkaline phosphatase (Reagent B)

After 30 minutes, the slides were washed in phosphate buffer.

The chromogen 3,3-diaminobenzidinetetraclorur (DAB) was enhanced with nickel sulphate in 1M acetate buffer, as follows:

5mg DAB in 0.5ml phosphate buffered saline added to

0.5g nickel sulphate in 50 mls of buffer followed by

filtration into a Hallendahl jar and

150µl 6% hydrogen peroxide

This solution was applied to the sections for 10 minutes and washed thoroughly with water.

2.43 Optimisation of staining

2.43.a Image enhancement

Chromogen was applied to slides with nickel sulphate enhancement, without nickel sulphate enhancement and with copper sulphate instead of nickel sulphate.

2.43.b Counterstaining

Vessel staining needed to be optimised to maximise differentiation on image analysis between dark foreground stained vessels in the foreground and the light, background counterstaining of tumour tissue. Sufficient counter-stain was required to enable tissue identification and orientation on the slide.

Different counter-stains were assessed both manually and by the image analysis system for ease of navigation around the section and reproducibility. CD34 -labelled sections were counter-stained with Mayer's Haematoxylin, Methyl Green, Light Green, Neutral Red and Methylene Blue. Mayer's Haematoxylin and Methyl Green produced sharper images which were straightforward to navigate, so, contiguous sections were stained with Mayer's Haematoxylin and Methyl Green and 30 areas were counted in each using both manual counts and the Image Analysis System for comparison. Repeated staining with Methyl Green yielded considerable variability in the quality of staining so Mayer's Haematoxylin was selected as the counter-stain of choice.

2.44 Vessel patterns

The following categories were used to count the microvessels in each tissue section:

1) The hottest vessel spot at the edge of the tumour.
2) Contiguous vessel counts along the tumour edge
3) The hottest vessel spot in the tumour excluding the edge hotspots
4) Intratumour cumulative vessel counts

2.45 Computer Hardware and Software used

Images were viewed using a JVC KYF50 3-chip colour video camera connected to a Nikon microscope and captured with a Scion cg-7 frame-grabber attached to an Apple Macintosh G–3 computer. These images were imported into the program "NIH Image". This program was download free from the National Institute of Health website. This software enables computerised measurement of captured images.

A computer macro was written instructing the computer, on command, to convert the captured colour image to greyscale and subtract from it a blank background image. The macro then instructed the computer to count the number of dark foreground objects (vessels) on the screen.

2.46 Matching Computer counting to manual counting

The manual counting of vessels was considered the "gold standard" and image analysis was compared to this. As with any counting technique rules were established to allow reproducibility between users. A vessel was defined as any separate area of dark (CD34 positive) staining. The presence of a lumen or blood was not required. Any vessel falling on the lower edge or right hand border of the screen was not counted, but those falling on the top edge or left hand border were. Vessels were counted only in areas of tumour tissue. Manual counts were performed on the computer screen and in greyscale. To assist the operator and aid in data collection, a black dot was placed on each vessel using the computer mouse; the computer then recorded the number of dots. This application was written into the macro allowing the count to be performed with a single keystroke. As this did not involve any recognition analysis by the computer it was 100% accurate and did not influence the manual count in any way.

2.47 Calibrating the image analysis software

Within the macro, several variables were set to increase the accuracy of image analysis compared to manual counting. The Threshold determines a level of recognition, or contrast, for the computer to detect the difference between foreground objects and the background. A threshold level is set instructing the computer to differentiate between black and white at a set level of grey in a greyscale picture. After performing a manual count, the threshold was increased until the image analysis count was equal to the manual count. Once a threshold level was set this was repeated without further adjustment of threshold, in ten areas of tumour to assess reliability.

The size of foreground objects (vessels) that the computer recognises and counts was also set. The minimum particle size was set to exclude any background contamination whilst not excluding small vessels. The maximum particle size was set to an infinitely large number so as not to exclude a count when all vessels in a field were contiguous and thus counted as one.

As stated above, when measuring MVD, each separate CD34 stained object was counted as one vessel. Within the macro, a series of functions were defined to 'clean' the captured image by removing unwanted background staining which may produce a false positive count, and enhancing the individual vessel images in order to reduce false negative counts. These functions were called "erosions" and "dilatations" respectively. One erosion removed one layer of black pixels that lay next to white pixels, thus obliterating stray pixels; two erosions removed two layers of black pixels etc. A dilatation then restored pixels but only if they had black neighbours thereby

sharpening vessel edges. This, to some extent, prevented adjacent vessels being counted as one and single fragmented vessels being counted as many. This macro allowed for the manipulation of the number of erode and dilate functions performed before the final count was made. An area of intense neovascularisation was chosen at random and the erode and dilate functions altered until the manual count exactly matched the computer count. This formula was then applied to the section as a whole.

Once a model was created within the macro, its reliability and reproducibility was assessed by performing manual and image analysis counts in 30 randomly chosen areas, in 30 cases of superficial bladder cancer. A single image was captured for each area and digitally copied so manual and image analysis counts could be carried out in identical fields. The macro settings discussed above were not changed further throughout this experiment.

Once this model was functioning accurately with cases of superficial bladder cancer it was applied to cases of other solid tumours. Sections of 30 cases of non-small cell lung cancer and 30 cases of colonic liver metastases were stained with CD34 and Mayers' Haematoxylin as described above. For each tumour type, the image analysis macro designed for use with superficial bladder cancer was re-evaluated. Once again the threshold level and "erode" and "dilate" functions with optimised against manual counting for each tumour.

2.48 Counting vessels

The four methods of counting are illustrated graphically in Figs 2.1 and 2.2

2.48.a Tumour edge hotspot and contiguous vessel counts

The edge of the tumour (abutting normal liver tissue in a crescent- shaped manner) was identified x10 magnification. At high magnification (x200), the viewing field was adjusted so that only tumour edge was visible. Starting at the proximal end of the tumour edge contiguous areas of vessel staining were identified and counted using computerised image analysis as described above. Once the entire tumour edge was counted, the hottest vessel staining area was identified (the hottest hotspot).

2.48.b Intra-tumour hotspots

To ascertain the most intense intra-tumour vessel staining (hotspots) the entire specimen was scanned at x10 magnification, and the 10 areas of greatest vessel staining were identified. These ten areas were viewed at x200 magnification, and the vessels within each hotspot counted (as above) in the investigator's perceived order of intensity, namely, the most vascular hotspot first, and so forth.

2.48.c Cumulative vessel counts

In order to determine the intra-tumour vessel counts, three experimental phases were followed.

True random vessel counts were obtained by measuring the tumour specimen (excluding the tumour edge) using the micrometer on the microscope platform, for the length and breadth of the biopsy. These were then entered into a random number generator (Microsoft Excel '97, Microsoft inc USA) that produced microscope platform coordinates. The stage was moved to each coordinate in turn until 30 areas of viable tumour had been visualised at x200 and the vessels counted using the image analysis system described above. If less than 50% of the captured area was occupied by viable tumour then the next coordinate was used. It quickly became evident that

this method was not time efficient as a large number of captured areas had less than 50% tumour, due to fibrosis or necrosis being present.

Some specimens took over two hours to assess, and thus the method was adjusted to obtain semi-random vessel counts and cumulative mean counts. Semi-random counts were obtained by de-focusing the visual field (x200), moving the specimen longitudinally, re-focusing, and counting the vessels in the captured area. Once again, if tumour occupied less than 50% of the captured image, the process was repeated.

For cumulative mean counts, a pilot study was conducted by using 50 specimens selected at random and counted. The individual vessel count for every captured area was entered into a computerised package that calculated the cumulative mean and identified when the cumulative mean stopped changing by 10% (Microsoft Excel '97, Microsoft inc USA). In all cases five extra areas were assessed beyond that which had achieved a cumulative mean that stopped changing by 10%. This number of areas was identified as the number to be counted for the rest of the series.

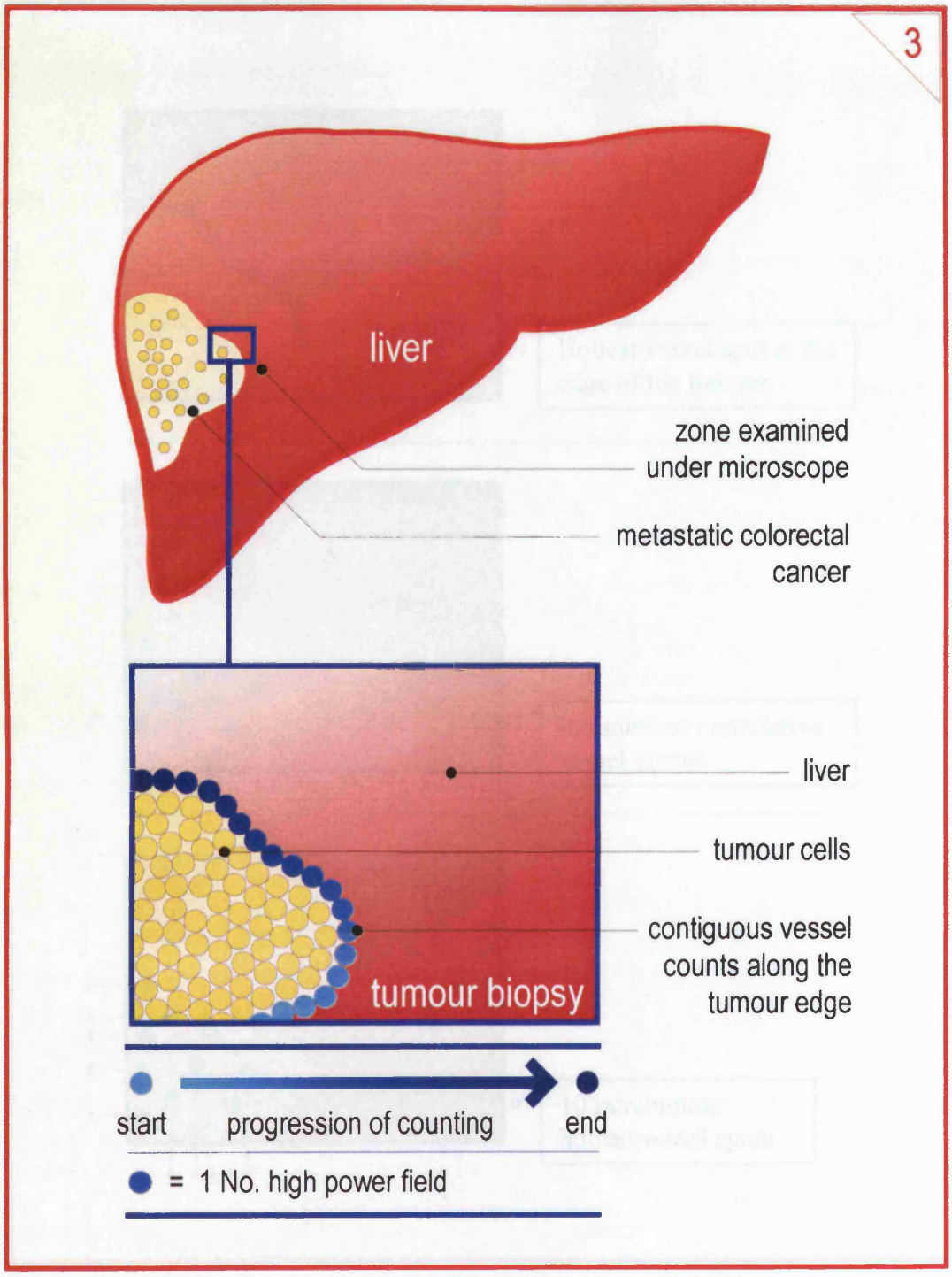


Figure 2.1 Graphic representation of the tumour biopsy and contiguous vessel counts

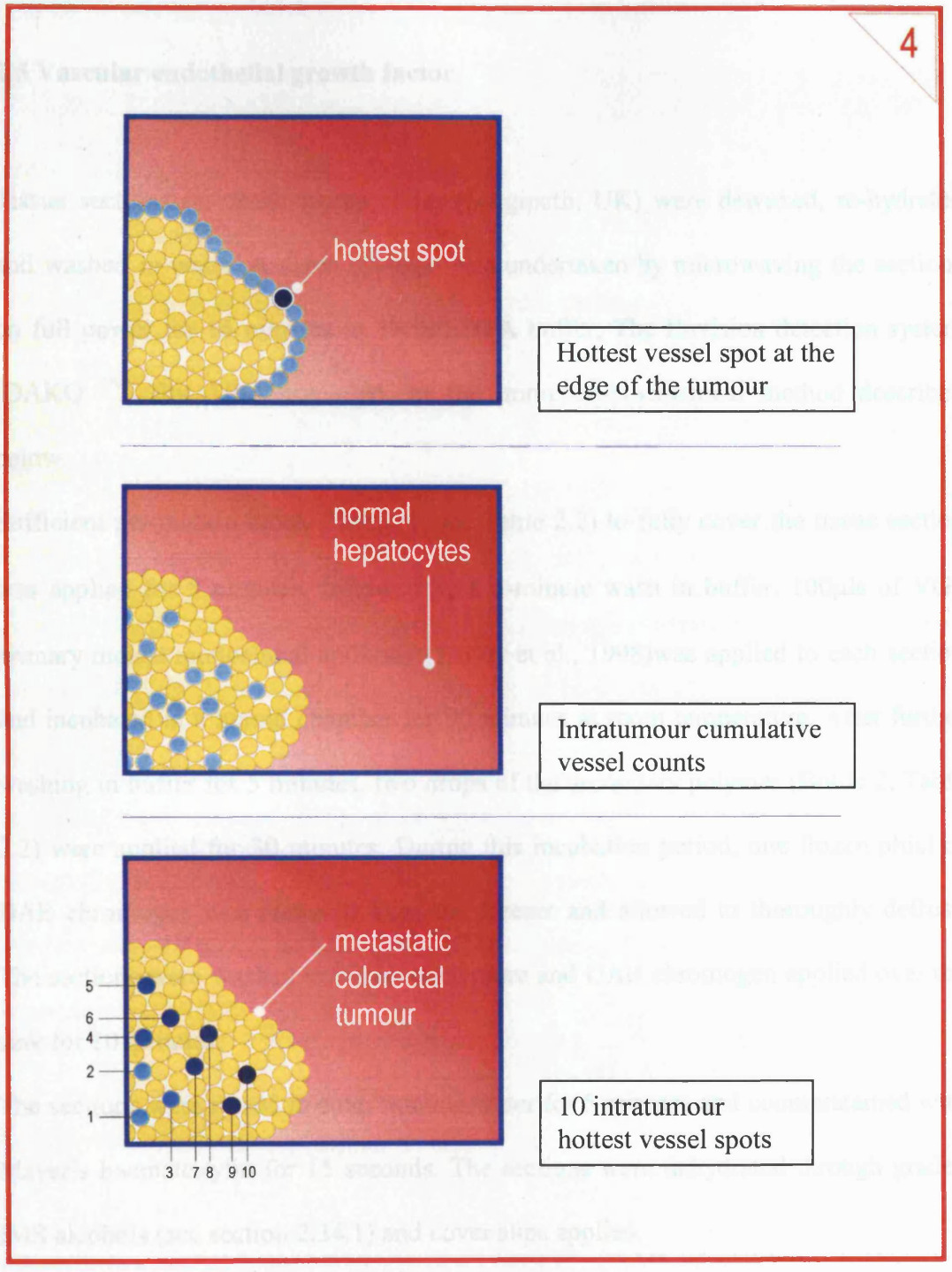


Figure 2.2 Tumour edge hot spot, intra-tumour cumulative mean and intra-tumour hotspots.

2.5 Vascular endothelial growth factor

Tissue sections on silane-coated slides (Surgipath, UK) were dewaxed, re-hydrated and washed in water. Antigen retrieval was undertaken by microwaving the sections on full power for 15 minutes in TRIS/EDTA buffer. The Envision detection system (DAKO ^{TM}, Ely, UK) was used for the immunohistochemical method described below.

Sufficient peroxidase block (Bottle 1, see Table 2.2) to fully cover the tissue section was applied for 5 minutes, followed by a 5-minute wash in buffer. 100μls of VG1 primary mouse monoclonal antibody (Turley et al., 1998)was applied to each section and incubated in a humid chamber for 30 minutes at room temperature. After further washing in buffer for 5 minutes, two drops of the secondary polymer (Bottle 2, Table 2.2) were applied for 30 minutes. During this incubation period, one frozen phial of DAB chromogen was removed from the freezer and allowed to thoroughly defrost. The sections were washed in buffer once more and DAB chromogen applied over the sink for 10 minutes.

The sections were rinsed in cold, running water for 5 minutes and counterstained with Mayer's haematoxylin for 15 seconds. The sections were dehydrated through graded IMS alcohols (see section 2.34.1) and cover slips applied.

2.51 Optimisation

The optimal dilution of VG1 was determined by varying the antibody concentration. Different antigen retrieval methods were also undertaken:

- Microwave for 15 minutes in citrate buffer
- Pressure cook for 3 minutes also in citrate buffer
- Pressure cook in microwave for 4 minutes

2.52 Interpretation of slides

A semi-quantitative grading scale devised with the help of a Consultant Histopathologist was used to determine the percentage expression of tumour cell and stromal VEGF by light microscopy at low power (x40 and x100). Slides were graded independently by the observer (CDS) and the Consultant. The inter-observer variability was calculated using a Kappa score.

The median percentage expression of VEGF in tumour cells was calculated.

The intensity of staining was scored as 1= no reaction, 2= weak reaction, 3= moderate reaction and 4 =strong reaction.

2.6 Thymidine phosphorylase

The Envision detection system was used (DAKO ^{TM}, Ely, UK).

Tissue sections on silane-coated slides were dewaxed, re-hydrated and washed in water. No antigen retrieval was necessary. Peroxidase block (Bottle 1, Table 8) was applied to each tissue section in a humid chamber for 5 minutes, followed by a 5-minute wash in buffer. 100µls of primary antibody (see Table 2.1) was applied to each section and incubated in a humid chamber for 30 minutes. After further washing in

buffer for 5 minutes, two drops of the secondary polymer (Bottle 2, Table 2.2) were applied for 30 minutes. During this incubation period, one frozen phial of DAB chromogen was removed from the freezer and allowed to thoroughly defrost. The sections were washed in buffer once more and DAB chromogen applied, over the sink, observing the safety protocol, for 10 minutes.

The sections were rinsed in cold, running water for 5 minutes and counterstained with Mayer's haematoxylin for 15 seconds. The sections were dehydrated through graded IMS alcohols and coverslips applied.

2.61 Optimisation

Dilution experiments were undertaken to confirm the optimal antibody dilution.

2.62 Interpretation of slides

A semi-quantitative grading scale was devised, with the assistance of a Consultant Histopathologist and with reference to the published literature. Slides were scanned by light microscopy at low power (x40 and x100) and high power fields (x200) to determine:

1) The overall distribution of positive stained tumour cells
2) Positively staining macrophages
3) Positively staining lymphocytes
4) Positively staining fibroblasts
5) An overall TP score

The percentage of tumour cells in the liver metastases positive for TP was recorded and the median percentage expression calculated.

The positive macrophages, lymphocytes and fibroblasts were graded using

0, +, ++, +++, for absent, minimal, moderate and extensive number of stained cells respectively. The median value was calculated.

2.7 Thrombospondin

The Envision detection system was used (DAKO ^{TM}, Ely, UK).

Tissue sections were dewaxed, re-hydrated and washed in water. Antigen retrieval was undertaken using proteolytic enzyme digestion with trypsin (see section 2.34.2). The peroxidase block (Bottle 1, Table 2.2) was applied to each tissue section for 5 minutes followed by a 5-minute wash in buffer. The primary antibody was applied to each section and incubated in a humid chamber. After further washing in buffer for 5 minutes, the secondary polymer (Bottle 2) was applied for 30 minutes. During this incubation period, one frozen phial of DAB chromogen was removed from the freezer and allowed to thoroughly defrost. The sections were washed in buffer once more and DAB chromogen applied, over the sink, observing the safety protocol, for 10 minutes. The sections were rinsed in cold, running water for 5 minutes and counterstained with Mayer's haematoxylin for 15 seconds. The sections were dehydrated through graded alcohols and coverslips applied.

2.71 Optimisation

Five different methods of antigen retrieval were undertaken using Ab1 (Oncogene Research Products), Ab4, and Ab7 (Labvision, UK) as antibodies:

Table 2.0 Thrombospondin antibody optimisation

Antibody	Concentration	Pre-treatment
Ab1	**1:10**	None Pronase digestion (2.34.b) Microwave in citrate buffer Pressure cook in citrate buffer Microwave pressure cook in citrate buffer Microwave in Tris/HCl buffer, pH1
Ab4	**1:50, 1:75, 1:100**	
Ab7	**1:100, 1:150, 1:200**	

2.72 Interpretation of slides

Slides were scanned by light microscopy at low power (x40 and x100) and high power (x200) to determine three principal patterns of staining:

1) Stromal
2) Peri-vascular
3) Tumour cell cytoplasm

A semi-quantitative grading scale was once again devised for each category, with the assistance of a Consultant Histopathologist, and with reference to the published literature. The pattern of expression was too complex to assign a % grading scale, therefore a binary score of present or absent was used. Slides were graded independently by the observer (CDS) and the Consultant. The inter-observer variability was calculated using a Kappa score.

2.8 p53

The Envision detection system was used (DAKO ^{TM}, Ely, UK).

Freshly cut tissue sections mounted on silane-coated slides were re-hydrated and washed in water. The sections were not dewaxed in the oven to avoid protein denaturation. Pressure-cooking (section 2.34.1) was used for antigen retrieval. The peroxidase block (Bottle 1, Table 2.2) was applied to each tissue section for 5 minutes followed by a 5-minute wash in buffer. The primary antibody was applied to each section and incubated in a humid chamber. After further washing in buffer for 5 minutes, the secondary polymer (Bottle 2) was applied for 30 minutes. During this incubation period, one frozen phial of DAB chromogen was removed from the freezer and allowed to thoroughly defrost. The sections were washed in buffer once more and DAB chromogen applied, over the sink, observing the safety protocol, for 10 minutes. The sections were rinsed in cold, running water for 5 minutes and counterstained with Mayer's haematoxylin for 15 seconds. The sections were dehydrated through graded alcohols and cover slips applied.

2.81 Optimisation

Dilution experiments were undertaken using different antibody concentrations.

2.82 Interpretation of slides

The number of positive and negative staining tumour cell nuclei within an intensely stained area were counted at x200 magnification and recorded. The high power field was captured in full colour using the JVC KYF50 3-chip colour video camera connected to the Nikon microscope and captured with a Scion cg-7 frame-grabber

attached the Apple Macintosh G–3 computer. Using Photoshop version 5.1, a 12x8 grid was superimposed onto the captured image and the number of positively stained nuclei and negative nuclei were counted in each square, until an overall count of 1000 nuclei was achieved.

2.9 Data analysis and statistics

Statistical analysis was performed using SPSS version 11. (SPSS Inc., USA).

Each cytokine was compared to microvessel counts, and to each other using the χ^2 and Pearson's correlation coefficient.

Bland Altman plots were used to determine the agreement between manual microvessel counts and counts generated by computerised image analysis.

The Kaplan Meier method and log rank survival curves were plotted to determine the relationship between the microvessels and each cytokine, and patient survival.

Multivariate analysis was performed using the Cox proportional hazard regression analysis.

2.10 Preparation of reagents

2.10.1 Citric acid buffer

Reagents:	42g citric acid monohydrate	(AnalR)
	500 mls distilled water	
	NaOH pellets	(Fisher Chemicals, UK)
	3M HCl	(Fisher Chemicals, UK)
	3M NaOH	(Fisher Chemicals, UK)

Method:

The citric acid was dissolved in the distilled water in a beaker on a magnetic stirrer.

The pH was adjusted using the calibrated pH meter (Hanna Instruments) to 6.0 using NaOH pellets.

The volume was made-up to 1L using distilled water in a 1L flask.

To obtain a 0.01M solution for use, a dilution of 1:20 was utilised.

For a three-litre pressure cooker, 150mls citrate buffer and 2850mls of distilled water were required.

2.10.2 Tris-buffered saline

Tris-buffered saline is utilised to wash unbound antibody from slides for 5 minutes on a magnetic stirrer, before incubation with the next antibody to prevent antibody-antigen complex precipitates. Excess buffer is wiped away from the corners of each slide with tissues. Incubation follows in a humid chamber. Please see Table 2.3 and section 2.10.2 for details of the preparation of TBS.

Quantity required:	2 litres for 25 slides	
Reagents per litre:	6g powdered Tris	(Roche Diagnostics, Indianapolis, USA)
	4.38g NaCl	(Roche Diagnostics, Indianapolis, USA)
	3M HCl	(Fisher Chemicals, UK)
	3M NaOH	(Fisher Chemicals, UK)
	1L of distilled water	

Method:

Powdered Tris was added to 50mls of distilled water in a beaker and stirred using a magnetic stirrer. Once dissolved, the powdered NaCl was added, and stirred. The pH was measured using a calibrated pH meter (Hanna Instruments) and adjusted to 7.6 by decanting drops of 3M HCl into the solution. 3M NaOH was used to reverse the effect of HCl, in the event of the solution being too acidic. The solution was made-up to 1L with distilled water.

Figure 2.3 Photograph of Nikon microscope and image analysis equipment used

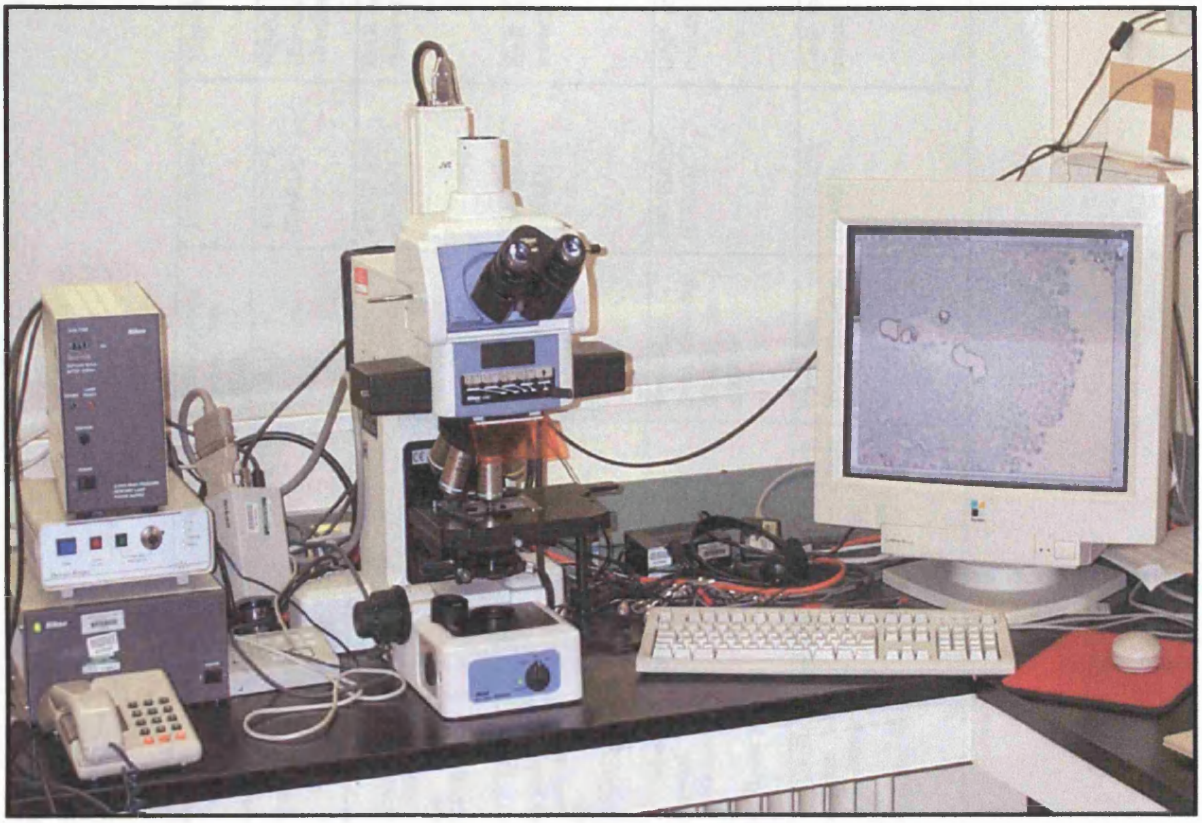


Figure 2.4 Photograph of haematoxylin and eosin stained tumour edge biopsy

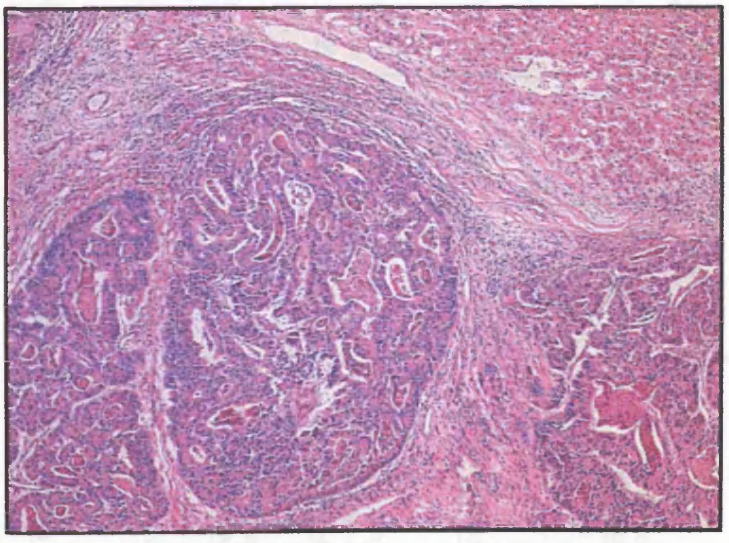


Table 2.1 Summary of microvessel and cytokine detection

Antigen	**Primary Antibody**	**Dilution of Antibody**	**Incubation**	**Diluent**	**Secondary Antibody**	**Positive control**	**Antigen retrieval**	**Endogenous peroxidase block**	**Technique**	**Visualisation**
Microvessel endothelium	Anti-CD34 mouse monoclonal antibody	1:50	Overnight at 4^0C	TBS	Biotinylated goat antimouse 1:200 for 30 minutes	Normal colon	Non required	6 % H_2O_2	ABC/HRP (DAKO)	$NiSO_4$ DAB complex (Envision DAKO) for 10 minutes
Vascular endothelial growth factor	VG1anti-VEGF mouse monoclonal antibody (gift from Oxford)	1:2	30 mins at room temperature	TBS	Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes	Kidney	Microwave	Peroxidase block (Envision, DAKO) for 5 minutes	ENVISION (DAKO)	DAB solution for 10 minutes
Thrombospondin	Ab-4 anti TSP-1mouse monoclonal antibody (Labvision, UK)	1:50	30 mins at room temperature	TBS	Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes	Tonsil	Pronase digestion 1μg/ml at 37^0C for 15 minutes	Peroxidase block (Envision, DAKO) for 5 minutes	ENVISION (DAKO)	DAB solution for 10 minutes
Thymidine phosphorylase	Anti-TP P-GF.44C mouse monoclonal antibody (Gift from Oxford)	1:20	30 mins at room temperature	TBS	Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes	Tonsil	None required	Peroxidase block (Envision, DAKO) for 5 minutes	ENVISION (DAKO)	DAB solution for 10 minutes
p53	Ab-6 anti-p53 mouse monoclonal antibody (Oncogene Research, UK)	1:100	30 mins at room temperature	TBS	Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes	Stomach	Pressure cook in citrate buffer for 2 minutes	Peroxidase block (Envision, DAKO) for 5 minutes	ENVISION (DAKO)	DAB solution for 10 minutes

Table 2.2 Visualisation systems

Kit	Reagent	Composition
ABC/AP (DAKO)	Reagent A	Streptavidin in 0.01mMol.L phosphate buffer, 0.15 sodium chloride, 15 mMol/L NaN_3. pH 7.2
	Reagent B	Biotinylated alkaline phosphatase in 0.05 mmol/L Tris/HCl, 0.1mol/L sodium chloride, 1mmol/L magnesium chloride, 0.1mmol/L zinc chloride, 15mmol/l NaN_3. pH 7.2
Envision (DAKO)	Peroxidase block (Bottle 1)	0.03% hydrogen peroxide with sodium azide
	Labelled polymer (Bottle 2)	Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins in Tris/HCl buffer with carrier protein and anti-microbial agents
	Buffered substrate solution (Bottle 3a)	Hydrogen peroxide and a preservative. PH 7.5
	DAB and chromogen (Bottle 3b)	3,3'-diaminobenzidine chromogen solution

Table 2.3 General Reagents

Reagent	Composition
Tris buffered saline (TBS)	0.05M Tris/Hcl, 0.15M NaCl at pH 7.5
Citrate acid buffer	10mM citric acid at pH 6.0
Acetate buffer	Solution A (3.8ml): 0.2M acetic acid 1.2 cm^3 glacial acetic acid in 100 cm^3 distilled water Solution B (47.2ml): 0.2 M sodium acetate 1.64g sodium acetate anhydrous in 100cm^3 distilled water pH6

Chapter 3

Clinicopathological Variables

3.1 Introduction

Colorectal cancer principally metastasises to regional lymph nodes and the liver. Up to 50% of all patients with colorectal cancer eventually develop liver involvement (Hughes et al., 1986). Of these, as many as 38% of people that eventually die from the disease may have the liver as the only site of metastasis (Fong et al., 1997). As a malignancy, colorectal cancer is unique amongst solid tumours as surgical resection of liver and lung secondaries can result in cure (Fong et al., 1997). Unfortunately, only 20-25% of patients with liver involvement are suitable for hepatic resection (Geoghegan and Scheele, 1999).

Surgical resection of colorectal liver metastases remains the only proven treatment that can offer cure, with five year survival rates quoted from 28% (Nordlinger B, 1996) to 40% (Choti et al., 2002). Up to 6 of the 8 anatomical liver segments may be removed without causing postoperative liver failure providing the remaining liver parenchyma is normal (Penna and Nordlinger, 2002). However despite radical surgery the majority of patients treated with liver resection eventually die due to recurrence of their disease (Geoghegan and Scheele, 1999). Therefore there have been moves to identify those factors that predict poor survival following liver resection.

The aim of this chapter was to assess the prognostic relevance of a number of clinico-pathological factors in terms of patient survival for a consecutive series of liver resections performed at the University Hospitals of Leicester and the Royal Liverpool Hospital.

3.2 Study population

The total number of patients who underwent liver resection for colorectal liver metastases at our institutions during the study period was 197 (Leicester n= 117, Liverpool n= 80). These patients did not have extra-hepatic disease. Patients with a 30-day operative mortality (n=7, 3.5%) and no or inadequate pathology specimens (n=8) were excluded.

The final number of patients included in the study was 182 (Table 3.1). With regards to patients treated in Leicester, 50 underwent adjuvant, post-operative chemotherapy with a combination of 5 fluorouracil and folinic acid or leucovorin and 35 patients did not receive chemotherapy.

3.3 Patient survival

Patients alive at 2 years post resection n=135 (75%)

Patients alive at 5 years post resection n=58 (32%)

3.4 Clinico-pathological variables

All clinico-pathological data analysed is presented in Table 3.1

The only clinicopathological factor prognostic for survival on univariate analysis was disease distribution: bilobar disease was significantly associated with poorer survival as compared with unilobar disease. This is illustrated in Figure 3.1a.

Table 3.1 Clinico-pathological variables and prognosis

Clinicopathological factor	Number	Log rank test
Age:		
range	25 – 81 (years)	
mean	61.6 (years)	p=0.8
median	62 (years)	
Gender:		
male	n=122	p=0.7
female	n=60	
Site of disease:		
Colon	n=99	p=0.2
rectum	n=83	
Dukes' stage:		
A	n=6	
B	n=79	p=0.7
C	n=97	
Metastases		
range	1-13	
median	n=2	
1-3 lesions	n=146	p=0.19
>3 lesions	n=36	
Metastases		
1 lesion	n=76	p=0.22
>1 lesion	n=106	
Disease distribution:		
unilobar	n=110	**p=0.01**
bilobar	n=72	
Temporal presentation of metastases:		
synchronous	n=89	p=0.37
metachronous	n=93	
Size of lesion		
range	4 - 230mm	
median	35mm	p=0.85
<50mm diameter	n=127	
>50mm diameter	n=53	
Resection margin:		
Clear	n=145	p=0.12
unclear	n=38	

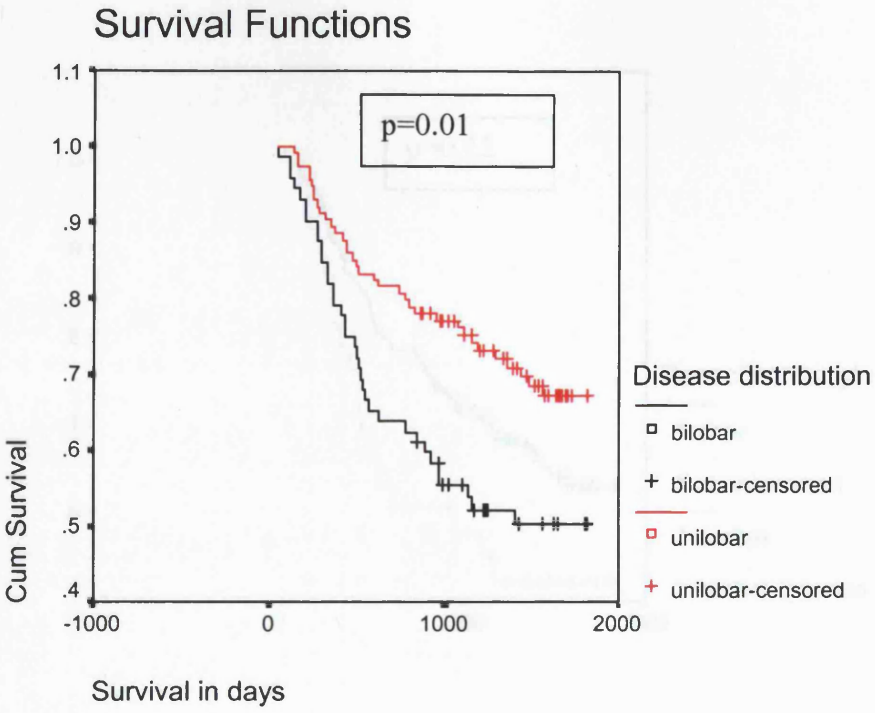


Figure 3.1a Disease distribution (unilobar versus bilobar) and survival

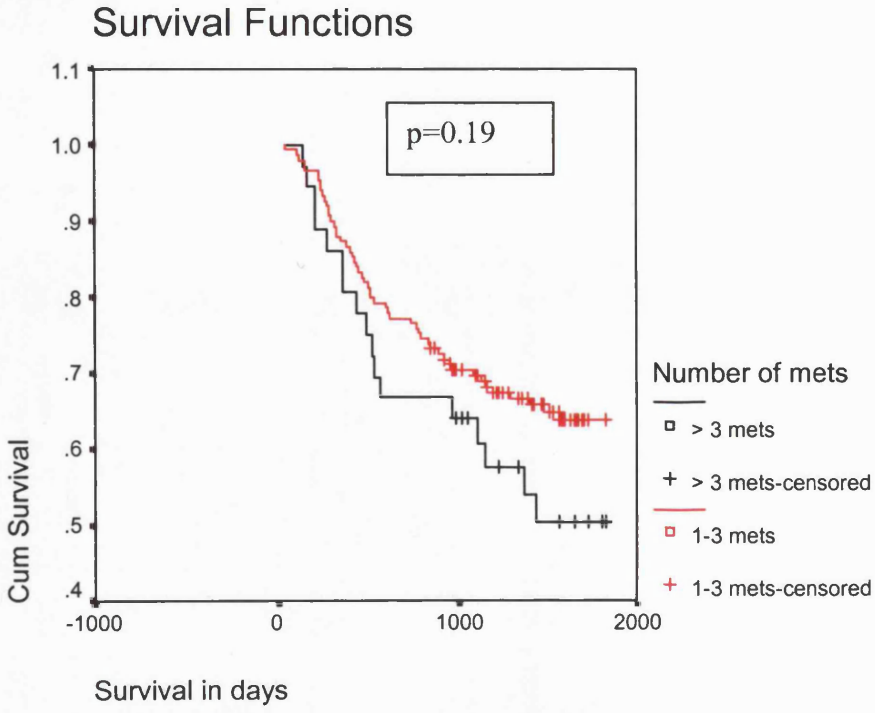


Figure 3.1b Number of metastases and survival

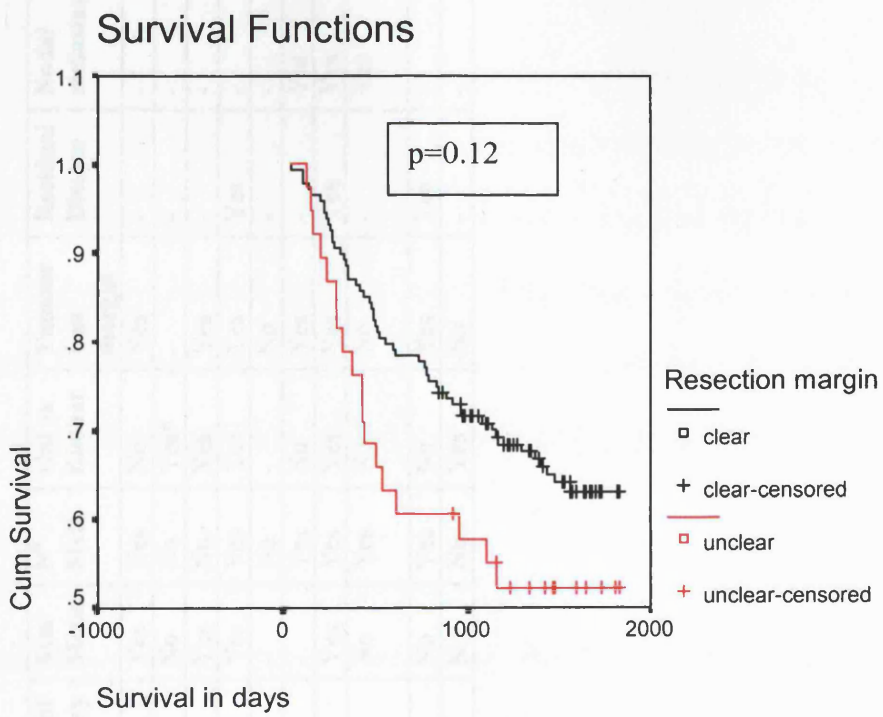


Figure 3.1c Disease at resection margin and survival

Table 3.2 Summary of studies examining clinico-pathological variables in colorectal liver metastases

Author	Year	N° Patients	Gender	Site of Primary	Pre-op CEA	Age	Tumour Size	Stage of Primary	Syn/ Meta.	N° Mets	Uni vs Bilobar	Tumour free margin	Residual Disease	Nodal metastases
(Hughes et al., 1988)	1988	859	No	-	No	Yes	Yes	Yes	Yes	Yes	No	Yes	-	-
(Doci R, 1991)	1991	100	No	No	No	No	-	Yes	No	No	Yes*	-	-	-
(Scheele et al., 1995)	1995	469	No	No	No	No	Yes	Yes	Yes	No	Yes	Yes	-	-
(Nordlinger B, 1996)	1996	1568	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-
(Jamison et al., 1997)	1997	280	-	-	-	-	No	No	-	No	-	No	-	-
(Cady et al., 1998)	1998	244	-	-	Yes	-	No	No	-	Yes	No	Yes	-	Yes
(Fong et al., 1999)	1999	1001	No	No	Yes	Yes	Yes	-	Yes	Yes	Yes	Yes	Yes	Yes
(Minagawa et al., 2000)	2000	280	No	No	No	No	No	Yes	No	Yes	No	No	-	Yes
((Choti et al., 2002)	2002	226	No	-	Yes	-	No	No	No	Yes	No	Yes	Yes	-
This study	2003	182	No	No	-	No	No	No	No	No	Yes	No	-	-

* = patients with bilobar disease and longer survival

3.5 Discussion

Information of clinico-pathological factors that influence patient survival following liver resection for colorectal metastases is interesting, and may guide patient selection for surgery. A selected series of some of the larger studies on clinico-pathological factors is summarised in Table 3.2. As can be seen from the table, the number of prognostic factors increases with increasing study populations.

The only significant clinico-pathological factor from our study was the presence of bi-lobar disease (Figure 3.1a). Other factors may have become significant if the study population had been larger; this is born out if we analyse Kaplan-Meier survival plots for the number of metastases present and survival Figure 3.1b. As can be seen from the graph, there was a trend for those patients with greater than three metastases to do worse than if less lesions were present. Surprisingly, the presence of disease at the resection margin and poor survival did not reach significance, although the trend was observed (Figure 3.1c).This may be due to the way the liver parenchyma was divided. In our study population, all patients underwent liver resection using a cavitational ultrasonic surgical aspirator (CUSA), which removes some liver parenchyma at the site of hepatic division producing a wider resection margin. Alternatively, it may be that our study was underpowered for this factor. Data on chemotherapy was not available from Liverpool, but no survival advantage was demonstrated when the Leicester patients were analysed separately ($p=0.46$).

Aggressive surgical policies may be justified as almost no patients live beyond three years when their liver disease is untreated (Fujimoto et al., 1985). In contrast, five-

year survivors occur even when a large number of negative prognostic factors are present (Fong et al., 1999; Nordlinger B, 1996). In an attempt to make the process more objective, Fong *et al* developed a scoring system based on five independently negative prognostic factors from 1001 liver resections (Fong et al., 1999), consisting of node-positive primary tumour, disease-free interval of less than twelve months, more than one liver metastases, diameter greater than 5cm and pre-operative CEA greater than 200ng/ml(Fong et al., 1999). Patients with all five negative prognostic factors still had a five-year survival rate of 14% (Fong et al., 1999), making it difficult to withhold surgery on these grounds alone. One possible exception to this may be patients with tumour spread to the hepatic pedicle lymph node, as in this group no long term survivors have been reported (Cady et al., 1998; Fong et al., 1999; Minagawa et al., 2000; Rodgers M.S and McCall J.L, 2000). However, Jaeck et al have questioned even this recently, in a prospective study. Patients with hepatic lymph node involvement were classified into two categories, those with lymph node involvement of the hepatoduodenal ligament and retropancreatic area (area 1) and those with involvement of nodes over the common hepatic artery and celiac axis (area 2). They concluded that hepatic pedicle lymph node involvement was the most significant prognostic indicator in their study, but patients with nodes limited to area 1 had longer survival than area 2 (Jaeck et al., 2002). Although prospective, the number of patients with lymph node involvement in this study was only 17 (Jaeck et al., 2002), when compared with the meta-analysis of fifteen studies that provided survival data on 145 node positive patients. Only five patients were alive at five years, two of whom had recurrent disease (Rodgers M.S and McCall J.L, 2000). In our study, one patient had hepatic lymph node involvement, and died of recurrent disease within six

months. Therefore, the presence of hepatic lymph node involvement should probably remain a contraindication for liver resection until further data are published.

Following liver resection for colorectal metastases, 60% to 70% of patients develop recurrence of their disease (Fong et al., 1999; Hughes et al., 1986; Scheele et al., 1995). Of these, approximately 30% will have metastases isolated to the liver. With advances in the approach to liver surgery repeat resections are being advocated for such patients (Nagakura et al., 2002; Petrowsky et al., 2002). In a bi-institutional retrospective analysis of 126 patients who underwent secondary resections for hepatic recurrence, 1, 3 and 5-year survival rates were 86%, 51% and 34% respectively (Petrowsky et al., 2002). Similar adverse prognostic factors were observed in these patients: more than one lesion and tumour size greater than 5cm diameter (Petrowsky et al., 2002). In our study population there were nine patients that underwent repeat resections (male n=6), two of whom are currently disease free.

In summary, the analysis of prognostic factors derived from the study of tumour biology may be useful as their presence will not necessarily preclude a surgical approach, but may help to select targeted chemotherapy as an adjunct to surgery.

Chapter 4

Microvessel Density

4.1 Introduction

Historically, Strohmeyer *et al* demonstrated that metastatic liver tumours up to a tumour diameter of 200μm derive their blood supply via liver sinusoids. To grow beyond this size, neovascularisation occurs (Strohmeyer et al., 1987). Teramaya claimed that microvessel density in colorectal liver metastases reaches a steady state when the tumour diameter was 3mm or above (Terayama et al., 1996).

Numerous publications have established angiogenesis as important prognostic factor in solid tumours. The most commonly used method to count microvessels was popularised by Weidner *et al*, who demonstrated that the most vascular area of a tumour, or hotspot, correlated to metastases in breast cancer (Weidner et al., 1991). A hotspot was subsequently found to be representative of whole tumour vascularity in breast (Martin et al., 1997). Similar findings have been documented for primary colorectal tumours, using Doppler assessment and microangiography (Konerding et al., 2001; Ogura et al., 2001) however, multiple hotspots were assessed. Work in breast tissue has postulated that in order to detect the hottest spot in every case, 10 hotspots need to be counted (Martin et al., 1997). Furthermore, computerised image analysis was found to decrease intra and inter-observer variability when assessing tumour MVD (Hansen et al., 1998).

Two centres have published data on the prognostic significance of vessel counts in colorectal liver metastases, and yielded contradictory results (Mooteri et al., 1996;

Nanashima et al., 2001; Nanashima et al., 1998). Therefore, the clinical relevance of angiogenesis in this situation remains to be clarified.

4.2 Aims of the study

1. To create a low cost image analysis system that was accurate and reproducible for multiple solid tumour types.
2. To use the image analysis system to determine the number of hotspots that need to be counted in order to ensure the 'hottest' is evaluated in a series of colorectal liver metastases
3. To determine the relationship between clinicopathological factors and MVD.
4. To assess the prognostic relevance of the microvessel counts from the tumour edge (both hotspot and mean of vessel counts for contiguous tumour edge areas), plus intratumoral vessel counts (hotspot and cumulative mean) in paraffin embedded tissue sections from tumour samples taken from a consecutive series of liver resections performed at the University Hospitals of Leicester and the Royal Liverpool Hospital.

4.3 Development of a low cost image analysis system

This was performed as described in the Chapter 2.3 (*vide supra).*

Sections of superficial bladder cancer were used to optimise the image analysis system. The papilliform nature of this tumour resulted in fragmented biopsies upon resection, which coupled with diathermy artefact, made formalin fixed paraffin embedded tissue sections of resected superficial bladder cancer difficult to assess. Indeed, some authors claim that MVD assessment is not possible in sections of superficial bladder cancer (Dickinson et al., 1994). The assumption was made that if

an accurate reproducible image analysis system could be produced using superficial bladder cancer it should work when evaluating other solid tumour sections.

The anti-CD 34 antibody was chosen as a pan-endothelial marker as it was reliable, strongly expressed on microvessels with minimal background and little or no tumour and inflammatory cell staining was seen (Martin et al., 1997).

4.31 Chromogen enhancers

Nickel sulphate was selected as an enhancer to chromogen visualisation of vessels as it yielded increased contrast, and the quality of staining was overwhelmingly superior to copper sulphate or no enhancement (Figure 4.1a-d).

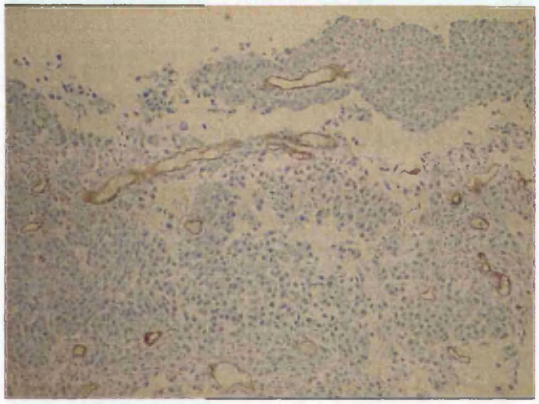


Figure 4.1a
Colour copper sulphate enhanced DAB

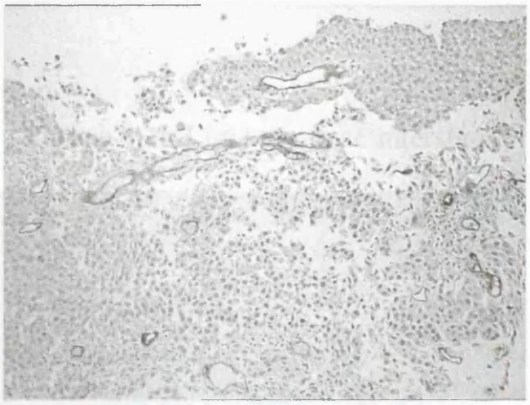


Figure 4.1b
Greyscale copper sulphate enhanced DAB

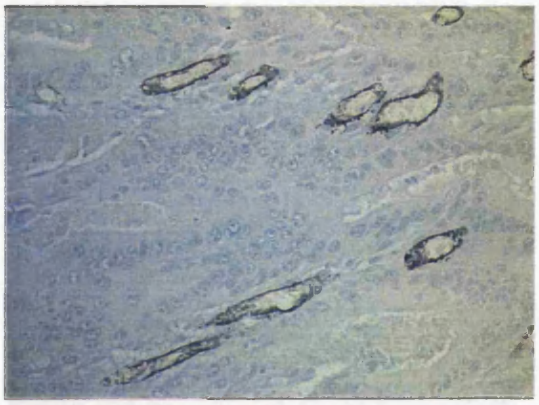


Figure 4.1c
Colour nickel sulphate enhanced DAB

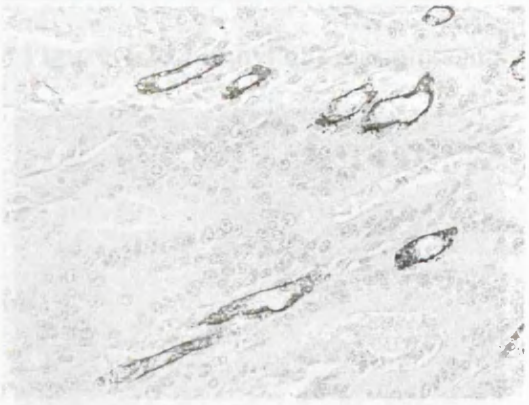


Figure 4.1d
Greyscale nickel sulphate enhanced DAB

4.32 Counterstaining

Mayer's haematoxylin was chosen as a counterstain as it produced clear and easy to navigate slides. In superficial bladder cancer, a one second exposure time was chosen as the correlation coefficient (r^2) between image analysis and manual counts using haematoxylin for one second $r^2 = 0.88$. This correlation decreased with greater exposure times (five seconds $r^2 = 0.002$ and for 10 seconds $r^2 = 0.089$). Of the remaining counterstains used during optimisation, methyl green, although also good for clarity and navigation, was not easily reproducible. Light green, neutral red and methylene blue were found to stain unevenly leaving "islands" of colour identified by the computer as vessels (Figures 4.2a-d).

Figure 4.2a Methyl green counterstain

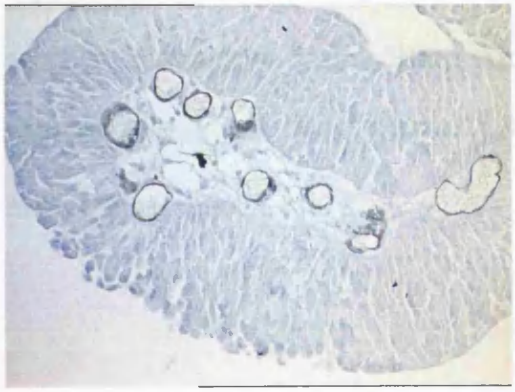


Figure 4.2b Light green counterstain

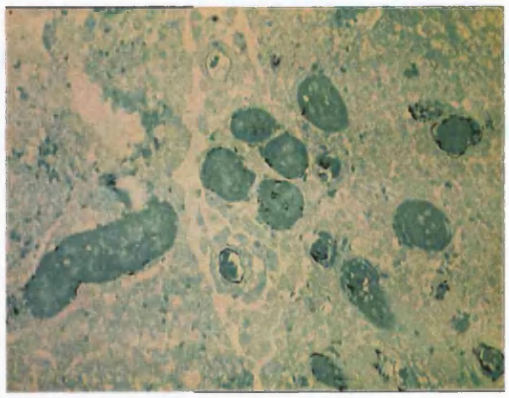


Figure 4.2c Neutral red counterstain

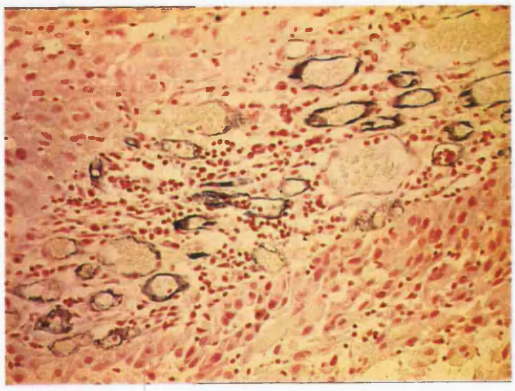
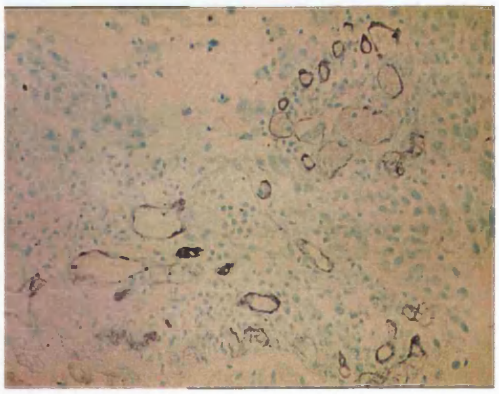


Figure 4.2d Methyl blue counterstain



4.33 Threshold levels

The most accurate threshold level was found to be 100 for superficial bladder cancer and 105 for colorectal liver metastases on a scale of 0-255. The minimum particle size representative of a vessel was found to be 200 pixels for superficial bladder cancer and 50 pixels for colonic liver metastases. The best erode and dilate series was erode n=1, dilate n=5 and erode n=3.

4.34 Correlation between manual and image analysis-generated microvessel counts

A pictorial summary of the process of manual and image analysis-generated vessel counts can be seen in Figure 4.3a-d. Using Pearson's correlation coefficient, comparison of image analysis and manual counts in the 30 areas of 30 cases of superficial bladder cancer gave a coefficient of 0.96. In colonic liver metastases the coefficient was 0.94. Using Bland Altman plots the mean difference for superficial bladder cancer was 1.37 (SD = 1.57) and 1.39 (SD 1.37) for colorectal liver metastases (Figure 4.4).

Figure 4.3a Colour image

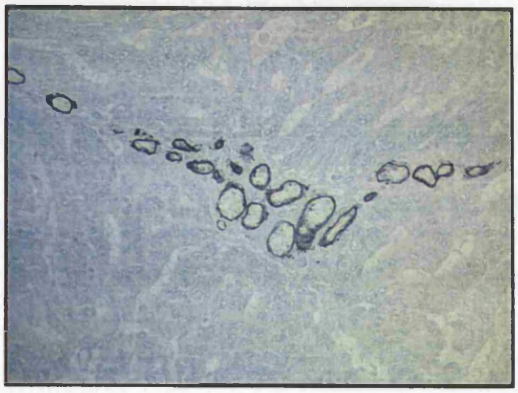


Figure 4.3b Greyscale image

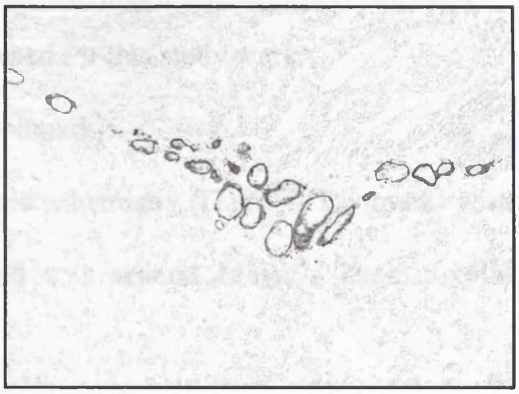


Figure 4.3c Manual count

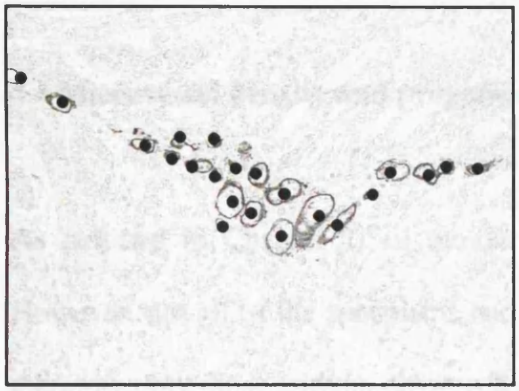


Figure 4.3d Computer generated count

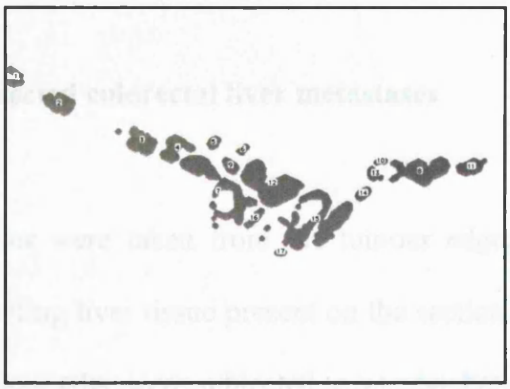
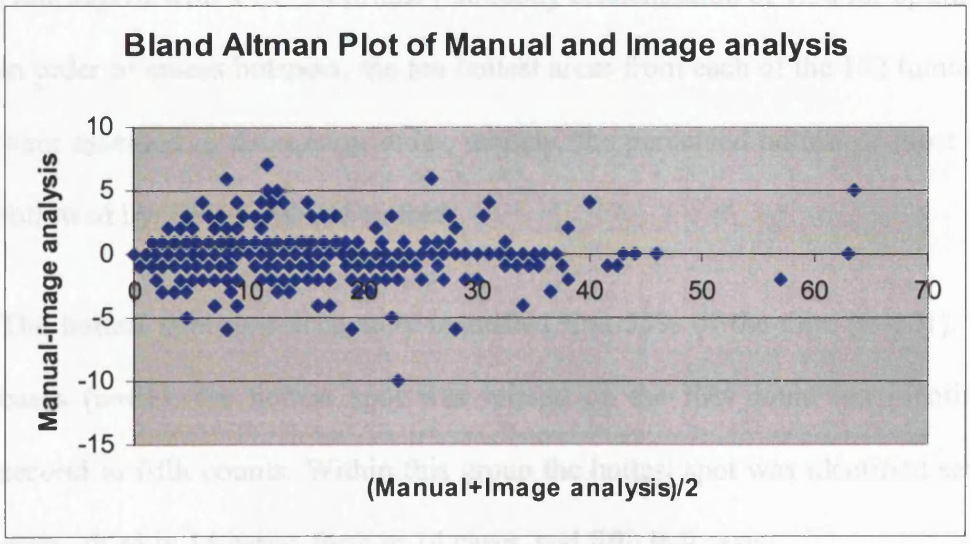


Figure 4.4 Bland Altman plot of colorectal liver metastases



4.35 Summary of image analysis

The final staining and image analysis process used for this study were:

1) Monoclonal antibody to CD 34 (Dako^{TM}, Denmark).

2) ABC streptavidin-biotin complex immunohistochemistry (Dako^{TM}, Denmark) with nickel sulphate chromogen enhancement and a 1 second Mayer's haematoxylin counterstain.

3) Threshold was set at 105, minimum particle size at 50, with the following erode-dilate settings: erode n=1, dilate n=5 and erode n=3.

4.4 Microvessel density and prognosis in resected colorectal liver metastases

As outlined in Chapter 2, all tumour biopsies were taken from the tumour edge. However, not all of the specimens had an abutting liver tissue present on the section, therefore only those with an obvious liver-tumour edge were subjected to an edge hot-spot and mean vessel count. The vessel counts were performed as already described *(vide supra)* with a CD 34 primary antibody concentration of 1:50 for optimal results. In order to assess hotspots, the ten hottest areas from each of the 182 tumour biopsies were assessed in decreasing order, namely, the perceived hottest or most dense area followed by the second and so forth.

The hottest spot was accurately identified first 55% of the time (n=101). In 34% of cases (n=61), the hottest spot was missed on the first count but identified in the second to fifth counts. Within this group the hottest spot was identified second in 24 cases, third in 14 cases, forth in 14 cases, and fifth in 9 cases.

The remaining 20 cases (11%) required up to 10 attempts before the hottest spot was identified: 6 n=5, 7 n=7, 8 n=4, 9 n=3, 10 n=1.

4.31 Relationship between edge and intra-tumour vessel counts

	Patients (n)	Vessel count range	Median vessel count
Tumour edge hotspots	149	10-69	31
Tumour edge vessels	149	5-39.4	15.6
Intra-tumour hotspots	182	13-119	43
Intra-tumour vessels	182	2-38	9

Intra-tumour hotspots were significantly more vascular than edge hotspots, with median vessel counts of 43 and 31 respectively ($p<0.0001$). Tumour edge contiguous vessels were statistically more vascular than intra-tumour vasculature, with median vessel counts of 15.6 and 9 respectively ($p<0.0001$).

4.32 Relationship between clinicopathological factors and MVD

Tumour diameter in mm was compared with the actual microvessel counts for each microvessel categories defined. No significant relationship was found between the size of the lesion and microvessel density (Table 4.1a). However, if lesions are divided about the median size for this series, greater than 35mm diameter versus less than 35mm, those tumours greater than 35mm diameter had significantly more median MVD counts in the tumour edge hotspots ($p=0.003$) and tumour edge contiguous areas ($p=0.02$) as compared with lesions less than 35mm diameter (Table

4.1b). Interestingly however, this result loses significance when applied to greater than 50mm versus less than 50mm, as published by Fong *et al*(Fong et al., 1999). No significant result was obtained for intra-tumour MVD.

The remaining clinicopathological factors, Dukes' stage, site of primary tumour, synchronous/ metachronous presentation, and number of lesions did not yield any statistically significant correlations with MVD.

Table 4.1a Microvessel density and size of tumour

		Edge hottest hotspot	**Mean of contiguous areas at edge**	**Intratumour Hottest hotspot**	**Intratumour random cumulative mean**
Size of lesion (mm)	Pearson's correlation	0.008	-0.41	0.11	0.058
	p value	0.948	0.75	0.306	0.59

Table 4.1b Microvessel density and size of lesion about the median diameter

Microvessel Density	**Size of lesion (n)**		χ^2
	below 35mm	**above 35mm**	
Tumour edge hotspots			
Below median	50	34	0.003
Above median	23	42	
Contiguous edge count			
Below median	44	32	0.02
Above median	29	44	
Intratumour hotspot			
Below median	49	44	0.09
Above median	36	53	
Cumulative mean			
Below median	49	54	0.7
Above median	36	43	

4.33 Univariate analysis and survival curves

The above four vessel counting strategies were analysed by dividing the patients into two groups: above and below the median counts. Kaplan-Meier survival curves for each group are shown in Fig 4.6, 4.7, 4.8, and 4.9 respectively. Prognostic significance for each group is summarised in Table 4.2

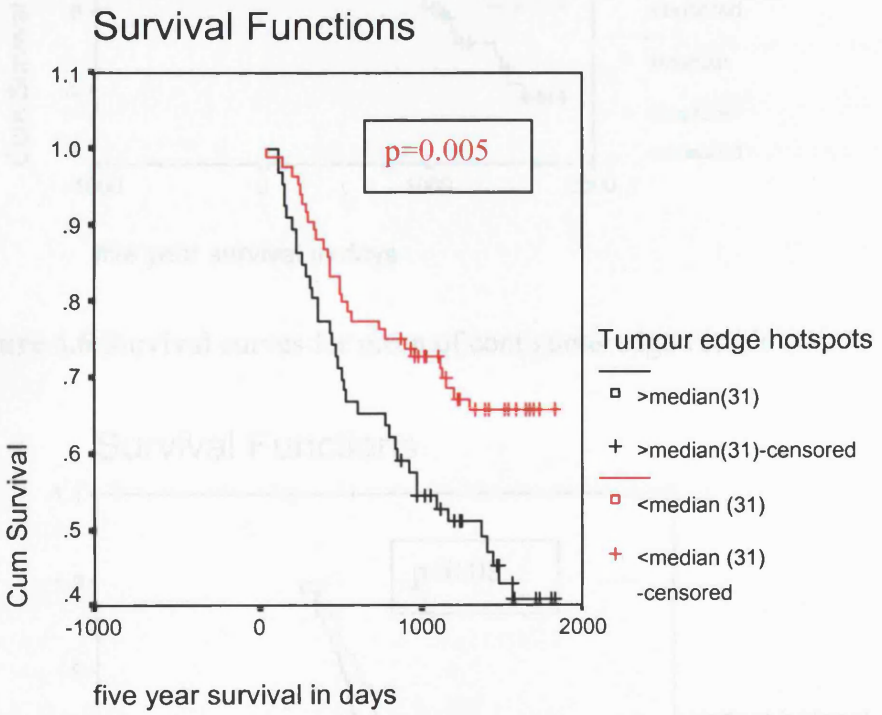


Figure 4.5 Survival curves for MVD tumour edge hot spot

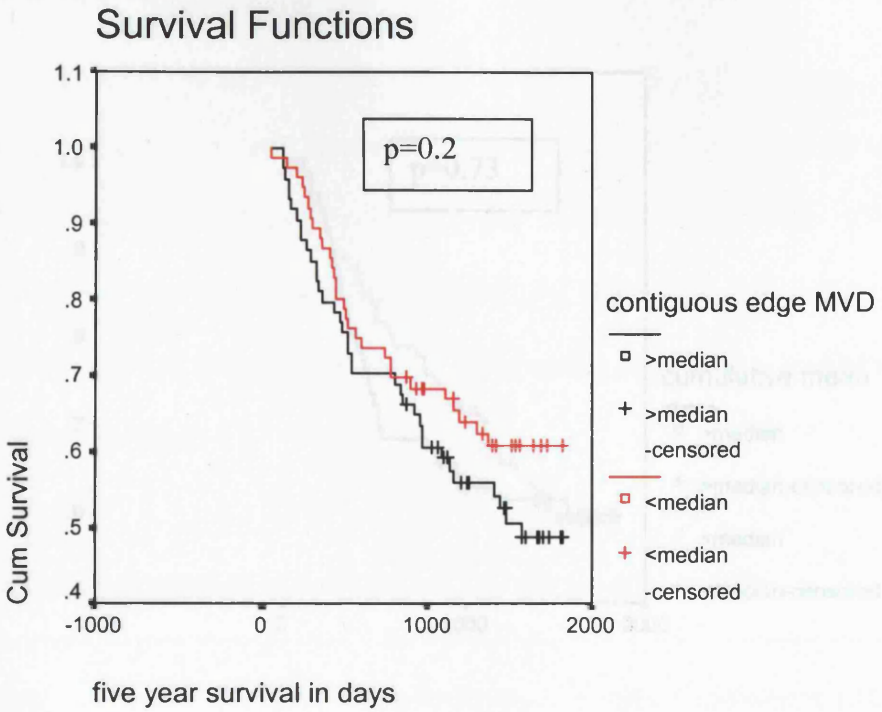


Figure 4.6 Survival curves for mean of contiguous edge vessels

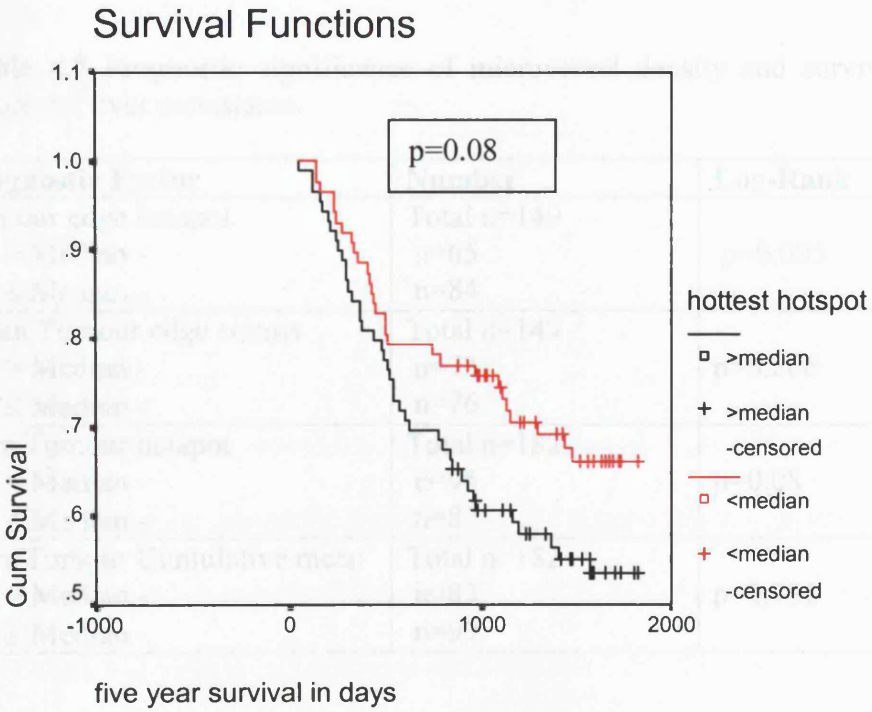


Figure 4.7 Survival curves for intra-tumour hot spots

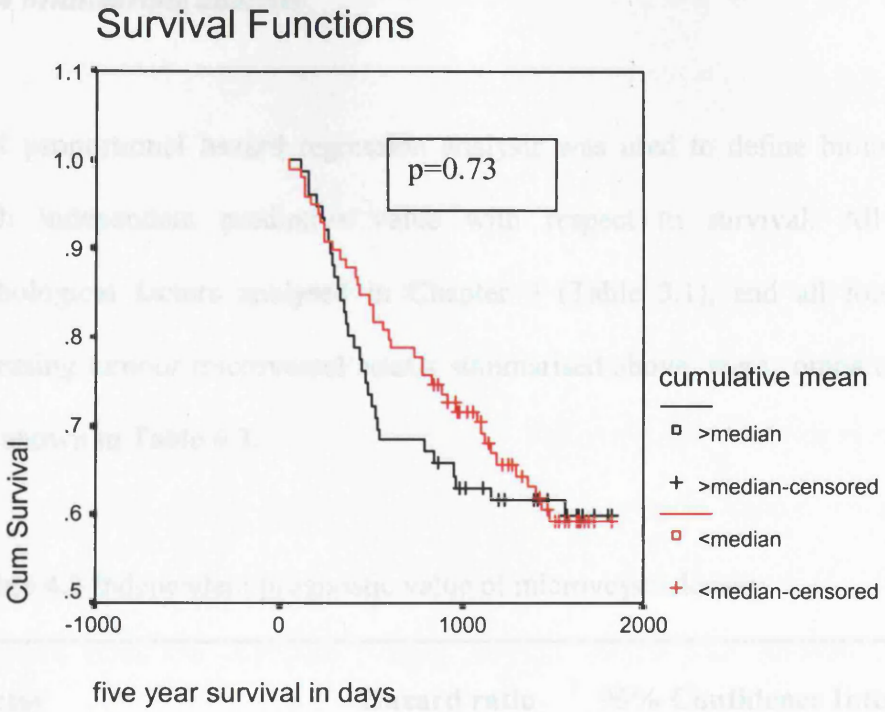


Figure 4.8 Survival curves for intra-tumour random cumulative mean

Table 4.2 Prognostic significance of microvessel density and survival in resected colorectal liver metastases.

Prognostic Factor	**Number**	**Log-Rank**
Tumour edge hotspot > Median - ≤ Median -	Total n=149 n=65 n=84	p=0.005
Mean Tumour edge counts > Median - ≤ Median -	Total n=149 n=73 n=76	p=0.206
Intra Tumour hotspot > Median - ≤ Median -	Total n=182 n=95 n=87	p=0.08
Intra Tumour Cumulative mean > Median - ≤ Median -	Total n=182 n=83 n=99	p=0.732

Multiple intra-tumour hotspots were not significant: the mean of 5 intra-tumour hotspots p= 0.6 and the mean of 10 intra-tumour hotspots p=0.12.

4.34 Multivariate analysis

Cox proportional hazard regression analysis was used to define biological markers with independent predictive value with respect to survival. All the clinico-pathological factors analysed in Chapter 3 (Table 3.1), and all four methods of assessing tumour microvessel counts summarised above, were compared. The results are shown in Table 4.3.

Table 4.3 Independent prognostic value of microvessel density

Factor	Hazard ratio	95% Confidence Interval	p value
Unilobar disease	1.00	1.15 – 3.57	**0.014**
Bilobar disease	2.02		
Edge hotspot ≤median	1.00	1.01 – 3.19	**0.045**
Edge hotspot > median	1.79		

4.4 Discussion

The first attempt at assessing MVD in colorectal cancer involved the laborious process of using photomicrographs of all stained tissue, manual counting and measurement of all vessels (Mlynek et al., 1985). Subsequently, Weidner's method of estimating the density of microvessels in subjectively chosen areas of obvious high vessel density (hotspots) was much less involved (Weidner et al., 1991). Despite the simplicity of this technique, its application in the study of angiogenesis of both colorectal primary tumours and their liver metastases has been heterogeneous, with

differing pan-endothelial markers, number of hotspots and tumour area biopsies being used as shown in Table 1.1.

With specific reference to colorectal liver metastases, of which there have been limited MVD studies (Table 6), we sought to develop a simple, reproducible image analysis system that could easily be utilised by any Pathology Department to count MVD. Image analysis systems have been used previously. Wester *et al* have used a system similar to the method described here in bladder cancer, with a manual to Image analysis correlation of 0.8 (Ranefall et al., 1998; Wester et al., 1999). Fox *et al* mapped areas on captured images with a mouse and measured the area of microvessels. They also used a pixel colour comparison analysis subsequently converted to greyscale for measurement (Fox et al., 1995). Kohlberger *et al* in breast tissue, used colour image analysis, again using the mouse to outline the area of interest and measuring area of staining (Kohlberger et al., 1996). Other investigators have used image analysis to create an Optimized Microvessel Density (OMVD) in prostate cancer tissue (Bigler et al., 1993; Bostwick et al., 1996). Biggler *et al* incorporated a manual correction, allowing deletion of falsely identified areas and inclusion of areas omitted.

In colorectal cancer, six papers have utilised a computerised image analysis system to measure MVD. These are summarised in Table 4.4. Five of these opted for a commercially available image analysis system produced by Zeiss, Germany (Kern et al., 2002; Kawakami T et al., 2001; Konerding et al., 2001; Tokunaga et al., 1999; Tsuchida T et al., 1999), whereas Pavlopoulos *et al* developed their own, colour, image analysis system (Pavlopoulos et al., 1998). Interestingly, only Kern *et al*

in their study of 11 colorectal liver metastases performed a comparison of the MVD measurements produced by the image analysis systems and manual counts, producing a similar result to this study (R=0.84)(Kern et al., 2002).

Table 4.4 Summary of papers using image analysis systems for counting MVD in colorectal cancer

Paper	N°	Bx Site	Stain	Areas counted	Analysis system	Mag	Field (mm^2)	MVD Value
(Kawakami T et al., 2001)	53	Edge	CD 34	2 Hotspots	Interactive Build Analysis, Zeiss	200	0.739	mean
(Kern et al., 2002)	11	NS	CD34	2 Hotspots (5 areas of each)	KS300, Zeiss	200	0.25	mean
(Konerding et al., 2001)	46	Multiple	VIII	1-6 Hotspots	KS300, Zeiss	ns	ns	ns
(Pavlopoulos et al., 1998)	90	Main lesion	VIII	1 Hotspot	SigmaScan v.2.0 (Jandel)	200	ns	max
(Tokunaga et al., 1999)	61	Edge	CD 34	1 Hotspot	Interactive Build Analysis, Zeiss	200	0.739	max
(Tsuchida T et al., 1999)	65	Centre	CD 34	>1 Hotspot	Interactive Build Analysis, Zeiss	200	0.739	mean

The technique that we have described differs from the previous published studies in its availability, applicability and accuracy. The software is freely available from the Word Wide Web (www) at http://rsb.info.nih.gov/nih-image/, instead of purchasing specialist software and hardware to analyse previously stained tissue sections. Knowing that the freeware package could easily be used to distinguish dark and light (positive and negative) fields the staining technique was optimised. Specific vessel staining with minimum background, tumour cell or inflammatory cell staining is extremely important when applied to an image analysis system, since extraneous staining will be counted as microvessels. Such staining was achieved in colorectal liver metastases using pan-endothelial marker CD34. The vessel staining was enhanced with nickel sulphate and the counterstain reduced to a minimum (yet still

allowing accurate navigation) thus enabling the affective use of the free standard image analysis package. The correlations between MVD using this method and manual counting (taken as gold standard) were extremely accurate, and using this image analysis system, Martin's conclusions that ten hotspots need to be counted in order to detect the hottest spot every time (Martin et al., 1997) were corroborated in the colorectal liver metastases (*vide supra*).

Since the measurement of MVD in colorectal cancer has been approached in different ways by different researchers, as previously discussed, this study aimed to perform a comprehensive assessment of MVD and relate the outcomes to prognosis.

All specimens measured were edge biopsies, therefore incorporating the most peripheral area of tumour, called the tumour edge, which has been postulated as the most biologically active (Fanelli et al., 1999; Vermeulen et al., 1996; Weidner et al., 1991; Konerding et al., 2001), and a central area of tumour, called the intra-tumour (Please refer to Chapter 2, Patients and Methods). The microvessel counts in these two areas were expressed as either hotspots or means of whole area vasculature (contiguous vessel counts at the tumour edge and random cumulative means in the intra-tumour). The hottest hotspot at the tumour edge was statistically more vascular than the intra-tumour hotspot ($p<0.0001$), and the only MVD measurement significantly correlated with poor survival on univariate ($p=0.005$) and multivariate analysis ($p=0.045$) . In this series, there was no relationship between the individual size of the tumours and the matched total number of microvessels counted (Table 4.1a). However, if the tumours were divided into two groups about their median size (35mm) and compared with the median MVD counts (Table 4.1b), those tumours

greater than 35mm diameter had significantly greater than the median edge hotspot and contiguous microvessels (p=0.003 and 0.02 respectively). This finding is in contrast to that published by Terayama *et al*(Terayama et al., 1996), who in a study of 13 colorectal liver metastases found no increase in total edge MVD in lesions above 3mm diameter. They did not, however, study the hotspot MVD at the tumour edge.
To my knowledge, this method of incorporating the first area of viable tumour viewed at high power field (x200) that abuts normal liver tissue has not previously been described in colorectal liver metastases. Although Nanashima *et al* (Nanashima et al., 1998) published findings relating to marginal and central biopsies of liver metastases, these actually represented two separate biopsy areas, so in actual fact, what they classified as their vessel counts of the marginal section, equates to the edge and intra-tumour hotspot described in this study. Furthermore, their results describe mean MVD for five hotspots that they associated with poor prognosis (p=0.05), whereas in this study, a mean of five hotspots and also a mean of ten hotspots failed to reach significance (p=0.6 and p= 0.14 respectively). Although the hottest intra-tumour hotspot also failed to reach statistical significance as a prognostic indicator of survival, it showed the strongest trend towards this correlation (p=0.08, Figure 4.7).

It can therefore be surmised that the tumour edge is the most biologically active area of a growing tumour, and that the hottest vascular area at the tumour edge is the one predictive of poor outcome.

In conclusion, the image analysis system described in this study is inexpensive, reliable and simple to use. The hottest hotspot of the edge of a colorectal liver metastasis independently predicts poor prognosis.

Chapter 5

Vascular Endothelial Growth Factor

5.1 Introduction

Angiogenesis is a multi-step process controlled by a balance of promoter and inhibitor substances. For neovascularisation to occur there must be an alteration in the balance between these factors favouring a pro-angiogenic environment that normally hold the vessels in a quiescent state (Bouck et al., 1996).

VEGF has been described as the one of the most potent angiogenic growth factors that induces endothelial cell mitogenesis. There are at least 5 distinct isoforms in humans: 121 (freely diffusible), 145 (partly bound by ECM, diffusible), 165 (partly bound by ECM, diffusible), 189 and 206 (fully bound by ECM thus not diffusible). (Tischer et al., 1991; Houck et al., 1991; Poltorak et al., 1997; Neufeld et al., 1994; Park et al., 1993). Numerous studies have aimed to examine the role of VEGF in primary colorectal cancer, but little work has been undertaken evaluating VEGF expression in metastatic colorectal cancer.

5.2 Aims of the study

1) To assess the expression of VEGF in colorectal liver metastases.

2) To correlate the expression of VEGF with the clinicopathological variables.

3) To assess VEGF and prognosis.

5.3 VEGF protein expression

Primary antibody concentrations of 1:1, 1:2, 1:4 and 1:8 were used.

Optimal results, as reviewed with a Consultant Histopathologist, were observed at the 1:2 dilution and was thus used. Tumour cell and stromal VEGF protein expression was semi-quantitatively analysed as described in Chapter 2. The inter-observer variability as measured by kappa scoring was 0.9.

The results are summarised in Table 5.1, and illustrated in Figures 5.1a&b.

Table 5.1 Protein expression of VEGF in colorectal liver metastases

	Tumour cell VEGF	Stromal VEGF
Total number of patients	182	182
Positive cases	166 (91%)	126 (69%)
Negative cases	16 (9%)	56 (31%)
Range of percentage expression (%)	0-100	0-70
Median expression (%)	77.5	10

Figure 5.1a Tumour cell VEGF

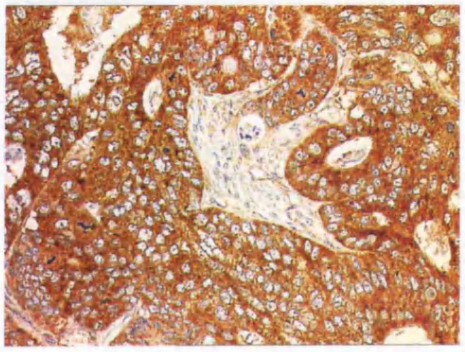
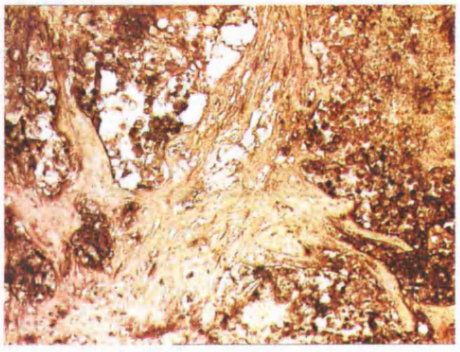


Figure 5.1b Stromal VEGF



5.4 Relationship between VEGF and clinico-pathological parameters

The relationship between stromal and tumour cell VEGF, and clinicopathological factors is summarised in Tables 5.2a&b.

Table 5.2a. Tumour cell VEGF compared with clinicopathological factors.

Pathological factor	Tumour cell VEGF below median	above median	χ^2	Tumour cell VEGF absent	present	χ^2
Gender						
Male	62	60	0.8	9	113	0.46
Female	29	31		7	53	
Dukes stage						
A	3	3		0	6	
B	34	40	0.9	9	70	0.40
C	54	48		7	90	
Synchronicity						
Synchronous	46	39	0.29	7	78	0.80
Metachronous	45	52		9	88	
Disease distribution						
Unilobar	46	64	0.006	12	88	0.21
Bilobar	45	27		4	68	
Size of lesion						
Below median	42	43	0.88	8	77	0.78
Above median	49	48		8	89	
Number of lesions						
One lesion	33	43	0.13	8	68	0.41
>1 lesion	58	48		8	98	
<3 lesions	72	74	0.71	13	133	0.94
>3 lesions	19	17		3	33	

Table 5.2b The relationship between stromal VEGF and clinicopathological factors.

Pathological factor	Stromal VEGF below median	above median	χ2	Stromal VEGF absent	present	χ2
Gender						
Male	59	63	0.8	39	83	0.73
Female	30	30		17	43	
Dukes stage						
A	1	5	0.06	3	3	
B	34	45		20	59	0.27
C	54	43		33	64	
Synchronicity						
Synchronous	41	44	0.86	23	62	0.32
Metachronous	48	49		33	64	
Disease distribution						
Unilobar	44	66	0.003	34	76	0.91
Bilobar	45	27		22	50	
Size of lesion						
Below median	43	42	0.67	21	64	0.09
Above median	46	51		35	62	
Number of lesions						
One lesion	30	46	0.03	25	51	0.59
>1 lesion	59	47		31	75	
<3 lesions	66	80	0.04	42	104	0.23
>3 lesions	23	13		14	22	

5.5 Relationship between VEGF and MVD

Tumour cell and stromal VEGF were compared with microvessel density as summarised in Tables 5.3a&b.

Table 5.3a The relationship between tumour cell VEGF and MVD.

Microvessel Density	Tumour cell VEGF		χ^2	Tumour cell VEGF		χ^2
	below median	above median		absent	present	
Tumour edge hotspots						
Below median	36	48	0.33	7	77	0.84
Above median	33	32		6	59	
Contiguous edge count						
Below median	36	40	0.79	7	69	0.83
Above median	33	40		6	67	
Intratumour hotspot						
Below median	50	43	0.29	10	83	0.33
Above median	41	48		6	83	
Intratumour cumulative mean						
Below median	49	54	0.45	7	96	0.27
Above median	42	37		9	70	

Table 5.3b The relationship between stromal VEGF and MVD.

Microvessel Density	Stromal VEGF		χ^2	Stromal VEGF		χ^2
	below median	above median		absent	present	
Tumour edge hotspots						
Below median	39	45	0.27	23	61	0.21
Above median	36	29		24	41	
Contiguous edge count						
Below median	34	42	0.16	20	56	0.16
Above median	41	32		27	46	
Intratumour hotspot						
Below median	41	52	0.18	28	65	0.84
Above median	48	41		28	61	
Cumulative mean						
Below median	49	54	0.68	28	75	0.23
Above median	40	39		28	51	

5.6 VEGF and patient survival

5.61 Univariate analysis

VEGF and survival is depicted in Figures 5.2a-d.

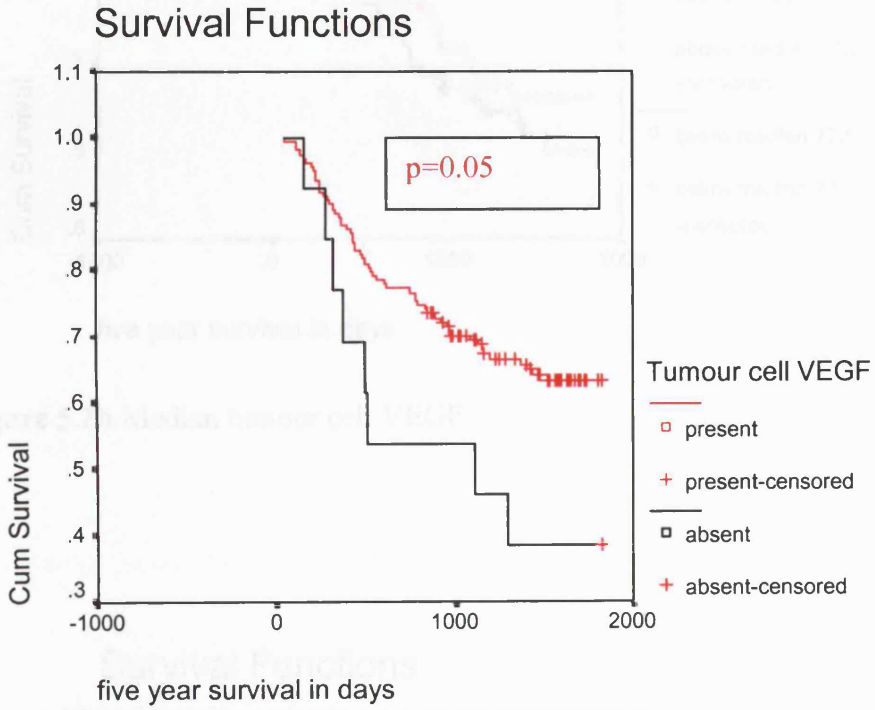


Figure 5.2a Present versus absent tumour cell VEGF

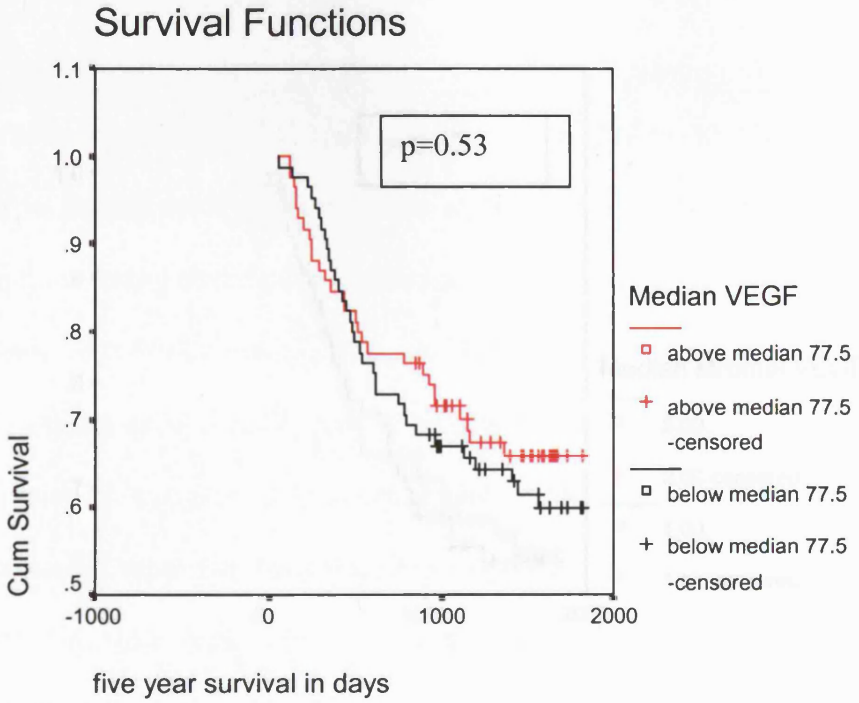


Figure 5.2b Median tumour cell VEGF

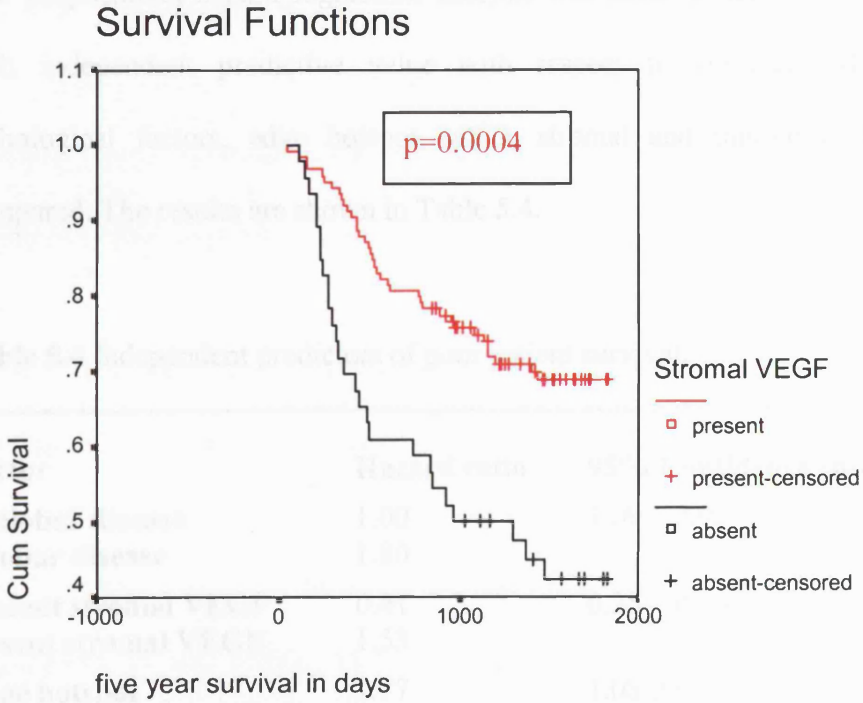


Figure 5.2c Present versus absent stromal VEGF

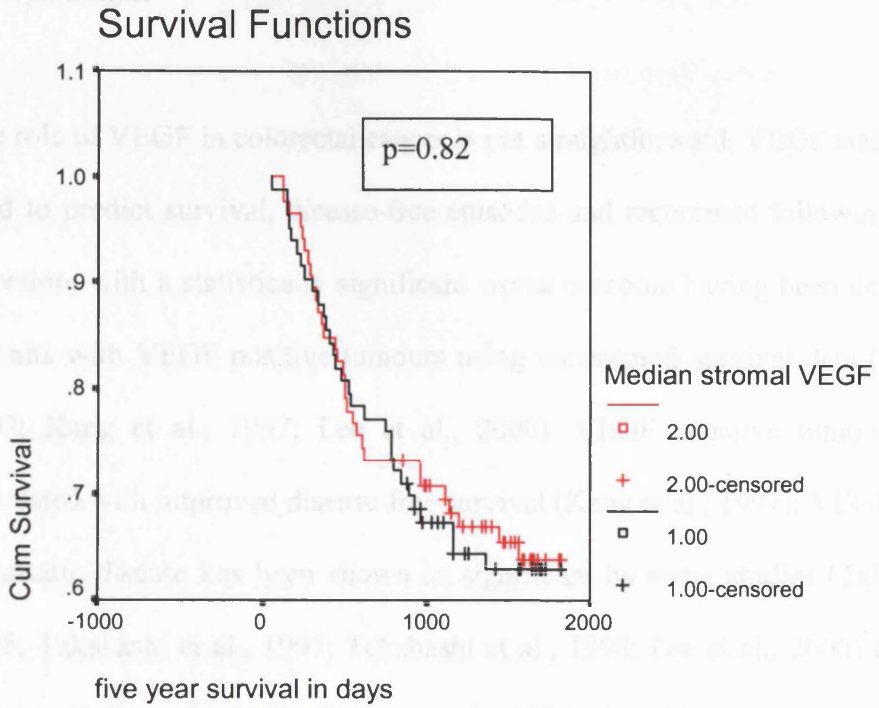


Figure 5.2d Median stromal VEGF

5.62 Multivariate analysis

Cox proportional hazard regression analysis was used to define biological markers with independent predictive value with respect to survival. All the clinico-pathological factors, edge hotspot MVD, stromal and tumour cell VEGF were compared. The results are shown in Table 5.4.

Table 5.4 Independent predictors of poor patient survival.

Factor	Hazard ratio	95% Confidence Interval	p value
Unilobar disease	1.00	1.09 – 2.95	p=0.02
Bilobar disease	1.80		
Present stromal VEGF	0.41	0.25 – 0.66	p<0.001
Absent stromal VEGF	1.53		
Edge hotspot	1.77	1.08-2.93	p=0.024

5.7 Discussion

The role of VEGF in colorectal cancer is not straightforward. VEGF staining has been used to predict survival, disease-free episodes and recurrence following a colorectal resection, with a statistically significant worse outcome having been demonstrated in patients with VEGF positive tumours using uncensored survival data (Amaya et al., 1997; Kang et al., 1997; Lee et al., 2000). VEGF negative tumours have been correlated with improved disease-free survival (Kang et al., 1997); VEGF staining and metastatic disease has been shown as significant by some studies (Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1998; Lee et al., 2000) but not others (Berney C R et al., 1999; Berney et al., 1998). In addition, there appears to be a consensus that high expression of VEGF correlates with high expression of MVD. However, few data can be found in the literature examining the role of VEGF in colorectal liver metastases. In this series, patients with unilobar disease had significantly more stromal and tumour cell VEGF compared with bilobar disease, whereas only stromal VEGF was associated with fewer liver metastases (see Table 5.2a&b). No correlation was found between expression of VEGF, tumour size and MVD (see Table 5.3a&b).

Nanashima's work, albeit a smaller study (patients n=44), did not significantly correlate VEGF with disease distribution, in contrast to this study's results, but in agreement with this series, also failed to find a relationship between the extent of VEGF expression and tumour size as examined by immunohistochemistry (Nanashima et al., 1998). Interestingly, other studies have found a relationship between VEGF and tumour size by examining the *soluble* isoforms of VEGF (121 and

165) and metastatic colorectal tumour load (Davies et al., 2000; Dirix et al., 1996). In one study of the growth kinetics of metastatic colorectal cancer, those patients with rapidly growing liver metastases, as measured on CT, had significantly higher levels of serum VEGF as compared with those with more slowly growing tumours (Dirix et al., 1996). Similarly, Davies *et al* (Davies et al., 2000) found a significant relationship between the soluble (plasma) isoforms of VEGF and volume of liver metastases as assessed by CT.

The discrepancies between these studies may be explained by the isoform of VEGF being measured. It may be postulated that soluble, circulating VEGF levels rather than immunohistochemically detected cellular VEGF expression, may be associated with the amount of tumour load. Alternatively, this may reflect an increase in platelet counts secondary to elevated cytokine expression in such platelets (O'Byrne et al., 1999).

Although this study failed to correlate VEGF expression with MVD, other studies have proposed such a correlation. Davies and Kern (Davies et al., 2000; Kern et al., 2002) both found that high VEGF significantly correlated with MVD. However, both involved small numbers (n=12 and n=11 respectively) and Kern's study could be further confounded by their much reduced, total expression of VEGF in the liver (63% as compared with 93% in our study and 84% in Nanashima's study), which could be as a result of a sampling error.

On univariate analysis, the presence of stromal VEGF was associated with significantly better patient survival as compared with absent stromal VEGF. Similarly, the presence of tumour cell VEGF was also associated with better patient

prognosis. However, as can be seen in table 5.1, the number of cases with absent tumour cell VEGF are small (n=13, 7%), so this prognostic result must be viewed with a degree of caution. The only other study to my knowledge that has examined survival failed to associate tumour cell VEGF with patient survival (n=44); stromal VEGF was not reported (Davies et al., 2000; Nanashima et al., 1998).

On multivariate analysis, the presence of stromal VEGF was an independently favourably prognostic factor, whereas bilobar disease and tumour edge MVD remained independently negative prognostic factors. In a recent study by Khorana *et al* examining primary colorectal tumours, the presence of stromal VEGF was associated with a significantly better outcome, comparable to the results from this study(Khorana et al., 2003). Interestingly, the improved prognosis observed was specifically associated with the expression of VEGF in tumour associated macrophages (TAM)(Khorana et al., 2003). The roles of TAM in neoplasia are antagonistic. They may be tumouricidal by producing tumour necrosis factor α, nitric oxide or inerleukins(Trinchieri, 1998; Urban et al., 1986), or promote tumour growth by secreting pro angiogenic growth factors such as fibroblast growth factor, transforming growth factor α and TP(Sunderkotter et al., 1994). This dual potential has been described as the macrophage balance hypothesis (Mantovani et al., 1992; Balkwill and Mantovani, 2001). The authors propose that stromal TAM producing VEGF tip the balance toward a tumoricidal environment (Khorana et al., 2003).

In summary, tumour cell VEGF was present in over 90% of colorectal liver metastases, and stromal VEGF in 69% of colorectal liver metastases. Absence of stromal VEGF was found to be a negative prognostic factor on both univariate and multivariate analysis.

Chapter 6

Thymidine Phosphorylase

6.1 Introduction

Thymidine phosphorylase (TP) is a member of the pyrimidine nucleoside phosphorylase family of enzymes that are essential for DNA synthesis. TP has been shown to stimulate angiogenesis and endothelial cell chemotaxis in animal models (Moghaddam et al., 1995), and its expression is elevated in several tumour types including carcinoma of the breast (Moghaddam et al., 1995; Toi et al., 1995), bladder (O'Brien et al., 1995), ovary (Reynolds and Redmer, 1998), stomach (Maeda et al., 1996) oesophagus, lung and pancreas (Takebayashi et al., 1996). TP appears to be involved in the development of colorectal cancer from an early stage (Enomoto et al., 2000; Takebayashi et al., 1995).

By evaluating tumour cell TP only, a significant relationship between MVD and TP expression has been shown (Takebayashi et al., 1995). Quantifying both stromal and tumour TP has also been associated with angiogenesis, higher MVD being found in tumours expressing TP in both (Matsumura et al., 1998). In addition, high TP expression has been found to be a poor prognostic factor for survival (Takebayashi et al., 1996) and an independent risk factor for the development of haematogenous metastases (van Halteren et al., 2001).

Thymidine phosphorylse is expressed in approximately 80% of colorectal liver metastases (Collie-Duguid et al., 2001). There is little, if any work available on the prognostic significance of TP expression by colorectal liver metastases.

6.2 Aims of the study

1) To assess the expression of TP in colorectal liver metastases.

2) To correlate the expression of TP with the clinicopathological variables.

3) To assess TP and prognosis.

6.3 Expression of TP in colorectal liver metastases

A 1:20 primary antibody concentration was utilised for optimal results.

A semi-quantitative grading scale was employed. Due to the complexity of the staining pattern produced, a Consultant Histopathologist and a Pathology Specialist Registrar graded the entire series using a double-headed microscope. These patterns were then reviewed by CDS and the Consultant Histopathologist.

Table 6.1 summarises the distribution of TP in colorectal liver metastases, categorised as tumour cell, fibroblast, lymphocyte and total TP expression.

Table 6.1 Protein expression of TP in colorectal liver metastases

	Tumour cell TP	Macrophage TP	Fibroblast TP	Lymphocyte TP	Total TP
Total number of patients	182	182	182	182	182
Positive cases	78 (43%)	181 (99.5%)	182 (100%)	181 (99.5%)	181 (99.5%)
Negative cases	104	1	0	1	1

Figure 6.1a Tumour cell TP

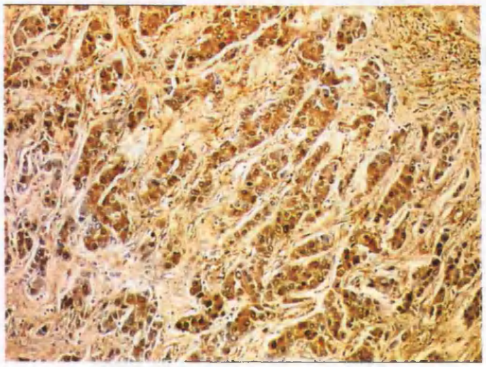
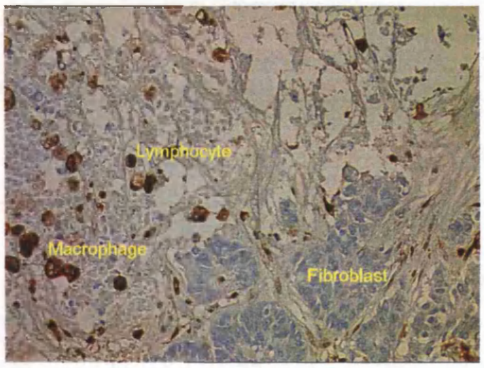


Figure 6.2 Cellular infiltrate TP



6.4 Relationship between TP and clinico-pathological variables

Median TP expression for each of tumour cell, lymphocyte, fibroblast, total and grouped TP categories, was correlated to the clinicopathological variables described in Chapter 2. The results are summarised in Tables 6.2a-c.

Table 6.2a Tumour cell TP expression and clinico-pathological variables.

Pathological factor	Tumour cell TP below median	above median	χ^2	Tumour cell TP absent	present	χ^2
Gender						
Male	63	59	0.87	36	86	0.6
Female	33	27		17	43	
Dukes' stage						
A	2	4		1	5	
B	43	36	0.6	27	52	0.37
C	51	46		25	72	
Synchronicity						
Synchronous	48	37	0.34	27	70	0.68
Metachronous	48	49		26	59	
Disease distribution						
Unilobar	52	58	0.06	27	83	0.09
Bilobar	44	28		26	46	
Size of lesion						
Below median	49	36	0.21	23	62	0.56
Above median	47	50		30	67	
Number of lesions						
One lesion	41	35	0.78	19	57	0.30
>1 lesion	55	51		34	72	
<3 lesions	80	66	0.26	45	105	0.31
>3 lesions	16	20		8	28	

Table 6.2b Macrophage, fibroblast and lymphocyte TP expression and clinico-pathological variables

Pathological factor	Macrophage TP < median	> median	χ^2	Fibroblast TP < median	> median	χ^2	Lymphocyte TP < median	> median	χ^2
Gender									
Male	82	40	0.07	97	25	0.84	65	57	0.75
Female	32	28		47	13		30	30	
Dukes' Stage									
A	3	3	0.2	4	2	0.37	4	2	0.6
B	55	24		66	13		43	36	
C	56	41		74	23		48	49	
Synchronicity									
Synchronous	50	35	0.32	65	20	0.41	45	40	0.85
Metachronous	64	33		79	18		50	47	
Distribution									
Unilobar	69	41	0.97	84	26	0.25	60	50	0.43
Bilobar	45	27		60	12		35	37	
Size of lesion									
< median	51	34	0.49	64	21	0.23	46	39	0.62
>median	63	34		80	17		49	48	
N° Lesions									
One lesion	43	33	0.15	59	17	0.67	39	37	0.84
>1 lesion	71	35		85	21		56	50	
< 3 lesions	93	53	0.55	117	29	0.49	74	72	0.41
> 3 lesions	21	15		27	9		21	15	

6.2c Total and grouped TP expression and clinico-pathological variables

Pathological factor	Total TP below median	Total TP above median	χ^2	Grouped TP Tumour or Macrophage	Grouped TP TP in Both	χ^2
Gender						
Male	85	37	0.6	30	92	0.59
Female	39	21		17	43	
Dukes stage						
A	3	3	0.96	1	5	0.85
B	59	20		20	59	
C	62	35		26	71	
Synchronicity						
Synchronous	53	26	0.11	47	49	0.50
Metachronous	71	32		46	39	
Disease distribution						
Unilobar	75	35	0.98	51	58	0.23
Bilobar	49	23		42	30	
Size of lesion						
Below median	60	25	0.51	48	37	0.28
Above median	64	43		45	51	
Number of lesions						
One lesion	51	25	0.80	40	36	0.66
>1 lesion	73	33		53	52	
<3 lesions	105	41	0.02	77	68	0.57
>3 lesions	19	17		16	20	

6.5 Relationship between TP and MVD

The correlation between TP and MVD are shown in Table 6.3a-c

Table 6.3a Tumour cell TP and MVD

Microvessel Density	Tumour cell TP below median	Tumour cell TP above median	χ^2	Tumour cell TP absent	Tumour cell TP present	χ^2
Tumour edge hotspots						
Below median	49	35	0.04	23	61	0.97
Above median	27	38		18	47	
Contiguous edge count						
Below median	49	27	0.001	25	51	0.13
Above median	27	46		16	57	
Intratumour hotspot						
Below median	48	45	0.75	26	61	0.72
Above median	48	41		27	62	
Intratumour cum. mean						
Below median	57	46	0.42	32	71	0.51
Above median	39	40		21	58	

Table 6.3b Macrophage, fibroblast and lymphocyte TP expression and MVD

Microvessel Density	Macrophage TP < median	> median	χ^2	Fibroblast TP < median	> median	χ^2	Lymphocyte TP < median	> median	χ^2
Edge HS									
Below median	52	32	0.96	68	16	0.92	46	38	0.39
Above median	40	25		53	12		31	34	
Edge count									
Below median	47	29	0.98	60	26	0.47	42	35	0.37
Above median	45	28		61	12		35	38	
Tumour HS									
Below median	56	37	0.49	73	20	0.83	53	40	0.19
Above median	58	31		71	18		42	47	
Cum. mean									
Below median	63	40	0.64	81	22	0.87	55	48	0.71
Above median	51	28		63	16		40	39	

Table 6.3c Total and grouped TP expression and MVD

Microvessel Density	Total TP <median	>median	χ^2	Grouped TP Tumour or Macrophage	TP in Both	χ^2
Tumour edge hotspots						
Below median	64	20	0.02	48	36	0.05
Above median	38	27		27	38	
Contiguous edge count						
Below median	57	19	0.07	48	28	0.001
Above median	45	28		27	46	
Intratumour hotspot						
Below median	64	29	0.84	48	45	0.59
Above median	60	29		45	43	
Intratumour cum. mean						
Below median	69	34	0.71	56	46	0.38
Above median	55	24		37	42	

6.6 Relationship between TP and patient survival

6.51 Univariate analysis (Figures 6.2a-6.2g)

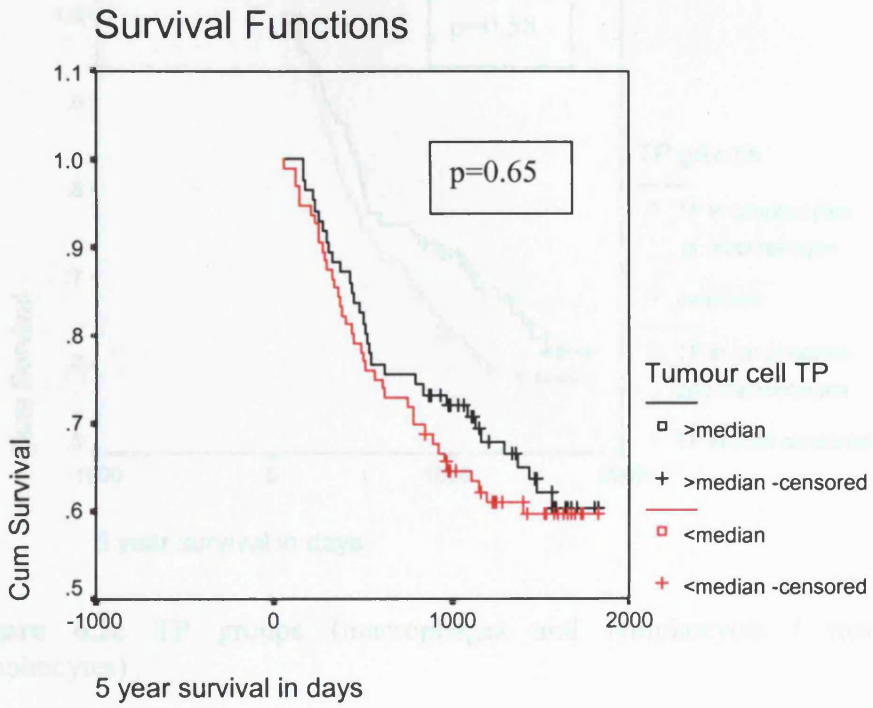


Figure 6.2a Tumour cell TP about the median.

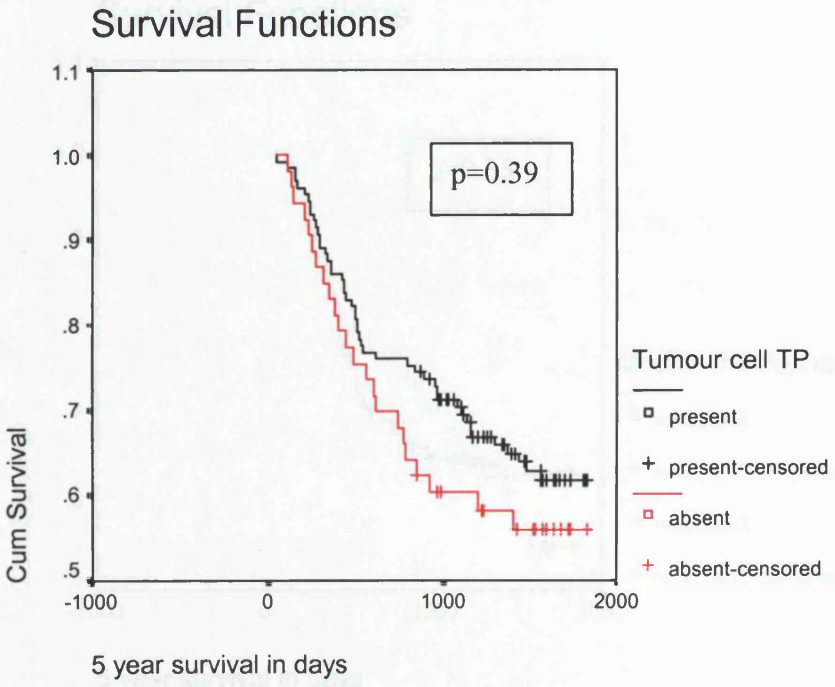


Figure 6.2b Present versus absent tumour cell TP.

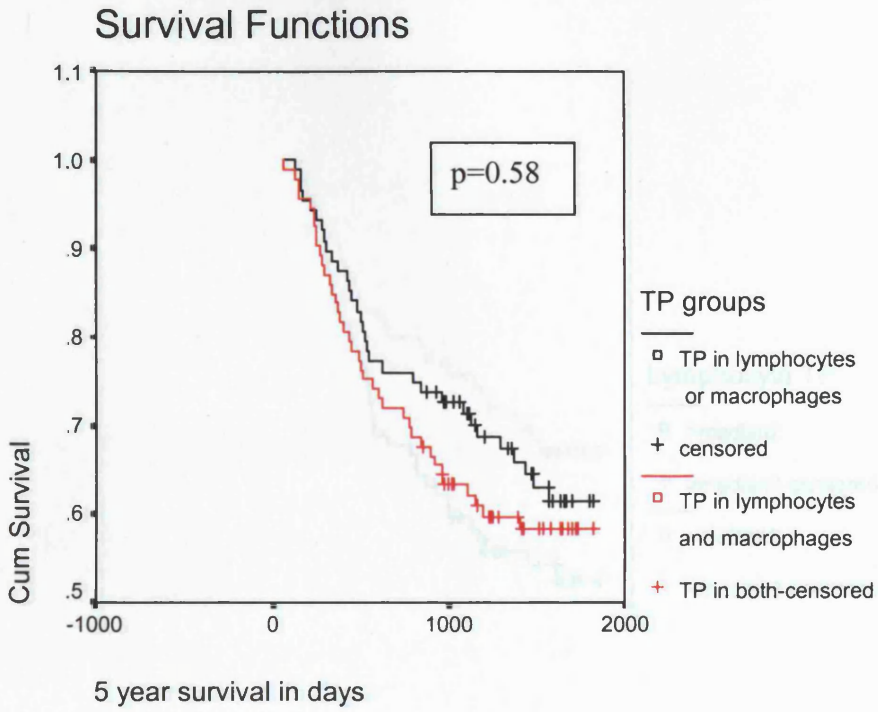


Figure 6.2c TP groups (macrophages and lymphocytes / macrophages or lymphocytes)

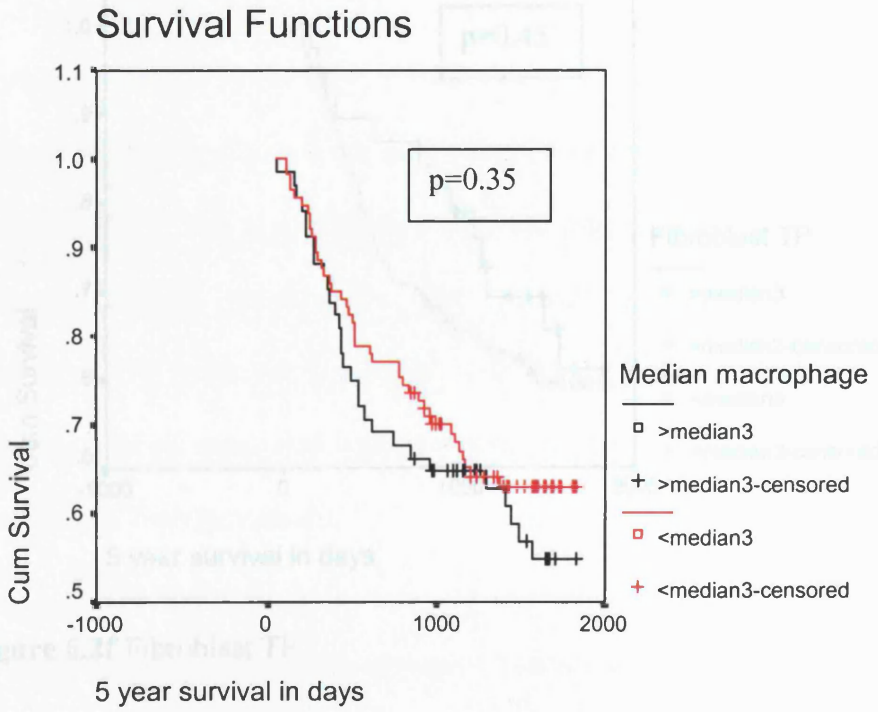


Figure 6.2d Median macrophage TP

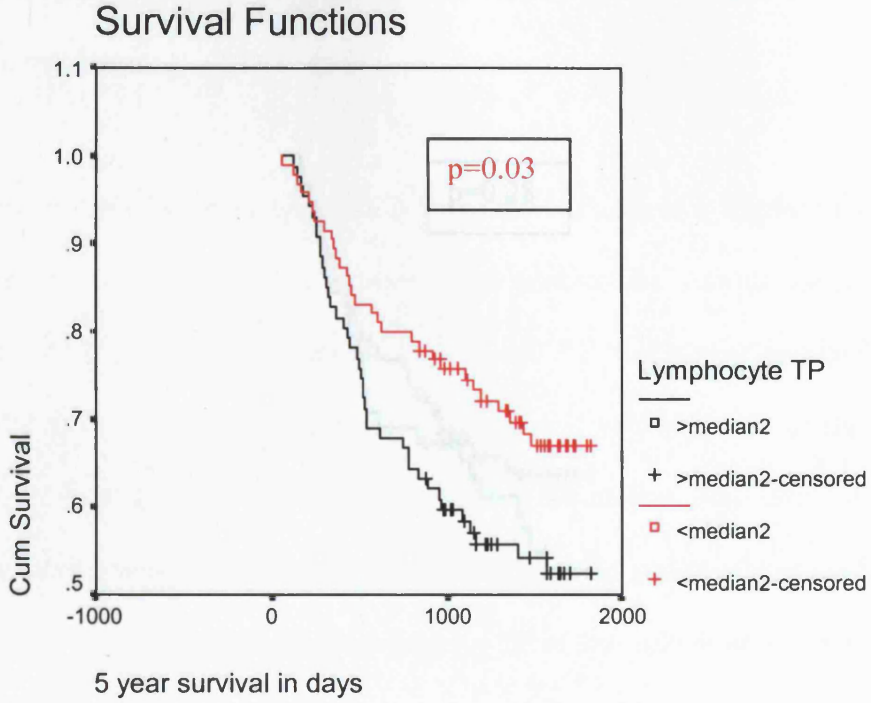


Figure 6.2e Lymphocytic TP

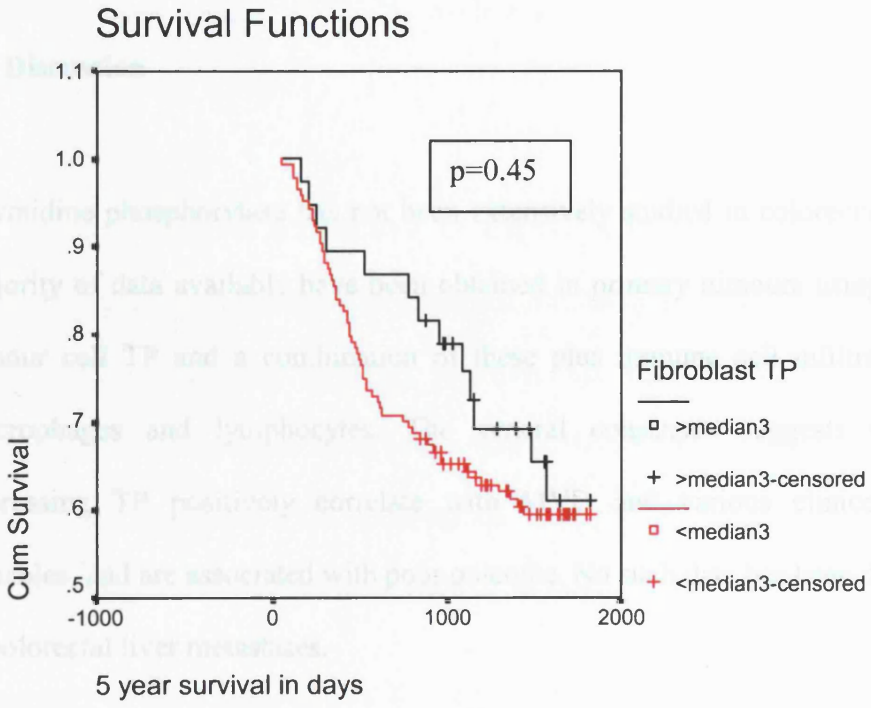


Figure 6.2f Fibroblast TP

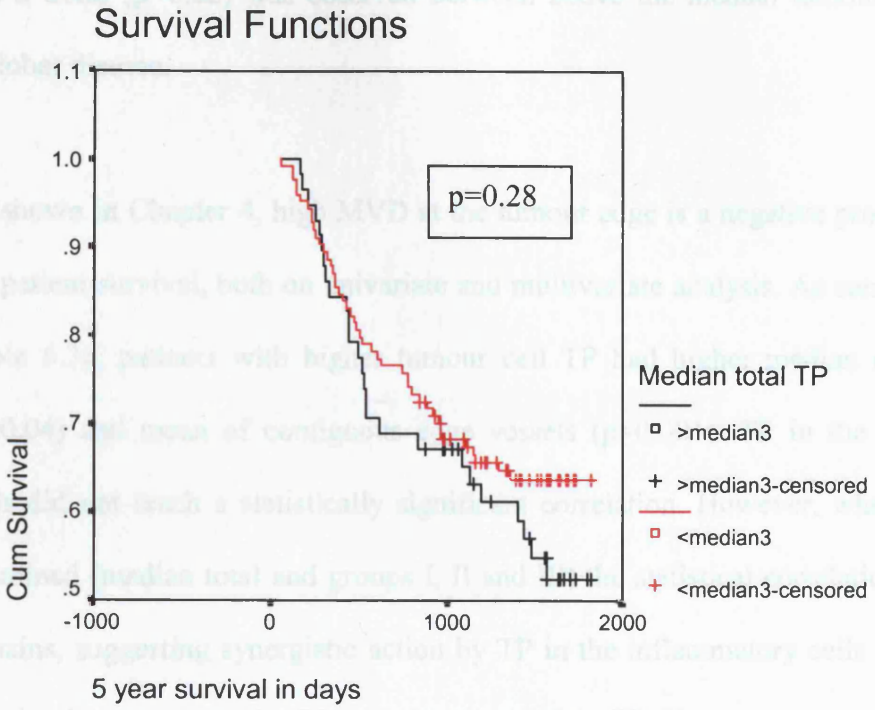


Figure 6.2g Total TP

6.7 Discussion

Thymidine phosphorylase has not been extensively studied in colorectal cancer. The majority of data available have been obtained in primary tumours using stromal TP, tumour cell TP and a combination of these plus immune cell infiltration, notably macrophages and lymphocytes. The general consensus suggests that tumours expressing TP positively correlate with MVD and various clinico-pathological variables, and are associated with poor outcome. No such data has been documented in colorectal liver metastases.

In this series, 43% of samples expressed tumour cell TP, whereas 99% of all samples expressed TP in the inflammatory infiltrate. Below the median total TP was significantly associated with less than three lesions (p=0.02). However in contrast to

this a trend (p=0.06) was observed between above the median tumour cell TP and unilobar disease.

As shown in Chapter 4, high MVD at the tumour edge is a negative prognostic factor for patient survival, both on univariate and multivariate analysis. As can be seen from Table 6.3a, patients with higher tumour cell TP had higher median edge hotspots (p=0.04) and mean of contiguous edge vessels (p=0.001). TP in the inflammatory cells did not reach a statistically significant correlation. However, when total TP is examined (median total and groups I, II and III) the statistical correlation with MVD remains, suggesting synergistic action by TP in the inflammatory cells in addition to that in the tumour cells. This may suggest that TP is an important modulator of angiogenesis in the tumour periphery, also demonstrated in lung cancer (Giatromanolaki et al., 1997). No such association was found with intra-tumour hotspots or vessels counts.

On univariate analysis, tumour cell TP did not correlate with poor survival. When lymphocytic TP was examined in isolation, a statistically significant correlation with poor survival was found (p=0.03). However, this may represent the inflammatory response initiated by the tumour, as opposed to the TP carried by the lymphocytes *per se* exerting this effect. On multivariate analysis, TP was not an independent prognostic factor for survival in patients with colorectal liver metastases.

In summary, TP appears to be an important pro-angiogenic factor in colorectal liver metastases with a specific association to MVD at the tumour edge. Inflammatory infiltrate in colorectal metastatic tumours is worthy of further investigation.

Chapter 7

Thrombospondin

7.1 Introduction

TSP-1 is a multi-domain glycoprotein that is secreted in response to platelet-derived growth factor and incorporated into the extracellular matrix. Thrombospondin-1 modulates platelet aggregation, wound healing, protease activity and cellular functions such as adhesion, motility and growth (Lawler, 1986), however, the precise function of TSP-1 in angiogenesis and tumour growth is controversial. Evidence suggests that TSP-1 has both pro-angiogenic and anti-angiogenic properties, possibly related to different actions of various fragments within the TSP-1 molecule, which may confer opposing angiogenesis-modulating properties: there is a 25kD heparin-binding fragment which was shown to induce a notably stronger angiogenic response in a rabbit cornea assay for neovascularisation compared with the TSP-1 molecule as a whole (Tarabolleti et al., 2000), whereas, another 140kD fragment, completely inhibited the angiogenic response (Tarabolleti et al., 2000). TSP-1 has also been shown to promote cell migration at high concentrations but inhibit migration at low concentrations if the whole TSP molecule is considered, thus emphasizing that different domains within the TSP-1 molecule may account for different tumour modulating properties (Weinstat-Saslow et al., 1994).

In colorectal cancer, TSP-1 has been shown to have an anti-angiogenic effect (Maeda K et al., 2001; Maeda et al., 2000) yet conversely be associated with venous invasion and tumour progression (Yamashita et al., 1998). To date there has not been a study of TSP-1, angiogenesis and prognosis in a series of colorectal liver metastases. The aim of this study was to examine the relationship of TSP-1 on MVD in tumour biopsies taken from a consecutive series of liver resections performed at the University Hospitals of Leicester and the Royal Liverpool Hospital.

7.2 Aims of the study

1) To assess the expression of TSP-1 in colorectal liver metastases.

2) To correlate the expression of TSP-1 with the clinicopathological variables.

3) To assess TSP-1 and prognosis.

7.3 TSP-1 protein expression

7.31 Antibody optimisation

As described in Chapter 2, three different antibodies were initially used to detect TSP-1 in paraffin embedded liver tissue sections, Ab1, Ab4 and Ab7 (See Table2.1).

No recognisable immunohistochemical visualisation of TSP-1 was achieved using the first antibody, Ab1, despite extensive antigen retrieval techniques (as detailed in Chapter 2).

Ab7 and Ab4 yielded similar patterns of expression, namely peri-vascular and stromal. However, detection with Ab7 was only observed using highly acidic antigen retrieval solution (pH1) in the microwave, which distorted the architecture of the section, leading to grading difficulties. Ab4 simply required pronase digestion, which was not only safer to use, but did not distort the architecture of the section, producing good quality staining. Antibody concentrations of 1:10, 1:50 and 1:100 were used, with an optimal dilution of 1:50.

7.32 Localisation of TSP-1

TSP-1 was detected perivascularly and in the stoma, but not within tumour cells. (Table 7.1 and Figures 7.1a&b). There was no inter-observer variability (Kappa score=1).

Table 7.1 Protein expression of TSP-1 in colorectal liver metastases

	Perivascular TSP-1	**Stromal TSP-1**
Total number of patients	182	182
Positive cases	45 (25%)	59 (33%)
Negative cases	138 (75%)	124 (67%)

Figure 7.1a Perivascular TSP-1

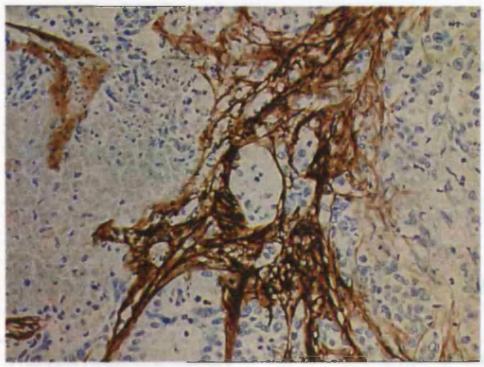
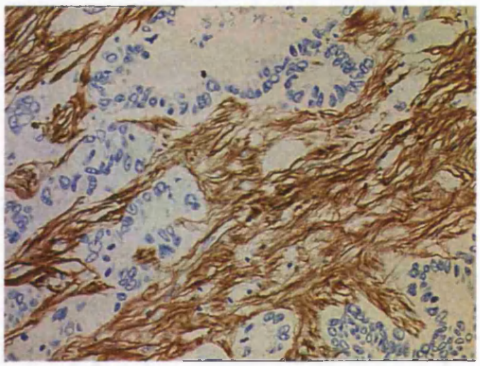


Figure 7.1b Stromal TSP-1



7.4 Relationship between TSP-1 and clinico-pathological parameters

Thrombospondin-1 was correlated with the clinicopathological factors, as summarised in Table 7.2

Table 7.2 TSP-1 and clinico-pathological parameters

Pathological factor	Perivascular TSP-1 absent	present	χ^2	Stromal TSP-1 absent	present	χ^2
Gender						
Male	91	31	0.85	80	42	0.5
Female	46	14		43	17	
Dukes stage						
A	4	2	0.28	4	2	0.5
B	64	15		57	22	
C	69	28		62	35	
Synchronicity						
Synchronous	71	26	0.48	60	34	0.41
Metachronous	66	19		63	25	
Disease distribution						
Unilobar	86	24	0.26	79	31	0.13
Bilobar	51	21		44	28	
Size of lesion						
Below median	61	24	0.34	57	28	0.88
Above median	76	21		66	31	
Number of lesions						
One lesion	59	17	0.53	52	24	0.84
>1 lesion	78	28		71	35	
<3 lesions	110	36	0.96	102	44	0.18
>3 lesions	27	9		21	15	

7.5 Relationship between TSP-1 and MVD

The relationship between TSP-1 and MVD is presented in Table 7.3

Table 7.3 Perivascular TSP-1, stromal TSP-1 and MVD

Microvessel Density	Perivascular TSP-1 absent	present	χ^2	Stromal TSP-1 absent	present	χ^2
Tumour edge hotspots						
Below median	65	19	0.18	59	25	0.14
Above median	44	21		38	27	
Contiguous edge count						
Below median	60	16	0.10	54	22	0.12
Above median	49	24		43	30	
Intra-tumour hotspot						
Below median	74	19	0.17	71	22	0.01
Above median	63	26		52	37	
Intra-tumour cumulative mean						
Below median	79	24	0.61	74	29	0.16
Above median	58	21		49	30	

7.6 Relationship between TSP-1 and patient survival

7.51 Univariate analysis (Figures 7.2a&b)

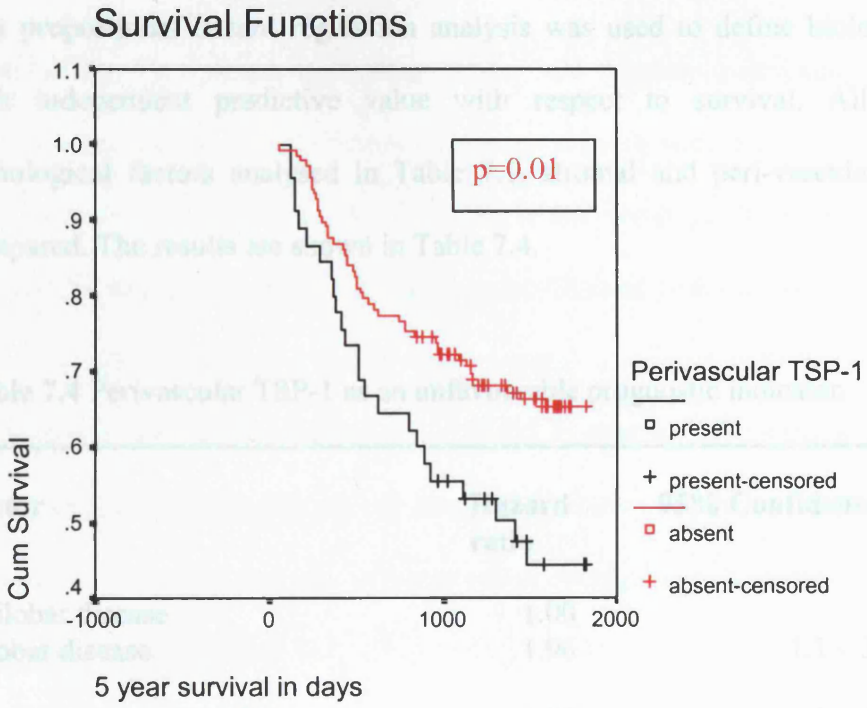


Figure 7.2a Present versus absent perivascular TSP-1

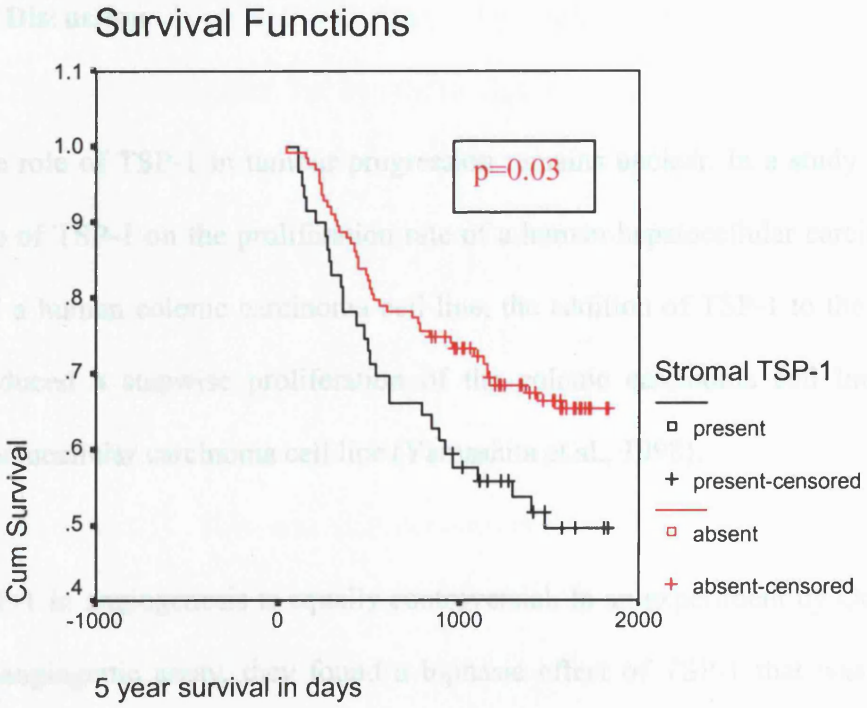


Figure 7.2b Present versus absent stromal TSP-1.

7.62 Multivariate analysis

Cox proportional hazard regression analysis was used to define biological markers with independent predictive value with respect to survival. All the clinico-pathological factors analysed in Table 7.1, stromal and peri-vascular TSP-1 were compared. The results are shown in Table 7.4.

Table 7.4 Perivascular TSP-1 as an unfavourable prognostic indicator.

Factor	Hazard ratio	95% Confidence Interval	p value
Unilobar disease	1.00		P=0.009
Bilobar disease	1.96	1.1 – 3.2	
Present perivascular TSP-1	1.82	1.0 – 3.1	P=0.01
Absent perivascular TSP-1	1.00		

7.7 Discussion

The role of TSP-1 in tumour progression remains unclear. In a study evaluating the role of TSP-1 on the proliferation rate of a human hepatocellular carcinoma cell line and a human colonic carcinoma cell line, the addition of TSP-1 to the culture media produced a stepwise proliferation of the colonic carcinoma cell line but not the hepatocellular carcinoma cell line (Yamashita et al., 1998).

TSP-1 in angiogenesis is equally controversial. In an experiment by Quan *et al* using an angiogenic assay, they found a biphasic effect of TSP-1 that was concentration dependent: low levels of TSP-1 (<10μg/ml) produced a pro-angiogenic effect, whilst

with higher levels (>15μg/ml) an anti-angiogenic effect was produced. They also demonstrated that TSP-1 increased production of MMP-9.(Qian et al., 1997)

Observations in human colorectal cancer are equally confusing. Maeda *et al* demonstrated TSP-1 as an inhibitor of angiogenesis by decreasing tumour vascularity, in association with reduced risk of recurrence and better patient survival (Maeda K et al., 2001). Another study by the same group showed that tumours with high VEGF and low TSP-1 had higher microvessel counts, associated with worse prognosis on multivariate analysis (Kawahara et al., 1998). In contrast, Tsuchida *et al* showed that TSP-1 gene expression did not correlate with tumour vascularity, whereas CD36 gene expression correlated not only to lower vessel counts, but to improved patient survival (Tsuchida T et al., 1999).

Yamashita *et al*, in a study of 115 patients with colorectal cancer, measured the concentration of plasma TSP levels by enzyme-linked immunoadsorbent assay (ELISA) and correlated the results to Dukes' stage and venous invasion. Plasma levels were statistically increased with increasing Dukes' stage and with those tumours exhibiting venous invasion (Yamashita et al., 1998).

In this series, TSP-1 appears to be pro-angiogenic in the centre of the tumour as those patients with absent stromal TSP-1 had significantly lower intra-tumour hotspot counts ($p=0.01$). This was not demonstrated at the tumour edge. On univariate analysis, the presence of both peri-vascular and stromal TSP-1, were significantly associated with poor survival ($p=0.01$ and $p=0.03$ respectively).On multivariate

analysis, the presence of peri-vascular TSP-1 was an independent prognostic factor for poor patient survival.

As indicated by Qian (Qian et al., 1997), low levels of TSP-1 have been associated with pro-angiogenic activity. It may therefore be postulated that the centre of liver metastases have low levels of TSP-1 functioning in a pro-angiogenic manner, thus accounting for the association of TSP-1 and intra-tumour hotspots.

With regards to TSP-1 as a negative prognostic factor for patient survival, it may be argued that TSP-1, in addition to being pro-angiogenic, is also promoting tumour invasion and proliferation, which is in agreement with a recently proposed model by Sargiannidou *et al* in which TSP-1 up-regulates MMP-9 via a complex pathway (Sargiannidou et al., 2001).

In summary, in colorectal liver metastases, TSP-1 is pro-angiogenic at the centre of the metastasis and perivascular TSP-1is an independent prognostic factor for poor patient survival.

Chapter 8

p53

8.1 Introduction

p53 is a tumour suppressor gene which acts to prevent genetic damage by detecting damaged DNA and up-regulating genes that either arrest the cell cycle to allow for DNA repair or promote cellular apoptosis (Lane D P, 1994). p53 is known to be anti-angiogenic *per se,* and by promoting the up-regulation of TSP-1 and down-regulation of VEGF.

The most common genetic change in solid tumours is mutation of the p53 gene (Lane D P, 1994; Smith M L and Fornace A J Jr, 1996). Mutated p53 is found in approximately 50% of spontaneous cancers: loss of cell cycle controls promotes uncontrolled cell growth. In those tumours with wild type p53, other mechanisms have been suggested that impair its tumour suppressor effect, such as increased tumour glycolysis (Chiarugi V et al., 1998). The most common genetic alteration in colorectal cancer is loss of the p53 tumour suppressor gene (Fearon and Vogelstein, 1990). More than 50% of sporadic colorectal cancers have p53 missense mutations. Point mutations of p53 have also been identified in liver metastases (Heide I et al., 1997; Yao J et al., 1996; Tullo A et al., 1999) which have been found to either correlate with (Heide I et al., 1997; Yao J et al., 1996; Tullo A et al., 1999) or exceed (Kastrinakis W V et al., 1995) the number of mutations in the primary tumour.

Mutated p53 in primary colorectal cancer has been correlated with increased risk of developing liver metastases and poor outcome (Okano K et al., 1999; Berney C R et al., 1999). Mutated p53 in liver metastases has been correlated with increased

recurrence and poor survival in some studies (Tullo A et al., 1999; Nitti et al., 1998) and with better survival in others (De Jong K P et al., 1998; Yang Y et al., 2001).

8.2 p53 protein expression

Primary antibody concentrations of 1:50, 1:75, 1:100 and 1:150 were used, with optimal results being obtained using 1:100 (Figure 8.1). Greater than 20% positive nuclei was classified as a p53 positive tumour, and <20% was classified as negative. (Table 8.1)

Figure 8.1 p53 Staining

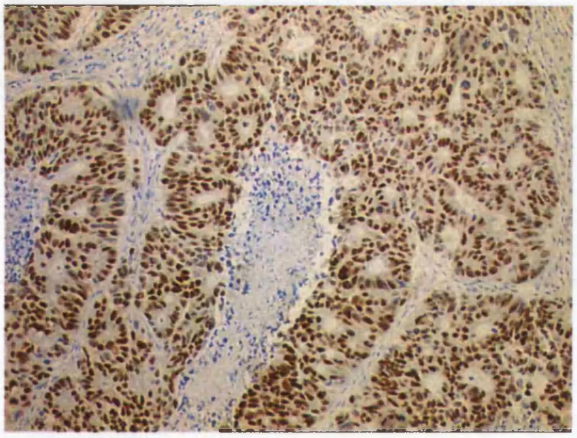


Table 8.1 Expression of p53 in colorectal liver metastases

	p53
Total number of patients	182
Positive cases	116 (64%)
Negative cases	66 (36%)

8.3 Relationship between p53 and clinico-pathological parameters

p53 was compared with the clinico-pathological variables (Table 8.2).

Table 8.2 Clinico-pathological variables and p53

Pathological factor	p53 below median	p53 above median	χ^2	p53 absent	p53 present	χ^2
Gender						
Male	61	61	1.0	47	75	0.36
Female	30	30		19	41	
Dukes' stage						
A	3	3	0.69	2	4	0.78
B	38	41		30	49	
C	50	47		34	630	
Synchronicity						
Synchronous	49	48	0.88	33	64	0.50
Metachronous	42	43		33	62	
Disease distribution						
Unilobar	53	57	0.55	33	77	0.03
Bilobar	38	34		33	39	
Size of lesion						
Below median	39	46	0.22	26	59	0.13
Above median	52	45		40	57	
Number of lesions						
One lesion	38	38	1.00	24	52	0.26
>1 lesion	53	53		42	64	
<3 lesions	75	71	0.45	54	92	0.68
>3 lesions	16	20		12	24	

8.4 Relationship between p53 and MVD

p53 was compared with MVD, as shown in Table 8.3

Table 8.3 p53 and MVD

Microvessel Density	p53 below median	p53 above median	χ^2	p53 absent	p53 present	χ^2
Tumour edge hotspots						
Below median	35	49	0.14	24	60	0.20
Above median	35	30		26	40	
Contiguous edge count						
Below median	35	41	0.81	23	53	0.49
Above median	35	38		27	47	
Intra-tumour hotspot						
Below median	39	54	0.02	28	65	0.07
Above median	52	37		38	51	
Intra-tumour cumulative mean						
Below median	44	59	0.02	33	70	0.17
Above median	47	32		33	46	

8.5 Relationship between p53 and VEGF, TSP-1 and TP

The results are shown in Table 8.4

Table 8.4 p53 and mediators of angiogenesis

Microvessel Density	**p53**		χ^2	**p53**		χ^2
	below median	**above median**		**absent**	**present**	
Stromal VEGF						
Below median	46	43	0.66	35	54	0.40
Above median	45	48		31	62	
Stromal VEGF						
Absent	25	31	0.34	20	36	0.91
Present	66	60		46	80	
Tumour cell VEGF						
Below median	51	40	0.10	37	54	0.27
Above median	40	51		29	62	
Tumour cell VEGF						
Absent	9	7	0.60	5	11	0.66
Present	82	84		61	105	
Stromal TSP-1						
Absent	60	63	0.64	42	81	0.39
Present	31	28		24	35	
Perivascular TSP-1						
Absent	70	67	0.61	49	88	0.80
Present	21	24		17	28	
Tumour cell TP						
Below median	45	51	0.37	31	65	0.23
Above median	46	40		35	51	
Tumour cell TP						
Absent	21	32	0.07	18	35	0.67
Present	70	59		48	81	

8.6 Relationship between p53 and patient survival

8.61 Univariate analysis (Figures 8.2a&b)

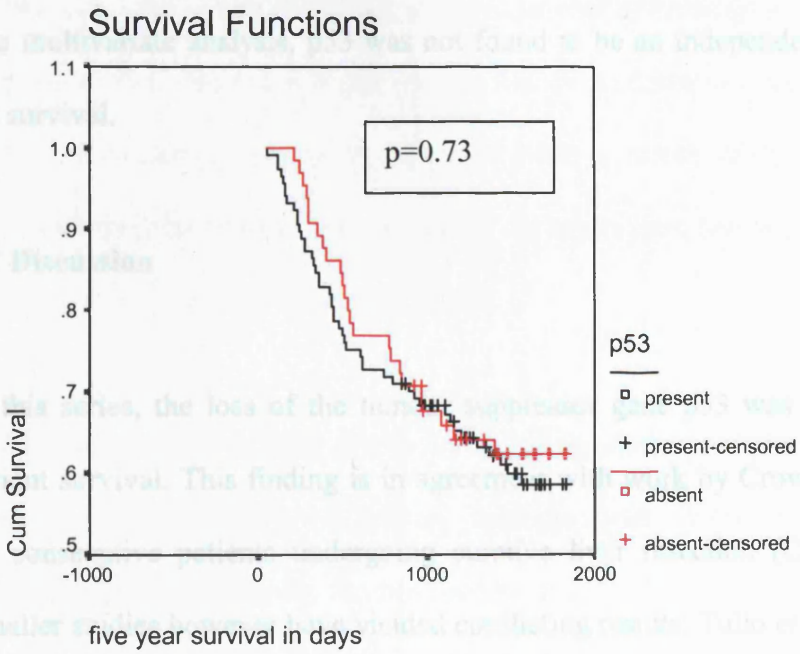


Figure 8.2a Present versus absent p53

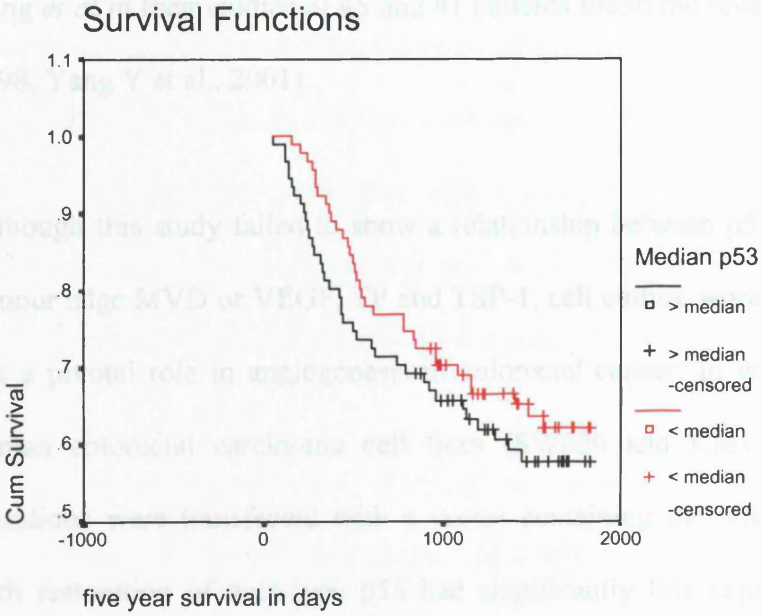


Figure 8.2b Median p53 p=0.38

8.62 Multivariate analysis

By incorporating all the clinico-pathological factors analysed in Table 8.1 and p53 into multivariate analysis, p53 was not found to be an independent prognostic factor for survival.

8.7 Discussion

In this series, the loss of the tumour suppressor gene p53 was not associated with patient survival. This finding is in agreement with work by Crowe *et al* who studied 71 consecutive patients undergoing curative liver resection (Crowe et al., 2001). Smaller studies however have yielded conflicting results: Tullo *et al* and Nitti *et al,* in studies of 40 and 69 patients respectively, describe the loss of p53 as a negative prognostic event (Tullo A et al., 1999; Nitti et al., 1998), whereas De Jong *et al* and Yang *et al* in their studies of 45 and 41 patients found the reverse (De Jong K P et al., 1998; Yang Y et al., 2001).

Although this study failed to show a relationship between p53 expression and either tumour edge MVD or VEGF, TP and TSP-1, cell culture work has suggested that p53 has a pivotal role in angiogenesis in colorectal cancer. In work from Bouvet *et al*, human colorectal carcinoma cell lines (SW620 and KM12L4) with known p53 mutations were transfected with a vector containing the wild-type p53 gene. Cells with restoration of wild-type p53 had significantly less expression of VEGF RNA compared to their controls. In addition, when both forms of cells were transplanted into the dorsal air sac of nude mice, those with wild-type p53 had significantly less

angiogenesis than those with the mutated form of p53 (Bouvet et al., 1998). This relationship appears to hold true for early colorectal cancers in a study comparing colorectal carcinomas confined to the mucosa with those that had invaded the submucosa. The former group showed a relationship between loss of p53 and VEGF expression that was not demonstrated in the more advanced tumours (Kondo et al., 2000). In keeping with work presented here, a recent study of 11 patients with colorectal liver metastases, demonstrated no association between the loss of p53 and VEGF over-expression (Kern et al., 2002).

p53 is not thought to play a role in the up-regulation of TP in colorectal polyps (Enomoto et al., 2000), or primary tumours (van Triest et al., 2000). To my knowledge, this relationship has not been previously documented in the literature with regards to liver metastases. The findings from this study agree with the above concept, since no relationship between p53 and TP was found, thus suggesting that the up-regulation of TP occurs by a p53-independent process. Similarly, there appears to be no information regarding the relationship between p53 and TSP-1 in colorectal liver metastases, although cell line work using cultured fibroblasts from patients with Li Fraumeni Syndrome transfected with mutant p53 showed up-regulation of TSP-1 (Dameron et al., 1994). Data from this study indicates that there is no significant correlation between p53 and TSP-1 (Table 8.4).

The only seemingly positive findings in this study marginally correlate higher expression of p53 in unilobar disease, and in association with lower median intra-tumour hotspots. The significance of these is uncertain.

In conclusion, loss of wild-type p53 was not associated with patient survival, angiogenesis or increased expression of angiogenic regulatory factors in this study. Indeed, p53 expression appeared, if anything, to correlate with more favourable clinicopathological variables including unilobar disease and low intra-tumour vessel counts.

Chapter 9

Conclusions and Future Directions

9.1 Multivariate analysis of clinicopathological variables, angiogenesis and angiogenic regulating factors

On multivariate analysis, bilobar disease, absence of stromal VEGF and hotspot MVD at the tumour edge are independent negative prognostic factors for survival in patients with colorectal liver metastases. This is shown in table 9.1

Table 9.1 Multivariate analysis of a series of 182 resected colorectal liver metastases

Factor	Hazard ratio	95% Confidence Interval	p value
Unilobar disease	1.00	1.18 – 3.33	0.01
Bilobar disease	1.98		
Hotspot MVD at the tumour edge	1.73	1.03 – 2.91	0.038
Present stromal VEGF	0.43	0.25 – 0.72	0.002
Absent stromal VEGF	1.57		

9.2 Summary and conclusions

9.21 Colorectal adenoma carcinoma cycle

In colorectal primary tumours, VEGF and TP are up regulated, and high levels of these correlate with high MVD counts. In turn, high levels of all three factors have been found to be negative prognostic factors both on univariate and multivariate analyses. TSP-1 has been described as an anti-angiogenic factor with high levels correlating with low MVD. Loss of tumour suppressor gene p53 is thought to be associated with up-regulation of VEGF and TP and down-regulation of TSP-1.

9.22 Behaviour of liver metastases in this series

In colorectal liver metastases high hotspot MVD at the tumour edge was found to be an independent negative prognostic factor. TP appears to be influencing angiogenesis at the tumour edge, with high levels correlating with both mean edge vessel counts and hotspot vessel counts.. Loss of p53 tumour supression does not appear to be regulating the behaviour of VEGF, TSP-1 or TP. VEGF is over-expressed in over 90% of tumours in this series, and does not appear to be associated with MVD. Curiously, absent stromal VEGF is a negative prognostic factor on univariate and multivariate analysis; this obviously requires further investigation. TSP-1 does not appear to be acting as an anti-angiogenic factor, but may be acting via its invasion "site", as increased levels of TSP-1 were significantly associated with poor survival on univariate analysis.

9.3 Future work

Having demonstrated that resected colorectal liver metastases over-express VEGF and the angiogenic potential seems to be driven by TP, it would be of interest to examine factors that modulate their behaviour. VEGF has been found to be up-regulated by hypoxia through Hypoxia-Inducible Factor-1(Levy et al., 1995). Hypoxia also upregulates factors involved in modulating cell metabolism, such as carbonic anhydrase IX (Swinson et al., 2003), to promote an acidic extracellular environment required for tumours to invade. Such invasion results from the breakdown of the extracellular matrix by the matrix metalloproteinases (Cox et al., 1999).

Future work will thus comprise the evaluation of the role of hypoxia and angiogenesis in resected colorectal liver metastases. This will be achieved by the

immunohistochemical detection of HIF-1 (as a modulator of VEGF), CAIX (as a modulator of tumour cell metabolism), MMP-2 and MMP-9 in this series of colorectal liver metastases.

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