UNIVERSITY OF SOUTHAMPTON

AN INVESTIGATION INTO THE ROLE OF MATRIX METALLOPROTEINASES IN LIVER METASTASES FROM COLORECTAL CANCER

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ABSTRACT

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MATRIX METALLOPROTEINASES AND LIVER METASTASES FROM COLORECTAL CANCER

By Robert Duncan Howell

A review of the literature of the natural history, treatment and survival of patients with colorectal cancer and liver metastases from colorectal cancer has been undertaken. Special reference has been made to the metastatic process and the role of various clinicopathological and biological markers in determining outcome. A critique of the role of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in cancer and metastasis is made and a potential role in liver metastases from colorectal cancer discussed.

Immunohistochemistry has been used to investigate the expression of matrix metalloproteinases -1, -7 & -9 and tissue inhibitors of matrix metalloproteinases -1 & -2 in archival tissue from 105 patients who underwent hepatic resection for colorectal cancer metastases. The expression of the matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases were analysed both for a descriptive picture and with respect to patient outcome.

Variability in expression levels of these enzymes is demonstrated and the correlation with patient outcome is explored. In addition the clinicopathological variables for each patient are also examined for prognostic significance.

The description of matrix metalloproteinases -1, -7 & -9 and tissue inhibitors of matrix metalloproteinases -1 & -2 in colorectal liver metastases is novel. The hypothesis that these enzymes have prognostic significance in colorectal liver metastases is partially supported and the need for further research is emphasised.

For

Harriet, Katherine and Nicholas

&

Margaret and Peter Howell, my loving parents,
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Chapter One

The Hypothesis

Hypothesis: matrix metalloproteinases are prognostic markers in colorectal liver metastases

Colorectal cancer is the second most common cancer in the UK with an incidence of approximately 31000 new cases per year. In England and Wales approximately 21000 people die from colorectal cancer every year and this prevalence is mirrored in most Western countries. Epidemiological studies have documented a steady increase in the number of patients developing colorectal cancer over the past 50 years and this trend looks set to continue (Vukasin et al, 1990). The overall 5-year survival remains low at around 40 per cent despite improvements in diagnosis, surgical technique, anaesthesia, postoperative care and adjuvant therapy.

Liver metastases are common and frequently the cause of death in colorectal cancer. At the time of presentation with the primary colorectal cancer approximately 25 per cent of patients will have hepatic metastases (synchronous metastases) and a further 50 per cent of patients will develop them (metachronous metastases) within 5 years of the initial 'curative' resection (Taylor, 1996). Some 90 per cent of patients who die from colorectal cancer have liver metastases and there are few cancers in which the metastatic pattern is so predictable. The natural history of untreated metastatic colorectal cancer to the liver is dismal with prolonged follow-up of untreated patients showing that median survival is between 6 to 12 months (Allen-Mersh et al, 1994) and survival beyond 5 years is rare (Wagner et al, 1984; Scheele et al, 1990).

The two main treatment options for colorectal liver metastases are surgery or chemotherapy. Results with chemotherapy alone have so far been disappointing and surgical resection provides the only opportunity for cure with 5-year survival rates of 25-35 per cent being reported (Jatzko et al, 1995; Wanebo et al, 1996b; Rees et al, 1997). However adjuvant chemotherapy may show increased survival and may cure patients with micrometastatic disease. It is estimated that surgical resection is suitable in 10-27 per cent of patients with proven hepatic metastases (August et al, 1984; Adson, 1987). Mortality rates of up to 15 per cent and morbidity rates of up to 45 per cent have been reported, although current mortality rates should now be less than 5 per cent (Hughes et al, 1988; Gayowski et al, 1994; Doci et al, 1995). Surgical

intervention is thus a major undertaking and therefore the aim is to identify those patients who are unlikely to benefit from surgery.

Several clinicopathological factors, including stage of the primary lesion, number of metastases, tumour diameter, positive surgical margin, coexistence of extrahepatic recurrence or metastases, and the presence of satellite lesions have been described as prognostic markers in liver metastases. However many of these negative prognostic factors have been found in long-term survivors and currently the decision to resect hepatic metastases remains subjective and on an individual patient basis.

Metastatic spread of colorectal cancer continues to be the greatest barrier to cure and an understanding of the molecular basis of metastasis is essential for the design and effective use of new treatment options. One class of molecules that has been repeatedly implicated in metastasis is the matrix metalloproteinases (MMP's). The matrix metalloproteinases are a family of zinc-dependant endopeptidases that collectively have the capacity to degrade all the major components of the extracellular matrix. In the extracellular matrix the activity of matrix metalloproteinases is tightly regulated by various control mechanisms, which include the naturally occurring tissue inhibitors of matrix metalloproteinases (TIMP's). The precise regulation of the MMP/TIMP balance seems to be essential in regulating many physiological processes associated with tissue remodelling, such as trophoblast-decidua invasion, angiogenesis, endometrial proliferation, embryogenesis and lung development. Furthermore, disruption of the MMP/TIMP balance leads to profound changes in extracellular matrix integrity observed not only during neoplastic invasion, but also in other disease processes such as emphysema, periodontitis and arthritis. The early data implicating matrix metalloproteinases in metastasis was based on correlation between levels of the matrix metalloproteinases and metastatic potential in model systems (Duffy, 1992).

The original proposal that proteinases might be used as markers of prognosis in human cancers came in 1987. Since then matrix metalloproteinases have been linked with a variety of cancers: breast cancer, gastric cancer (Mori et al, 1997), urothelial cancers and colorectal cancer. High expression of MMP-9 and MMP-1 have both been associated with poor prognosis in colorectal cancer.

The naturally occurring tissue inhibitors of matrix metalloproteinases inhibit active matrix metalloproteinases by forming strong 1:1 complexes. Decreased levels of tissue inhibitors of matrix metalloproteinases have been found to be related to tumour progression. However, the effect of tissue inhibitors of matrix metalloproteinases on the growth of primary tumours and metastatic lesions is further complicated by the observation that TIMP-1 and TIMP-2 also display growth promoting activity for a variety of cell types (Nemeth & Goolsby, 1993). Increased levels of TIMP-1 have been shown to predict poor outcome in breast cancer and also high levels of TIMP-2 have been associated with poor prognosis in both breast and bladder cancer.

Expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in colorectal cancer liver metastases is not widely reported in the literature. It has been shown that both MMP-9 and TIMP-1 are expressed by liver metastases and indeed that they seem to have a particular pattern of localisation, with MMP-9 found at the interface between tumour stroma and normal liver and TIMP-1 found throughout the malignant tumour stroma (Zeng & Guillem, 1995). More recently, it has also been shown that there is increased expression of MMP-1, -7 and -9 within the liver metastases and decreased expression of TIMP-1 and -2 in the immediately adjacent liver tissue to the metastases (Kelly et al, 1999).

It is our primary hypothesis that the expression of matrix metalloproteinases -1, -7 and -9 and tissue inhibitors of matrix metalloproteinases -1 and -2 will have prognostic significance in patients with colorectal liver metastases.

Aims

1. To investigate, by immunohistochemistry, the expression patterns of MMP-1, MMP-7, MMP-9, TIMP-1 and TIMP-2 in a series of patients who have undergone hepatic resection for colorectal liver metastases.

2. To investigate the correlation between the expression of matrix metalloproteinases and their inhibitors and patient survival

Chapter Two

Introduction

Colorectal Cancer

Epidemiology

Colorectal cancer is the second most common cancer with an incidence of 31,320 new cases per year in the United Kingdom (Cancer Research Campaign, 1998). In England and Wales approximately 21,000 people die from colorectal cancer every year (Government Statistical Service, 1995).

Worldwide, there were 875,000 new cases of colorectal cancer in 1996 accounting for 8.5 per cent of all new cases of cancer (World Health Organisation, 1997). The incidence of colorectal cancer varies 20 fold around the world (Parkin et al, 1992), with high incidences seen in industrialised western countries such as Northern and Western Europe, United States and Canada, and lower rates seen in Asia and Africa. Whilst the world-wide incidence has remained stable over the last 30 years, epidemiological studies have documented a steady increase in actual numbers owing to an expansion in the population size (Myers & Ries, 1989; Vukasin et al, 1990; National Institute of Health Consensus Conference, 1990). Colon cancer is the only cancer with almost equal frequency between males and females (McMichael & Potter, 1980), although in areas of high incidence and rapidly increasing rates e.g. Japan and Italy male incidence is greater than female incidence by as much as 20 per cent (Potter, 1999).

Aetiology

The aetiology of colorectal cancer is not fully understood, although many risk factors have been identified: having a first degree relative with colorectal cancer (Fuchs et al, 1994), a medical history of chronic inflammatory bowel disease (Goldbohm et al, 1993), and a history of cigarette smoking (Giovannucci et al, 1994) are all associated with an increased risk of colorectal cancer.

Diet is probably the most important environmental factor and a positive correlation has been demonstrated between the incidence of colorectal cancer and total fat intake.

This association is clearly highlighted in Japan where fat intake has increased from 10 per cent to 25 per cent of total energy intake over the last 50 years, accompanied by a striking rise in mortality from colon cancer (Willett, 1989). The international differences, migrant data, and recent rapid changes in incidence rates in Italy, Japan, urban China and male Polynesians in Hawaii (Parkin et al, 1992), show that colorectal cancer is highly sensitive to changes in environment. Among immigrants and their descendants, incidence rates rapidly reach those of the host country, sometimes within the migrating generation (Haenszel, 1961; McMichael & Giles, 1988). The 20 fold international difference may be explained, in large part, by dietary and other environmental differences. Although incidence rates in Japan have been low even until quite recently, the highest rates in the world are now seen among Hawaiian Japanese (Parkin et al, 1992).

Colorectal cancer has long been known to occur more frequently in certain families (Macklin, 1960) and there are several rare genetic syndromes that carry a markedly elevated risk (Gardner, 1951; Veale, 1965; Utsunomiya & Lynch, 1990). Recognition of the genetic component of colorectal cancer is growing. Mutations are present as inherited germline defects or arise in somatic cells secondary to environmental insult. There are two main inherited predisposition syndromes: familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC); remaining cases are attributed to so-called sporadic colorectal cancer. Although the timescale of appearance and the propensity for recurrence of these various forms of colorectal cancer differ, they share the common random pathway of the adenoma to carcinoma sequence.

Most colorectal cancers arise within pre-existing adenomatous polyps or adenomas. These lesions are common but necropsy studies have shown a prevalence of about 35 per cent in Europe and the USA, with lower rates (10-15 per cent) in Asia and Africa (Coons et al, 1941). Adenomas are classified by histological architecture as tubular, tubulovillous, or villous. Villous change is associated with a higher malignant potential, as are large adenomas (up to 25 per cent of adenomas are >1 cm in diameter) and high-grade epithelial dysplasia (severe dysplasia is found in 5-10 per cent of adenomatous polyps). Approximately 5 per cent of adenomatous polyps are

estimated to become malignant, which takes 5-10 years (Coons et al, 1941; Midgley & Kerr, 1999).

A multistep model for the genetic events in the progression of sporadic colorectal cancer has been proposed (Vogelstein et al, 1988). Colorectal cancer occurs mainly in the elderly, which is consistent with the theory that a cell must accumulate a combination of four or five defects, including mutational activation of oncogenes and inactivation of tumour suppressor genes, to undergo full malignant transformation. If one or more of these defects are present at birth as germline abnormalities, fewer further mutations are required to complete transformation and the disease will appear earlier. Such abnormalities are likely in families whose members do not fulfil the criteria for the inherited predisposition syndromes, but have an increased risk of colorectal cancer at a younger age than the general population.

Familial adenomatous polyposis accounts for 0.2 per cent of colorectal cancers (Cunningham & Dunlop, 1996). It is an autosomal dominant disorder in which multiple adenomatous polyps develop in the colon during the second and third decades of life. These polyps are histologically identical to those preceding sporadic colorectal cancer, and, although each has little risk of malignant transformation, the numbers (reaching thousands in some patients) mean that the risk of colorectal cancer is almost 100 per cent by 40 years of age (Cawkwell & Quirke, 1996).

Hereditary non-polyposis colorectal cancer criteria are fulfilled in up to 5 per cent of patients with colorectal cancer (Lynch & Smyrk, 1996). It describes an autosomal dominant predisposition to colorectal cancer that lacks the excess polyposis seen in FAP. HNPCC is characterised by early age of cancer onset (average age of 45 years), proximal predominance of colorectal cancer (approximately 70 per cent proximal to the splenic flexure), multiple synchronous and metachronous colorectal cancers (approximately 45 per cent within 10 years of incomplete colonic resection), and an association with other tumours (endometrium, ovary, stomach, renal). The colon cancers may show special histology, including an excess of poorly differentiated carcinoma, mucinous carcinoma, signet cell carcinoma, and medullary, or undifferentiated histology (Lynch & Smyrk, 1996).

HNPCC is defined according to a set of strict criteria known as the Amsterdam criteria :

1. At least three family members with colorectal cancer, two of whom are first-degree relatives
2. At least two generations represented
3. At least one individual younger than 50 years at diagnosis

Colorectal cancer is therefore causally related to both genes and the environment.

Treatment and Outcome

Surgery

Surgical resection has been and remains the mainstay of treatment for colorectal cancer. This type of surgery began in the early 19th century when Lisfranc reported the first successful perineal resection of rectal carcinoma (Lisfranc, 1828). Resection and primary anastomosis, although first performed in 1823 (Reybard, 1844), was thought to carry an unacceptable mortality rate from anastamotic dehiscence, and therefore primary resection with colostomy formation was preferred. In the United States at the Lahey clinic, resection was never combined with primary anastomosis until the 1940's (Lahey, 1941).

Kronlein reported some of the earliest results in the treatment of rectal cancer in 1900 to The Berlin Surgical Congress (Kronlein, 1900). A series of 881 patients operated on by several eminent surgeons of the day including Kocher, von Mickulicz and others was collated. The operative mortality was 19.7 per cent and the 5-year survival rate of the 640 patients followed up was 14.8 per cent. Over the past century improvements in surgical technique and anaesthetic management have led to an increase in tumour resection rates and a reduction in operative mortality (Grinnell, 1953; Lockhart-Mummery et al, 1976). The survival of patients with colorectal cancer mirrored these improvements for the first half of the century but over the last 50 years there has only been a marginal improvement in survival. This improvement has been reduced by an increase in incidence of colorectal cancer and presentation with late stage disease remains common. The overall 5-year survival rate from population data is 37 per cent (Black et al, 1993).

The prognosis of patients following surgery depends on the tumour stage. Cuthbert Dukes, a pathologist at St. Marks Hospital, initially proposed a classification for rectal cancer that was later also adopted for colon cancer (Dukes, 1932). Nearly 60 years later, staging by Dukes' method remains the single most important predictor of outcome for patients with colorectal cancer. Dukes' described three stages:

- Stage A: tumour is confined within the serosa of the bowel
- Stage B: tumour extends through the serosa or into the extrarectal tissue but not into lymph nodes
- Stage C: tumour involves adjacent structures or lymph nodes

Dukes' used this classification to stage 2447 patients treated for rectal carcinoma, and reported corrected 5-year survival rates of 97.7 per cent for stage A lesions, 77.6 per cent for stage B and 32 per cent for stage C (Dukes & Bussey, 1958). Dukes' classification was modified by Gabriel to differentiate stage C cases with only regional lymph node involvement (C_1) from those with nodal involvement extending to the apical ligatures on the blood vessels of the resected specimen (C_2): 5-year survival for stage C_1 was 40.9 per cent and for stage C_2 13.6 per cent (Gabriel et al, 1935). Although not originally described by Dukes', stage D disease is now commonly used to identify those patients with metastatic disease beyond adjacent structures or lymph nodes.

Despite advances in methods of detection and treatment of colorectal cancer, approximately 50 per cent of patients develop recurrent disease, usually within one to five years following an apparently curative resection. This recurrence may either be local i.e. within the area of the operative field, the wound or anastomosis, or distant i.e. most commonly in the liver or lungs. The liver is involved in approximately 80 per cent of patients who develop recurrent colorectal cancer (August et al, 1984). The main cause of morbidity and mortality in colorectal cancer is the formation of distant metastases and it is this metastatic potential that remains the greatest barrier to cure.

Cancer Metastases

The formation of metastases is distinct from carcinogenesis and can be regarded as the final step in the evolution of cancer. The formation of metastases is a multistep process involving a number of linked sequential events (Fidler, 1990). The main steps are as follows:

1. Growth of primary tumour
2. Vascularisation of primary tumour
3. Invasion of surrounding host tissue
4. Release of tumour cells into circulation
5. Interaction of tumour cells in circulation with fibrin and platelets
6. Dissemination throughout the body
7. Arrest in capillary bed of distant organs
8. Extravasation of tumour cells through blood vessel wall into secondary organ
9. Growth at secondary site

The time period between the initial diagnosis of a primary cancer and the first detection of metastases varies widely between patients. In addition to different tumours having different metastatic capacities, the malignant cells within a given tumour have varying abilities to complete the metastatic cascade. Thus, the presence of malignant cells in either the circulation or a distant site does not necessarily mean that metastases will result. Using radiolabelled B16 mouse melanoma cells, Fidler demonstrated that, after 24 hours in the circulation, less than one per cent of the cells were viable and less than 0.1 per cent survived to form metastases (Fidler, 1970).

None of the events in the formation of metastases is specific to malignant cells. Normal cells such as leucocytes, lymphocytes and monocytes share many properties with metastatic cells including invasive behaviour, release into the circulation and extravasation. Indeed, these normal cells appear to use the same molecular process as malignant cells to cross tissue boundaries and move throughout the body. In contrast to malignant cell invasion, physiological invasion is highly regulated and ceases after

removal of the relevant stimulus. Invading tumour cells appear to have lost the control mechanism that prevents normal cells from moving into neighbouring tissue at inappropriate times and places (Duffy, 1998).

The genes responsible for metastases are distinct from those involved in carcinogenesis. The key genes involved in carcinogenesis include the c-oncogenes, tumour suppressor genes and DNA repair enzymes. The genes primarily involved in invasion and metastasis code for angiogenic factors, adhesion proteins, proteases and motility factors (Duffy, 1998).

Angiogenic factors

One of the early events in the progression of cancer is angiogenesis, or formation of new blood vessels (Mighell et al, 1997). These new capillaries arise from pre-existing capillaries or venules but never from arteries, arterioles or veins. Without the formation of its own vasculature, a tumour would be unable to grow beyond 2mm in size and thus would remain confined to the primary site. The new blood vessels embedded in a tumour provide an entry point for malignant cells into the circulation and spread to distant sites.

The initiation of angiogenesis can be mediated by both tumour cells and host inflammatory cells at the tumour site. These cells produce a variety of angiogenic mediating peptides such as VEGF, TNF-α, PDGF, acidic and basic PGF, TGF-α and angiogenin. There are also molecules that inhibit angiogenesis (Talks & Harris, 2000). Some of the most recently described antiangiogenic factors are proteolytic products of large molecules not known to be involved in angiogenesis. Angiostatin is a proteolytic degradation product of plasminogen that suppresses vascularisation and growth of lung metastases (O'Reilly et al, 1994). Purified angiostatin has been shown to induce apoptosis in metastases and sustain the dormancy of several human tumours implanted subcutaneously into nude mice (O'Reilly et al, 1996).

Proteolytic enzymes

During the process of cancer invasion and metastasis a number of natural tissue barriers have to be degraded. These barriers include both basement membranes and interstitial connective tissue (Duffy, 1992). Basement membranes are continuous extracellular structures that separate organ parenchyma from the underlying stroma. These structures are the first extracellular barriers to be crossed by invading cancer cells. Basement membranes consist of a number of different proteins and glycoproteins that form a highly cross-linked structure. Quantitatively, the most important protein in basement membranes is type IV collagen. Other components include laminin, proteoglycans (heparin sulfate and chondroitin sulfate), entactin and osteonectin. During invasion and metastasis, malignant cells cross basement membranes at least 3 times. They first pass through during their escape from the primary site, and subsequently they invade basement membranes during both entry into and exit from the blood stream.

Unlike the acellular basement membranes, the interstitial connective tissue consists of cells distributed in a meshwork of collagen fibres, glycoproteins (e.g. fibronectin), proteoglycans and hyaluronic acid. The main forms of collagen found in interstitial connective tissue are known as type I, II and III or interstitial collagen. Degradation of these natural barriers by invading cancer cells is believed to be brought about by the release of a number of different proteases from the invading tumour. The proteases implicated in degradation of the extracellular matrix include the urokinase form of plasminogen activator (uPA), cathepsin B, cathepsin D and various matrix metalloproteinases (Duffy, 1992). These proteases appear to act in a cascade manner to mediate metastasis (Duffy, 1996). The role of matrix metalloproteinases in cancer metastasis is considered in more detail later.

Adhesion proteins

During the process of cancer spread, malignant cells are continually breaking and forming new attachments to surrounding structures. Thus, in the early stages of invasion, metastatic cells must escape from their neighbouring cells and adhere to basement membranes. In the circulation, malignant cells initially bind to platelets,

leucocytes and fibrin. Later in the metastatic cascade, malignant cells adhere to the microvascular endothelium in the target organ. The molecules that allow these different cell-cell and cell-extracellular matrix interactions are known as adhesion proteins.

The adhesion proteins are divided into a number of different groups, i.e. the integrins, cadherins, immunoglobulin superfamily group, selectins and CD44. Compared to normal tissue, the levels of these adhesion proteins in malignancy can be elevated, down regulated or structurally altered.

Motility factors

Cell motility or migration is necessary for malignant cells to undergo invasion and metastasis. Migration involves extension at the leading edge and retraction at the trailing edge of the cell. Inside the migrating cells, the actin-based cytoskeleton is continuously being reconstituted, by assembly of actin filaments at the leading edge and disassembly at the trailing edge. The extension of the leading edge is associated with adhesion to the extracellular matrix that is thought to provide guidance and traction for pulling the cell body forward, the necessary mechanical force being generated by contraction of actin filaments (Duffy, 1998).

Hepatocyte growth factor (or scatter factor) may play a significant role in the pathogenesis and biology of human cancers. When combined with its receptor the resultant signalling has multifunctional effects on mammalian cells that include stimulation or inhibition of cellular proliferation, promotion of cell movement, invasion into extracellular matrix, and induction of glandular/tubular morphogenesis by epithelial cells (To & Tsao, 1998).

Pathophysiology of Colorectal Cancer Metastases

Colorectal cancer has a clonal origin (Vogelstein et al, 1985) but by the time most patients become symptomatic, the tumours are heterogeneous and consist of multiple subpopulations of cells with different metastatic potential (Talmadge & Fidler, 1982). The process of invasion and metastasis involves a series of linked sequential steps as described previously. In order for a metastasis to develop, the malignant cell must first detach from the primary tumour, invade through the extracellular matrix, and after travelling through either the vascular or lymphatic system must re-attach in a new environment and grow independently.

The predilection for colorectal cancer to metastasise to the liver is partly related to the fact that the gastrointestinal tract is drained by the portal vein from where tumour emboli may arrive via the mesenteric veins. Experimental work suggests that the majority of circulating tumour cells survive the mechanical trauma and host defence mechanisms encountered during their passage through the vascular system (Kerbel, 1995). If they are to grow and develop they must take on the characteristics of a micrometastasis and hence stimulate the development of a neovascular blood supply (angiogenesis). It is inconceivable that every malignant cell entering the portal circulation will successfully develop into a micrometastasis. Indeed, in animal models utilising portal vein injection of malignant cells, the liver is demonstrated to be a very effective 'trapper' of malignant cells as 'the organ first encounter' but is comparatively inhospitable to subsequent metastatic development (Weiss et al, 1981; Murphy et al, 1986). Animal liver metastases will only develop if more than 10^6 malignant cells are injected into the portal venous system. Once trapped, however, the malignant cells fail to recirculate and subsequent metastases (e.g. to the lungs) only develop from liver metastases (Murphy et al, 1988). In other words, it is likely that extrahepatic metastases arise from liver metastases. This pattern is recognised in the clinical situation where extrahepatic metastases are rare in the absence of synchronous liver metastases.

There is evidence to suggest that in most, if not all, patients with primary colorectal cancer, micrometastases exist in the liver in a dormant state (Fisher & Fisher, 1959;

Fisher & Fisher, 1965). It is probable that only a very small proportion of micrometastases develop into clinically overt disease; although trapped in the liver they are presumably prevented from growing owing to failure of mechanisms that determine metastatic growth. It is the development of a neovascular circulation that appears to be of major importance (Folkman, 1985) and it is suggested that negative regulators of angiogenesis, possibly arising from the primary tumour itself, determine whether metastases have the ability to grow (Folkman, 1995).

In comparison to the haematogenous spread of cancer, the lymphatic pathways probably have only a very limited role in the development of colorectal cancer metastases (Weiss et al, 1986). Although there are no lymphatic channels draining directly to the liver from the colon or rectum, it is possible a metastatic deposit may arise by sequential spread from pericolic to the para-aortic lymph nodes; however, this probably occurs in less than 20 per cent of cases (Weiss et al, 1986). Tumour cells that invade lymphatics may also spread haematogenously to the portal or systemic circulation via veno-lymphatic communications or directly to the superior vena cava via the thoracic duct. The factors that determine by which route colorectal tumours spread to the liver are still unknown. In patients with hepatic metastases it is uncommon to find tumour infiltration of lymph nodes along the hepatic artery, irrespective of the extent of disease within the liver, if there was no lymph node involvement at the primary tumour site (Dworkin et al, 1995).

The portal venous system is the vascular compartment most commonly invaded by liver metastases and there is considerable evidence that intra-hepatic spread of tumour (tertiary metastasis) occurs via the portal vein. In experimental tumour models it has been shown that metastatic cells first come into contact with, and implant in, the portal endothelium within a limited portion of the sinusoid (Barberaguillem et al, 1989). At that point, tumour cells may break off into a portal radical and re-seed close to the initial lesion or may spread by peri-portal lymphatic pathways. This intra-hepatic spread of tumour (satellite formation close to a large liver metastasis) may be referred to as 'tertiary metastasis' and is a fairly common finding in patients with secondary tumours of the liver (Foster & Lundy, 1981). Invasion of the intrahepatic Glisson's capsule, which includes the bile duct, the portal and hepatic veins and the periportal nerves, is also common in metastases from colorectal cancer.

Prevention of Colorectal Cancer Recurrence

Adjuvant chemotherapy and/or radiotherapy may be given to patients after resection of the primary colorectal cancer to try and prevent both local recurrence and the formation of distant metastases.

The anatomical differences between colon and rectal cancer and the ease of surgical intervention determine the pattern of recurrence, which defines the most appropriate adjuvant and relapse treatments. About 50 per cent of recurrences of rectal cancer occur in the pelvis and are, therefore, amenable to local radiotherapy. By contrast, relapse of colon cancer is generally at distant sites (liver, lungs and bone) and systemic chemotherapy seems more appropriate.

Chemotherapy

Approximately 75 per cent of all patients with colorectal cancer present at a stage when all macroscopic disease can be surgically resected (Kewenter et al, 1988). Despite this approximately 50 per cent of all colorectal cancer patients develop recurrence, presumably because of disseminated micrometastases present at the time of surgery. Adjuvant chemotherapy is used to eradicate these circulating cancer cells before they become established and refractory to intervention.

5-Fluorouracil has been the mainstay of chemotherapy for colorectal cancer for four decades. It is a prodrug that is converted intracellularly to various metabolites that inhibit synthesis of thymidine, DNA, and RNA. Insights into its molecular pharmacology have led to several strategies to modulate its cytotoxic effects. The most important is coadministration with folinic acid (leucovorin), which increases the degree of inhibition of thymidylate synthase, depletes cellular thymidine, and induces apoptosis. 5-fluorouracil is both cycle and phase specific thus showing greater efficacy against dividing cells in the S-phase, therefore a logical prediction would be that long-term infusional exposure to 5-fluorouracil would be more active. The type and severity of side effects seen with 5-fluorouracil based chemotherapy vary according to the regimen, but include nausea, vomiting, myelosuppression, mucositis,

diarrhoea, desquamation of the palms and soles, and, more rarely, cardiac and neurological toxic effects. This occurs to a mild extent in 70 per cent of all patients treated for advanced colorectal cancer, but is of sufficient severity to require a cessation or reduction in treatment in only 30 per cent of patients (Glimelius et al, 1994).

Although the history of adjuvant chemotherapy for colorectal cancer spans over 30 years, only since the mid-1980's have several clinical trials shown reproducible positive results. The Intergroup trial compared adjuvant chemotherapy after surgery with surgery alone (Moertel et al, 1990). 352 patients with Dukes B colorectal cancer and 971 with Dukes C disease were randomised to receive either surgery alone or surgery with 5-fluorouracil and levamisole for 1 year. In patients with Dukes C disease there was a significant reduction of 41 per cent in the risk of cancer recurrence (mainly within the liver) and a corresponding reduction in mortality of 33 per cent. This initial improvement corresponded with an absolute survival benefit of approximately 5 per cent, which was maintained at 5 years. The survival benefit for patients with Dukes B disease did not reach conventional levels of significance and there was no reduction in the incidence of liver metastases.

Three large prospective randomised trials have also shown increased disease free and overall survival after adjuvant treatment with 5-fluorouracil/folinic acid regimens, which suggests a decrease in the odds of dying from colon cancer by 25-30 per cent, or an absolute survival benefit of 5-6 per cent compared with controls (Wolmark et al, 1993; IMPACT trial, 1995; O'Connell et al, 1997).

All prospective trials to date have shown significant benefit in Dukes C colon patients, and there is continuing controversy about the effectiveness of systemic adjuvant chemotherapy for rectal and Dukes B colon cancers. Current trials should contribute useful information to this debate.

5-Fluorouracil is an S-phase specific drug and yet its active metabolites have half-lives of about 10 minutes, which limits its target, in the bolus form, to the small fraction of cells in the S-phase at the time of administration. Infusional therapy can, therefore, increase the proportion of cells it affects. The most common site for

micrometastases after resection of a colorectal tumour is the liver. By contrast with the macroscopically identifiable secondary tumours in advanced disease that derive their blood supply from the hepatic artery, these micrometastases are thought to be supplied by the portal vein. Therefore, administration of chemotherapy directly through the portal vein should produce the highest concentrations at the most vulnerable sites and lead to substantial first-pass metabolism, which would lessen systemic toxic effects. Portal vein infusion was developed for adjuvant 5-fluorouracil for 5-7 days after surgery. Data from ten trials (including 4000 patients and 1557 deaths) of portal vein infusion compared with surgery alone showed that survival with and without portal vein infusion were similar for the first 2 years. At 5 years, however, there was an absolute survival improvement of 4.7 per cent with portal vein infusion (Liver Infusion Meta-analysis Group, 1997). There is no clear evidence of a decrease in the number of patients who developed liver metastases. The AXIS study, in which 3583 patients were randomised to portal vein infusion or observation, reported preliminary results and concluded that portal vein infusion may increase 5-year survival by 4-5 per cent in colonic tumours (Taylor, 1999). Long-term follow-up of this trial and an update of the meta-analysis should help to provide a definitive answer.

Radiotherapy

The natural history and patterns of recurrence following 'curative' resection for colon cancer differ from those for rectal carcinomas. Locoregional failure as the only or major site of recurrence is common in rectal cancer, whereas colon cancer tends to recur throughout the peritoneum, in the liver and at other distant sites and has a lower rate of local failure. Consequently, pelvic irradiation has a significant role in the treatment of rectal but not colonic tumours. However, certain patients with colon cancer (tumours associated with abscess or fistula formation or those having residual disease or invasion into adjacent organs) may also benefit from postoperative radiation therapy in addition to systemic therapy (Willet et al, 1993).

In advanced rectal cancer, radiotherapy can improve pain, staunch haemorrhage, and lessen tenesmus. In patients with locally advanced disease which is inoperable, radiotherapy can down stage 35-75 per cent sufficiently to allow resection to be

performed (Pahlman, 1997). Radiotherapy has been assessed as an adjuvant therapy and the largest trial of preoperative radiotherapy showed a relative decrease in local recurrence rate of 61 per cent and an improvement in overall survival (58 per cent versus 48 per cent) (Swedish Rectal Cancer Trial, 1996). More recently the Dutch Colorectal Cancer Group have conducted a multi-centre prospective randomised trial to investigate total mesorectal excision with or without preoperative radiotherapy in the treatment of primary rectal cancer (Kapiteijn et al, 2001). A total of 965 patients were randomised and results showed a reduction in local recurrence from 8.2 per cent in the surgery only group to 2.4 per cent in the group also receiving preoperative radiotherapy. CRO7 is an MRC randomised controlled trial that is also investigating the role of preoperative radiotherapy in local recurrence rates and survival for rectal cancer, and this trial is due to recruit until 2003.

Radiotherapy after surgery seems to be less effective, even at higher doses, possibly because of rapid tumour cell repopulation after surgery and wound induced hypoxic weakening of the radiotherapeutic response. Only one trial has compared radiotherapy before and after surgery (Jansson-Frykholm et al, 1993). Despite a higher dose of radiotherapy after surgery, there was a significant reduction in the local recurrence rate among patients treated preoperatively. Interestingly, animal studies have suggested that 5-fluorouracil may prime tumour cells and sensitise them to subsequent irradiation. Indeed, one trial has shown substantially improved outcome for patients with rectal cancer treated with radiotherapy plus 5-fluorouracil infusion compared with radiotherapy alone (O'Connell et al, 1994).

Colorectal Liver Metastases

The addition of chemotherapy and radiotherapy to surgery in the treatment of colorectal cancer has led to a reduction in disease recurrence, but despite this, a significant number of patients will still develop metastatic disease.

Epidemiology

Twenty-five per cent of patients with a diagnosis of colorectal cancer will have synchronous liver metastases (Obrand & Gordon, 1997). Approximately 50 per cent of all patients with colorectal cancer will develop tumour recurrence (metachronous) following apparently curative surgery, usually within 5 years of the primary resection (D'Angelica et al, 1997). The liver is involved in up to 80 per cent of patients who develop recurrent colorectal cancer (August et al, 1984). Furthermore, 25 per cent of patients will have recurrence confined to the liver (Adson, 1987).

Anatomical Considerations

The predilection for liver metastases in colorectal cancer arises because of the portal venous drainage from the primary tumour. The right lobe of the liver is involved more frequently than the left although the reason for this remains unclear. There may be heterogeneity of blood flow distribution within the liver at a given time, but overall there is no gross difference in the volume of arterial or portal venous flow received by each lobe (Holbrook et al, 1995). It may simply be that the proportional segmental volume of the right lobe is larger than the left, and thus is more likely to contain metastases. However, right lobe involvement may also be a consequence of portal vein 'streaming', resulting in tumour emboli preferentially entering the right branch of the portal vein. Approximately one third of patients with colorectal cancer metastases have disease limited to one lobe of the liver (Cady & Stone, 1991).

Clinical and experimental studies have demonstrated that liver metastases derive their blood supply principally from the hepatic artery (Ackerman, 1982). There is some supply to micrometastases from the portal vein but the relative contribution of the arterial supply increases as the tumour enlarges (Lin et al, 1984). In an experimental

animal model, Ackerman showed that liver tumours form new blood vessels when their diameter exceeds 1mm, after which they are encircled by newly formed capillaries derived randomly from either the arterial or portal circulation (Ackerman, 1982). Others, however, have shown that metastases as small as 0.5 mm in diameter may have an established internal vasculature perfused predominantly by the hepatic artery (Lin et al, 1984). Small liver metastases initially grow elliptically, but as tumour expansion occurs they become spherical possibly because of a reduction in growth rate (Finlay et al, 1988). Peripheral portal vessels are compressed as the tumour enlarges, although arterio-portal communications exist at the periphery and may explain a partial portal 'take-over' if arterial occlusion occurs (Lin et al, 1984).

Arteriographic studies of secondary liver tumours show that there are great variations in vascularity; some tumours show minimal contrast uptake while others are hypervascular, with accompanying hypertrophy of the hepatic arterial supply to the involved lobe (Kim et al, 1977; Ridge et al, 1987). These variations in blood supply of liver metastases have implications for local treatment modalities such as regional chemoperfusion, hepatic dearterialisation and embolisation (Lin et al, 1984).

Natural History

Liver metastases develop within the liver during the period of primary tumour growth prior to diagnosis (Allen-Mersh, 1991). These are frequently undetected (occult) at the time of primary tumour resection, but subsequently grow until they reach a size when approximately 30 per cent of the liver is replaced (Earlam et al, 1996). It has been estimated from tumour growth kinetics that the sub-clinical phase of a liver metastasis (i.e. from metastatic implantation to clinical appearance) may be 2.5-5 years (Finlay et al, 1988; Purkiss & Williams, 1993). However, liver metastases sometimes appear many years after apparently curative resection of a primary tumour and this long delay in the development of clinically detectable disease suggests that in such patients host defence mechanisms have induced dormancy following initial treatment (Foster & Lundy, 1981; Kerbel, 1995). It is our lack of understanding of these mechanisms that frustrates attempts to predict and influence survival of patients with metastases.

The survival of patients with untreated colorectal metastases confined to the liver is closely related to the extent of liver replacement by tumour (Nagorney, 1987; Scheele et al, 1990). Various studies have examined survival in untreated patients with colorectal cancer metastases. In 1968 a study examined 177 patients and found a median survival of 5 months with no 5-year survivors (Jaffe et al, 1968). A review of the natural history of patients with untreated liver metastases considered the outcome of 172 Swedish patients, all of whom had proven colorectal cancer. The original laparotomy demonstrated synchronous metastases in 155 (91 per cent) of these patients and none of the patients had further treatment other than resection of the primary tumour. Median survival was 4.5 months with no survival beyond 3 years (Bengtsson et al, 1981). Similar data have been reported by others (Wood et al, 1976; Adson et al, 1984), although a 3-year survival rate of 14 per cent was reported in untreated patients, who in retrospect, had resectable disease (Wagner et al, 1984).

The computerised files of the US Department of Veterans Affairs Hospitals were analysed from 1988 to 1992. This represented over one million admissions per year and identified 887 patients whose codes indicated that they had a single liver metastasis that was not resected. Mean survival was 11 months, with a projected 5-year survival of 2 per cent (Wade et al, 1996).

It is clear that whilst there is variation in the reported survival times for patients with untreated liver metastases, survival beyond 5 years is rare.

Treatment and Outcome

Conventional treatments for liver metastases have either been purely palliative or have involved a range of techniques including systemic chemotherapy, regional chemotherapy, embolisation, cryotherapy or surgical resection. With the exception of surgery, none of these treatments has appeared to provide any real impact on overall survival and long-term cure.

Chemotherapy

The majority of liver metastases are not suitable for resection and therefore standard treatment is with systemic chemotherapy, which offers the only hope of palliation, prolongation of symptom free survival and possibly increased survival (Scheithauer et al, 1993).

Chemotherapy may be administered systemically or regionally via the hepatic artery or portal vein. Chemotherapy may be given prior to resection (neoadjuvant) or following resection (adjuvant) or may be given as the sole form of treatment in patients with unresectable disease (palliative). Adjuvant chemotherapy may also be used in conjunction with various cytoreductive procedures.

Systemic chemotherapy

Cytotoxic chemotherapy has been shown to confer a survival benefit and improvement in quality of life compared to best supportive care and this treatment is best started whilst patients are asymptomatic (Haller, 1995; Seymour, 1998).

Until recently the majority of chemotherapy regimens for metastatic colorectal cancer have been 5-fluorouracil based and when administered systemically as a single agent an objective tumour response rate is seen in 5-18 per cent of cases, although this has little effect on survival (Rougier et al, 1992). Improved response rates are achieved by combining 5-fluorouracil with folinic acid. Mean response rates with this combination are approximately 30 per cent with median survivals of approximately 12

months, which is significantly better than seen in untreated patients or those treated with 5-fluorouracil alone (Rougier et al, 1992).

The majority of patients currently undergoing liver resection will previously have been treated with 5-fluorouracil combined with folinic acid following resection of the primary tumour. Tumour resistance to repeated use of the same cytotoxic agents is a factor that needs to be considered in the selection of chemotherapy for colorectal liver metastases as response rates can be markedly reduced in previously treated patients. A number of options are emerging.

Irinotecan inhibits DNA topoisomerase I leading to replication arrest and breaks in single strand DNA. The overall response rate in advanced colorectal cancer resistant to 5-fluorouracil is 11.6 per cent, with a median duration of response of 6.7 months. Response rates in chemotherapy-naive patients range from 25-32 per cent (Conroy, 1997). Dose related side effects include severe diarrhoea, neutropenia, and alopecia. Two studies have compared irinotecan with best supportive care or high dose infusional 5-fluorouracil/folinic acid in patients with advanced colorectal cancer that had progressed despite previous therapy with 5-fluorouracil/folinic acid. There was a survival advantage for irinotecan in both studies (Cunningham et al, 1998). More recently a randomised multi-centre trial compared irinotecan combined with 5-fluorouracil/folinic acid versus 5-fluorouracil/folinic acid alone as first line treatment for metastatic colorectal cancer (Douillard et al, 2000). 387 patients were randomised and the results showed that those patients who received irinotecan combined with 5-fluorouracil/folinic acid fared better than those receiving 5-fluorouracil/folinic acid alone. The response rate was higher in the irinotecan group (49 vs 31 per cent), with longer time to disease progression (6.7 vs 4.4 months) and overall survival was higher (17.4 vs 14.1 months). These results have led to calls for irinotecan to be considered as a standard therapy for patients with metastatic colorectal cancer. Given the side effect profile of irinotecan, however, patients should be selected carefully.

Oxaliplatin is a third generation platinum analogue that cross-links DNA and induces apoptotic cell death. This agent becomes active in the presence of 5-fluorouracil/ folinic acid. A randomised trial showed significantly improved response rates of up to

40 per cent and progression free survival after the addition of oxaliplatin to 5-fluorouracil/folinic acid (Levi et al, 1994). The dominant toxic effect associated with oxaliplatin is cumulative neurotoxicity.

Tomudex is a quinazoline antifolate that directly inhibits thymidylate synthase. Tomudex has been compared with 5-fluorouracil/folinic acid in three randomised trials, including about 1500 patients with advanced colorectal cancer. Tumour response rates were similar between the groups in all three trials and overall survival was equivalent in two of three studies. Tomudex is better tolerated than 5-fluorouracil/folinic acid, with a significantly lower incidence of side effects (Kerr et al, 1995; Cunningham et al, 1995).

Regional chemotherapy

Hepatic metastases exclusively derive their blood supply from the hepatic artery and regional perfusion of established liver metastases allows delivery of high doses of cytotoxic agents directly to the tumour whilst avoiding high systemic levels because there is a high first-pass extraction of the drug. The two most frequently used agents for hepatic artery infusion are 5-fluorouracil and its derivative 5-fluoro-2-deoxyuridine. Intraportal chemotherapy is of no greater benefit than systemic chemotherapy and should be reserved for adjuvant treatment of micrometastases following primary colorectal excision.

Hepatic artery infusion is usually indicated in patients with unresectable metastases, which are confined to the liver. A catheter is inserted surgically into the gastroduodenal artery and thence into the hepatic artery to allow infusion chemotherapy directly into the liver. A cholecystectomy is usually required to avoid a chemical cholecystitis.

Tumour response and patient survival following regional chemotherapy have been shown to depend on the extent of liver involvement, with those patients who have less than 20 per cent liver involvement surviving longer (Kemeny, 1995). Response rates of intra-arterial chemotherapy are generally higher than those with systemic chemotherapy although only one study has reported marginal survival benefit over

systemic chemotherapy (Rougier et al, 1992). Another study has demonstrated that hepatic artery infusion of 5-fluoro-2-deoxyuridine may also contribute to improved quality of life, although death is more likely to occur from progression of extrahepatic disease (Allen-Mersh et al, 1994). In order to reduce the growth of extrahepatic metastases while liver metastases are controlled, a combination of regional and systemic chemotherapy has been tried. However, this was not found to be superior to hepatic artery infusion alone (Wagman et al, 1990).

Complications of regional chemotherapy such as biliary sclerosis, chemical hepatitis, arterial thrombosis, infections, catheter displacement, gastroduodenal inflammation and peritonitis are not uncommon.

Adjuvant chemotherapy

The role of chemotherapy in patients having a liver resection for colorectal metastases remains unresolved although current protocols of pre- and post-operative chemotherapy are common. No randomised trials comparing resection alone with resection followed by systemic chemotherapy have been completed although several studies have suggested that systemic adjuvant chemotherapy may improve survival (Hughes & Foster, 1991). A European study (the ENG study) was set up to examine the role of adjuvant chemotherapy following liver resection with curative intent, unfortunately, only 150 of 500 patients were recruited after 4 years and the study closed. However, analysis of the data acquired has not suggested benefit from adjuvant treatment.

Further recent studies have primarily investigated the role of regional chemotherapy following liver resection. A multicentre German trial of 226 patients comparing adjuvant postoperative hepatic artery chemotherapy with no treatment showed that the risk of death in the chemotherapy group was similar or worse than in the control group and so recruitment was terminated (Lorenz et al, 1998). The Intergroup prospective randomised study examined hepatic artery infusional therapy of floxuridine and systemic 5-fluorouracil versus resection alone in 109 patients who had liver resection for colorectal cancer. Preliminary results with a median follow-up time of 33 months show that the chemotherapy group had a decreased incidence of liver

recurrence and increased time to recurrence. However, whilst there was a trend to overall survival benefit in the chemotherapy treated patients this was not statistically significant (Kemeny et al, 1999). A third randomised study has compared hepatic arterial infusion plus systemic chemotherapy versus systemic chemotherapy alone in 156 patients following liver resection for colorectal cancer. This showed that hepatic arterial infusion plus systemic chemotherapy increased 2-year survival and hepatic disease free survival over systemic chemotherapy alone (Kemeny et al, 1999).

Neoadjuvant chemotherapy

Only 20-25 per cent of patients with liver metastases have surgically resectable disease. One of the strategies employed to increase resectability rates is neoadjuvant chemotherapy. For primarily unresectable hepatic metastases there have only been a handful of non-randomised retrospective studies and case reports that have described down staging to allow resection. The largest of these reported a series of 53 patients who underwent liver resection after neoadjuvant chemotherapy with systemic 5-fluorouracil, folinic acid and oxaliplatin (Bismuth et al, 1996). The 53 patients represented 16 per cent of the total number of patients with primarily unresectable colorectal metastatic disease seen at the authors' institution. The mean duration of chemotherapy before surgery was 8 months. The 5-year survival rate was 40 per cent for these 53 patients, which is comparable to the survival rates of patients undergoing resection alone. A large European Intergroup trial is currently assessing the role of pre- and post-operative chemotherapy with the above regimen.

Staging of Patients with Liver Metastases

The extent of liver involvement by metastatic tumour is an important determinant of survival (Wagner et al, 1984), and hence accurate staging of liver metastases may provide a guide to the possible success of surgical intervention. Several staging systems for colorectal liver metastases have been proposed, although unfortunately, no single system is universally accepted (Fortner et al, 1984; Gennari et al, 1986; Doci et al, 1991; Gayowski et al, 1994).

One system incorporated clinical and pathological information to classify patients with colorectal hepatic metastases into one of three stages but did not attempt to subclassify according to the extent of disease within the liver (Fortner et al, 1984). Another proposed staging system included the preoperative serum alkaline phosphatase level and performance status of the patient, and the extent of hepatic involvement and the presence of extrahepatic disease found at laparotomy (Petrelli et al, 1984). This system emphasised the importance of estimating the extent of hepatic involvement at laparotomy. However, improvements in imaging techniques have made preoperative assessment of the liver involvement more accurate than palpation during laparotomy. A further system also classified liver metastases into three stages but also took into account the multiplicity and distribution of metastatic lesions within the liver. The percentage hepatic replacement, as determined by preoperative CT scan, was used to measure the extent of tumour burden. Using this system it was noted that patients with Stage I and II disease (single metastasis involving less than 50 per cent of the hepatic parenchyma, or multiple metastases involving less than 25 per cent) had improved survival compared with patients with Stage III disease (multiple metastases involving between 25 and 50 per cent of hepatic parenchyma, or any number of metastases involving more than 50 per cent) (Gennari et al, 1985; Doci et al, 1991). However, measurement of percentage hepatic replacement rather than tumour size to assess extent of disease has been criticised on the basis that it may underestimate tumour volume. This is because normal liver parenchyma tends to be displaced rather than replaced by colorectal metastatic growth and also parenchymal volume may be preserved by liver regeneration (Gayowski et al, 1994; Dworkin et al, 1995).

A staging system for liver metastases modified from the International Union Against Cancer (UICC) and The American Joint Committee on Cancer (AJCC) recommendations for primary hepatobiliary tumours has been proposed (Gayowski et al, 1994). This system incorporates tumour size, tumour distribution, the number of metastatic lesions, and the extent of extrahepatic disease and patients are classified in Stages I-IV (Table 1). The problem with this system is the diaphragm, which is commonly resected with a good outcome, and yet strictly speaking should be classified as M1 disease.

The authors of this system analysed the outcome in 204 patients who underwent potentially curative resection of colorectal liver metastases. They confirmed that patients with nodal or extrahepatic involvement usually have a poor prognosis following resection of liver metastases although a small percentage of Stage IV patients (8 per cent) were still alive four years after resection (Gayowski et al, 1994). This data suggests that patients should not always be excluded from surgical treatment on the basis of the stage of liver metastases alone.

A prognostic scoring system was developed by Nordlinger based on the retrospective analysis of 1568 patients who had metastases confined to the liver and underwent resection with curative intent (Nordlinger et al, 1996). The analysis revealed seven variables that had prognostic value: age ($\leq$ 60 years vs. > 60 years), extension into the serosa of the primary cancer (absent vs. present), lymphatic spread of the primary cancer (absent vs. present), time interval from primary tumour to metastases ($\geq$ 2years vs. < 2 years), size of largest metastasis (< 5cm vs. $\geq$ 5cm), number of metastases (< 4 vs. $\geq$4), and liver resection margin ($\geq$ 1cm vs. < 1cm).

The relative risks of each of these seven variables were comparable and therefore a simple grading system was developed. Three risk groups were defined depending on the number of risk factors: low risk (0 –2), intermediate risk (3 or 4), and high risk (5 –7). The 2-year survival rates for the patients in the study decreased from 79 per cent in the low risk group to 60 per cent in the intermediate risk group, and 43 per cent in the high-risk group.

Table 1. Modified staging of hepatic colorectal metastases (see text)

Stage	Liver Metastases	Nodes	Other Metastases
I	mT1	N0	M0
II	mT2	N0	M0
III	mT3	N0	M0
IVa	mT4	N0	M0
IVb	Any mT	N1	M0/M1
		N0/N1	M1

mT1: solitary <2 cm

mT2: solitary >2 cm unilobar, multiple <2 cm unilobar

mT3: multiple >2 cm unilobar

mT4: solitary or multiple, bilobar, invasion of major branch of portal or hepatic veins or bile ducts

N1: abdominal lymph node

M1: extrahepatic metastases or direct invasion to adjacent organs

All of these variables are known prior to liver resection except the liver resection margin, but this can be estimated prior to surgery. When resection margin is not known the grading system can be modified accordingly with similar results: 0 –2 risk factors (2-year survival 78 per cent), 3 –4 risk factors (59 per cent), and 5 – 6 risk factors (35 per cent).

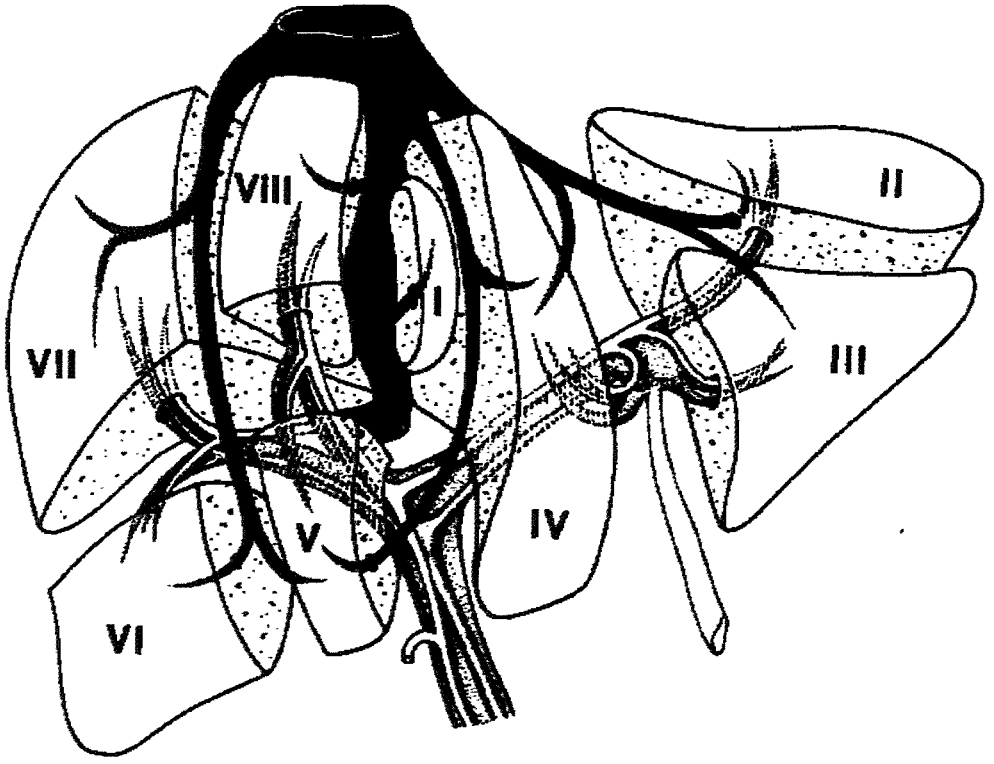
Surgery for Colorectal Liver Metastases

The earliest hepatic surgery was performed almost exclusively for trauma, but in 1888, it was Carl Langenbuch, of Berlin, who recorded the earliest successful resection for tumour (Langenbuch, 1888). In 1911 Wendel performed the first successful major hepatic resection using selective ligation of the vessels at the liver hilum (Wendel, 1911). In 1940 Cattell reported the first successful removal of a liver metastasis from a primary colorectal lesion (Cattell, 1940).

Francis Glisson from Cambridge first described the segmental anatomy of the liver in 1654 (Glisson, 1654). The era of planned resection of anatomically defined segments of the liver began in 1899 with Keen who performed the first left lateral segmentectomy (Keen, 1899). In 1948 Raven reported a left lateral segmentectomy for metastatic colon carcinoma (Raven, 1948). However, it was Lortat-Jacob who advanced the procedure by describing a technique for resection of the right lobe of the liver designed to control haemorrhage with ligation of the blood vessels and bile ducts to the right lobe (Lortat-Jacob & Robert, 1952). Others soon followed in reporting successful hepatic resections (Pack & Baker, 1953; Quattlebaum, 1953; McDermott, Jr. & Ottinger, 1966)

Advances in surgical technique have extended the boundaries of resectability for liver metastases over the last three decades. Recognition of the segmental basis of liver anatomy led to the evolution of segment based resection. The concept of functional anatomy based on the portal pedicles and the location of the hepatic veins evolved from Couinaud's study of vasculobiliary casts made by plastic injection followed by corrosion of the surrounding parenchyma (Couinaud, 1957). The liver is divided into four sectors by the three intrahepatic veins. Because the individual portal triad or pedicles supply both blood vessels and bile ducts to each sector they are called portal sectors. Each of the four sectors is further divided resulting in a total of eight segments (Figure 1). Theoretically each of the eight segments can be resected separately. As a result, the amount of functional hepatic impairment can be minimised. This has had a particular influence on surgery for colorectal

Figure 1. The surgical anatomy of the liver showing Couinaud's eight segments (Couinaud, 1957)



metastases because it allows excision of bilateral or multiple liver lesions that might previously have been deemed irresectable.

Vascular occlusion techniques, particularly the 'Pringle manoeuvre', have had a major impact in reducing the morbidity associated with liver resection. J.Hogarth Pringle, from Glasgow, first reported the 'Pringle manoeuvre' in 1908. He described arrest of hepatic haemorrhage due to trauma by compression of the portal triad between finger and thumb so that the liver surface could be seen and the appropriate haemostasis carried out (Pringle, 1908). Total vascular exclusion (in which the vena cava is also controlled above and below the liver) has become accepted as a means of minimising blood loss when operating on difficult lesions. Although few surgeons use total vascular exclusion routinely, it is a technique that facilitates excision of lesions

involving the vena cava or those lying near the junction of the hepatic veins and vena cava (Belghiti et al, 1996). The use of venovenous bypass in association with *in situ* hypothermic perfusion (Pichlmayr et al, 1990), and *ex situ* resection and autotransplantation (Hannoun et al, 1992), although used infrequently, have both been important additions to the liver surgeons armamentarium.

Surgical resection of hepatic metastases from colorectal cancer is the only treatment option that can produce long-term survival and offer the chance of cure (Scheele et al, 1995). Unfortunately, the proportion of patients with colorectal liver metastases who are deemed suitable for surgical resection is only about 10 per cent (Huang & Thomas, 1998; Beard et al, 1999), although resection rates of up to 25 per cent have been quoted (August et al, 1984; Adson, 1987; Geoghegan & Scheele, 1999). The indications for surgery are not absolute, but can be simplified to include all patients who have metastases that are resectable with clear margins. However, at least one-third of the normal liver parenchyma should be preserved, and in practice this means that at least 3 segments should be preserved. Resectable extrahepatic disease should also be removed at the same time. In the presence of unresectable hepatic or extrahepatic disease (especially nodal disease), hepatic resection should not be performed (Karanjia, 1999).

The 5-year survival rates following hepatic resection for colorectal liver metastases, that have been reported in studies with more than 100 patients, range from 21-44 per cent (Doci et al, 1991; Rosen et al, 1992; Van Ooijen et al, 1992; Gayowski et al, 1994; Scheele et al, 1995; Fuhrman et al, 1995; Nordlinger et al, 1996; Wade et al, 1996; Rees et al, 1997). The survival curve for patients undergoing hepatic resection for colorectal metastases levels out around 5 years (Wagner et al, 1984; Hughes et al, 1988; Scheele et al, 1991; Scheele et al, 1995). The surviving patients subsequently have a life expectancy similar to that of a matched non-cancer cohort (Adson, 1987).

Post-operative complications following liver resection are frequent and often serious. A review of morbidity and mortality after hepatic resection of colorectal liver metastases noted that intra-abdominal sepsis was the most frequent 'major' complication and pulmonary infection or atelectasis was the most frequent 'minor' complication (Doci et al, 1995). A large residual cavity is often left after partial

hepatectomy and this may fill with blood or bile. This collection combined with sloughing of devitalised liver at the resection line may lead to intra-abdominal abscess formation. Open drainage, large liver resections, long operative times, or large peri-operative blood losses may also be contributory factors.

Excessive bleeding during hepatic resection is a major complication associated with a mortality as high as 17 per cent (Holm et al, 1989; Nordlinger et al, 1992). However, technical improvements (ultrasonic dissectors, intraoperative ultrasound, argon beam coagulation, fibrin glue) and better vascular control has reduced blood loss. Attention to the intrahepatic control of the Glissonian sheaths and their contents in order to delineate precisely the hepatic segment to be resected has also been emphasised as a means of reducing the risk of perioperative bleeding (Launois et al, 1994).

Hepatic failure occasionally occurs following extensive liver resection for metastases and is frequently fatal. The presence of cirrhosis increases this risk due to a combination of reduced hepatic reserve and higher bleeding complications during the resection (Doci et al, 1995). Interestingly, metastases are unusual in cirrhotic livers, which may be indicative of the biological conditions necessary for successful metastasis e.g. the finding that tissue inhibitors of matrix metalloproteinases (see later) are upregulated 5-fold in cirrhotic compared with normal liver (Arthur et al, 1999). The likelihood of postoperative hepatic failure depends on the volume and function of the residual liver and there is evidence that a residual liver volume of at least 35 per cent is predictive of a good outcome (Soyer et al, 1992). In general, the larger the hepatic resection the greater the probability of postoperative complications. Although a clear resection margin is obviously desirable and may best be achieved by segment orientated resections, as much liver as possible should be preserved. This is important in metastatic disease since the extent of liver resection inversely relates to the outcome (Scheele et al, 1991; Doci et al, 1995).

Despite differences in patient prognostic grouping and overall patient numbers, the general pattern is one of improving overall survival rates, perioperative mortality and morbidity (Tsao et al, 1994). Influences of technical advances in such treatment would appear to explain these improvements particularly as resection has become progressively more aggressive. A series of 111 cases from 1971 to 1995 in a single

centre in Sweden showed that 5-year actuarial survival increased from 19 per cent in the period up to 1984 to 35 per cent thereafter (Ohlsson et al, 1998). However, others have reported only a marginal improvement (37 and 39 per cent) in 5-year survival of a group of 450 patients, although the latter group appeared to have more radical surgery (Scheele et al, 1995). Whilst it remains difficult to attribute definite causal relationships to improvements over time periods, there appears to be some justification for the argument that improvements in surgical technique and postoperative care are allowing a comparable level of survival in more complex patients (Beard et al, 1999).

It is also important to note that the longest 5-year survivals are from single centres. The main multi-centre studies have 5-year survivals of 21-28 per cent (Van Ooijen et al, 1992; Nordlinger et al, 1996; Wade et al, 1996). The more numerous single centre studies range from 21-44 per cent, which may indicate an effect of surgical expertise concentrated in these centres (Scheele et al, 1995; Fuhrman et al, 1995; Rees et al, 1997).

Improvements in anaesthesia, postoperative care, and a better understanding of hepatic anatomy have contributed to low operative mortality rates of 0-4 per cent (Scheele et al, 1995; Nordlinger et al, 1996; Rees et al, 1997). Some of the larger series have run over longer periods of time, and are therefore able to demonstrate significant reductions in mortality rate with time, despite a more aggressive approach to their surgery. In a series of over 300 patients dramatic improvements in mortality rates were reported from 11.5 per cent pre 1980, to 3.5 per cent post 1980, and more recently to 1.8 per cent during 1992-95 (Scheele et al, 1995). Correspondingly morbidity rates have improved although they still remain in the 10-30 per cent range (Scheele et al, 1995; Doci et al, 1995; Nordlinger et al, 1996; Rees et al, 1997). However, the majority of complications are completely reversible before discharge from hospital (Petrelli et al, 1991; Scheele et al, 1995; Vetto et al, 1990).

The outcome for patients following resection of colorectal liver metastases is significantly better than the expected outcome in untreated patients. Therefore, hepatic resection has been accepted as the standard treatment for resectable metastases from colorectal cancer both in Europe and the United States of America. The

questions that have to be addressed are which patients could benefit from hepatic resection and whether surgery combined with chemotherapy might be beneficial.

Factors Affecting Survival After Resection

Analysis of various clinicopathological factors provides a guide to patient selection for hepatic resection and may prevent unnecessary intervention in patients who will gain no survival benefit from surgery. It would be helpful to establish preoperatively which patients should be operated on and which should not. Several clinicopathological factors have shown prognostic value, however, there are discrepancies between different studies, and the selection of candidates for surgery remains subjective.

Patient age

Age is not considered to be a determining factor in survival, although two multi-institutional studies suggest that survival is age related (Hughes, 1988; Nordlinger et al, 1996). It is more appropriate to base the decision to offer liver resection to patients on biological rather than chronological age as it has been shown that there is no difference in peri-operative mortality or morbidity between the elderly and young patients following hepatectomy (Fong et al, 1995). The major determinants of success in the elderly (>80 years of age) include fitness for general anaesthesia and the volume of residual liver (liver adaptation following resection diminishes with age).

Gender

It has been suggested that females fare better than males (Holm et al, 1989), but most studies do not confirm this. Overall, there does not seem to be a gender difference in survival.

Site of primary tumour

Several groups have noted that the site of the primary tumour was an independent predictor of survival in patients with colorectal liver metastases. One group followed up patients whose liver metastases were left untreated (or received palliative

chemotherapy), and found that patients with primary tumours arising in the right colon fared worse than those whose primary tumour developed elsewhere in the colon or rectum (Rougier et al, 1995). These findings were confirmed in patients who underwent resection of liver metastases (Younes et al, 1991; Rees et al, 1997), although the converse was noted by another group who reported that patients with rectal tumours had worse survival than patients with colon tumours (Jatzko et al, 1995). Whether these results reflect late presentation of right sided cancers with a correspondingly advanced stage or higher local recurrence rates of rectal cancers is speculative, but in any case a number of authors have not found the site of the primary cancer to influence the long-term outcome following resection for liver metastases (Doci et al, 1991).

Grade of primary tumour

Survival of patients after resection of liver metastases from histologically high grade primary tumours is worse than those with low grade tumours (Scheele et al, 1995; Jatzko et al, 1995). This is not surprising, as the grade of the tumour is known to influence survival after resection of the primary tumour, independent of the presence of liver metastases. However, other studies have not found such an association (Cady et al, 1992; Gayowski et al, 1994; Rees et al, 1997). The majority of liver metastases are either moderately or poorly differentiated and usually arise from similarly differentiated primary tumours. As biopsy of metastases prior to hepatectomy is not advisable because of the risk of tumour seeding, information regarding microscopic differentiation of a liver metastasis is not usually available preoperatively.

Carcinoembryonic antigen (CEA)

Several studies have shown that an elevated preoperative serum level of CEA was an independent predictor of poor survival in patients who undergo hepatectomy (Cady et al, 1992; Gayowski et al, 1994; Nordlinger et al, 1996). Others, however, have found that the postoperative change in the serum CEA level was a better predictor of survival (Hohenberger et al, 1994). Whether an elevated level of serum CEA preoperatively relates to an inherently more aggressive tumour, or whether a fall in

postoperative serum CEA level to within the normal range reflects the adequacy of tumour clearance following liver resection is unclear.

Number and distribution of metastases

The number of metastases has a controversial impact on survival. Multivariate analysis of the number of metastases present has been shown in some studies to be a significant factor for long term survival (Hughes et al, 1988; Cady et al, 1992; Gayowski et al, 1994; Nordlinger et al, 1996), however, many others have not found this association (Fortner et al, 1984; Doci et al, 1991; Scheele et al, 1995; Rees et al, 1997).

If three or less metastases are present, the actual number of lesions resected has no influence on long term survival. Many authors advocate that the maximum number of metastases that can be resected with a likelihood of cure is three and long term survival if four or more metastases are removed is rare (Adson et al, 1984; Hughes et al, 1988; Nordlinger et al, 1996; Yamamoto et al, 1999). However, one study describes patients with up to five randomly distributed metastases who have survived more than 5 years following complete resection, and argue that the limiting factor to the number of lesions that can be resected is whether it is technically possible to remove all of the visible tumour (Scheele et al, 1995). This is supported by a further study, which shows that patients with four or more metastases fared as well as patients with three or less metastases (Rees et al, 1997).

The presence of multiple metastases, therefore, should not be an absolute contraindication to resection, and it is reasonable to consider resection for multiple metastases as long as the procedure can be undertaken with a low risk of liver failure. This is particularly the case where a cluster of similar sized metastases (suggestive of a common tumour embolic event) has clearly occurred in a segment or lobe, and the residual liver is disease free. It has been suggested that the presence of satellite tumour nodules indicates an adverse tumour biology with a higher risk of spread (Cady et al, 1992; Scheele et al, 1995).

The lobar distribution of metastases may also be a prognostic factor. While some studies suggest that patients with unilobar disease fare better after resection than those with bilobar disease (Gayowski et al, 1994; Rees et al, 1997), most reports show that the distribution of metastases to one or both anatomical lobes of the liver does not affect prognosis.

Size of metastases

There is conflicting evidence in the literature whether or not the size of an individual metastasis relates to outcome after hepatectomy. Several large studies have shown that metastases greater than 5cm are associated with worse survival than smaller metastases (Hughes et al, 1988; Scheele et al, 1991; Nordlinger et al, 1996; Rees et al, 1997), although other studies have not found an association (Younes et al, 1991; Cady et al, 1992; Jatzko et al, 1995). In some situations, extended (and occasionally non-anatomical) resections may be required to remove very large deposits. These large metastases may be indicative of an aggressive malignant process with a poor outcome, regardless of intervention. However, the contrary view is that a giant solitary metastasis may indicate a tumour biology in which the capacity for multiple metastases is limited and the outcome may therefore be good after resection. Thus, the size of an individual metastasis is not a good prognostic marker when deciding which patients should undergo surgery.

Synchronous versus metachronous disease

There is controversy over the value of resection of liver metastases found synchronously with the primary lesion. Several studies have reported a worse outcome in patients who undergo synchronous resection and, conversely, better survival in patients with apparent disease free intervals of at least one year after resection of their primary tumours (Hughes et al, 1988; Nordlinger et al, 1996). This may merely reflect favourable tumour biology in lesions detected at a later date. Interpretation of data concerning the benefits of resection of metachronous versus synchronous metastases needs to take into account development of better methods of early detection of metastases. However, multivariate analysis in a number of studies has shown that prognosis following resection is not dependent on the time of

detection of metastatic disease (Doci et al, 1991; Younes et al, 1991; Jatzko et al, 1995; Rees et al, 1997). Therefore, if patients have potentially resectable metastases detected during the investigations for the primary colorectal cancer and are otherwise well, they should be considered for hepatectomy. If hepatic metastases are discovered unexpectedly during resection of the primary tumour the decision about whether to resect the metastases during the same procedure may be difficult and would depend on the location of the tumour, the availability of intraoperative ultrasound to fully examine the liver, and the expertise of the surgeon.

Extrahepatic disease

The presence of extrahepatic disease significantly reduces the likelihood of long-term survival and is usually a contraindication to liver resection (Nordlinger et al, 1992; Millikan et al, 1997). Palliation of symptoms may be attained by resection of a large tumour but, in most cases, this has no beneficial effect on survival (Scheele et al, 1995). There are, however, certain circumstances where long-term survival has been achieved after liver resection in the presence of extrahepatic disease. Patients with resectable pulmonary or adrenal gland metastases may survive for more than 5 years after a successful liver resection, although most develop further recurrence during that time (Launois et al, 1994; Scheele et al, 1995).

Occasionally a subcapsular hepatic metastasis appears to invade an adjacent structure (usually the diaphragm). This attachment is often only fibrotic and represents an inflammatory response to the tumour that does not influence outcome and should be considered for resection (Bradpiece et al, 1987). Hepatic pedicle lymph node involvement may be present in 20-30 per cent of patients with hepatic metastases and is a bad prognostic indicator with almost none of these patients surviving 5 years following hepatectomy (Hughes et al, 1988; Fortner, 1988; Cady et al, 1992). Some studies suggest benefit from radical excision of nodes in the region of the hepatic pedicle although this is not widely practised (Nordlinger et al, 1992; Nakamura et al, 1992).

Resection margins

There is considerable debate concerning the ideal resection margin of tumour free liver tissue that must be obtained around the metastasis to maximise the benefit of resection. Data from the Registry of Hepatic Metastases, a multi-institutional database of liver resections, showed that a margin greater than 1 cm was associated with a 45 per cent 5-year survival, which fell to 23 per cent if the margin was less. However, information on the margin size was not available for many cases thus weakening the statistical value of this observation (Hughes, 1988). Conventional teaching advocates that a margin of at least 10 mm clear of microscopic disease is desirable and this is supported by results which document a statistically significant worse overall and disease free 5-year survival in patients with smaller margins (Holm et al, 1989; Cady et al, 1992; Nordlinger et al, 1996; Millikan et al, 1997). However, this view has been challenged by others who argue that a strict margin of at least 10 mm may not be important (Nakamura et al, 1992; Scheele et al, 1995; Yamamoto et al, 1999).

A margin that may appear to be adequate during resection may subsequently be reported as inadequate on microscopic examination, but it is possible that a field of liver parenchyma adjacent to the resection margin may have been destroyed with either electrocautery, argon beam coagulation or cryotherapy to a depth of several millimetres. A pathological study of resected colorectal liver metastases showed that many metastases have a thick fibrous pseudocapsule that allows adequate clearance of tumour by 'shaving' non-cancerous tissue. Substantial tumour spread was also noted along Glisson's capsule in many of the specimens examined suggesting that particular attention should be paid to resection of the portal structures close to the tumour (Yamamoto et al, 1995).

The higher recurrence rates noted with narrow margins may merely reflect the tendency for margins to be reduced by the surgeon in patients with more extensive disease, in order to preserve liver function; and these patients have an intrinsically worse prognosis because of the extent of their disease. The pattern of recurrence also suggests that narrow margins may not be crucial because it is usual for recurrence to occur at a distant site in the liver rather than in the surgical bed.

The type of resection carried out depends on both patient and tumour related factors. The number, size and site of metastases will dictate the type of resection that is possible, however, a resection margin of at least 10 mm should be aimed for, but if this is not technically possible then narrow margins should not be an absolute contraindication to resection.

Operative blood loss

Major operative blood loss has been related to a worse prognosis following partial hepatectomy for colorectal metastases even when technical factors such as difficult resections of large tumours were taken into account (Rosen et al, 1992). This relationship has been noted following resection of primary colorectal tumours and is supported by laboratory research suggesting that blood product transfusions have an immunosuppressive effect in the perioperative period thus allowing tumour growth (Rosen et al, 1992). Some studies have shown, however, that perioperative transfusion requirements are not an independent prognostic factor in survival (Cady et al, 1992; Gayowski et al, 1994).

Recurrence and Re-resection

The main cause of death after liver resection for colorectal metastasis is tumour recurrence which occurs in 55 to 80 per cent of patients (Fowler et al, 1993; Vaillant et al, 1993; Nordlinger et al, 1994). The recurrence is confined to the liver in one-third of cases with the remainder having either simultaneous hepatic and extra-hepatic disease (25-35 per cent) or extra-hepatic involvement only (30-50 per cent) (Adson, 1987; Vaillant et al, 1993; Nordlinger et al, 1994). Despite the frequency of relapse following surgery, the natural history of the disease is probably altered by hepatic resection, as evidenced by unusual sites of recurrence e.g. pulmonary and bone.

Repeat resections for recurrence are possible and follow the same selection criteria as for the initial liver resection i.e. that all macroscopic tumour is resectable. Long term survival rates are similar to those of first hepatectomies, with comparable mortality and morbidity (Nordlinger et al, 1994; Wanebo et al, 1996a; Adam et al, 1997). The liver has a unique capacity to regenerate after a large resection and this may partly explain why hepatic failure following repeat hepatectomy has not been widely reported. Moreover, the chance of further hepatic resection is enhanced if the first procedure was conservative and the recurrence confined to the contralateral lobe. Repeat hepatectomy is more difficult than the initial procedure because of adhesions within the abdominal cavity, particularly around the liver itself. This may make vascular control of the portal pedicle and retrohepatic vena cava difficult. In addition, external landmarks to guide the dissection are often distorted and the liver parenchyma may be more friable or fibrotic after regeneration or chemotherapy (Adam et al, 1997). Surprisingly, the morbidity and mortality rates remain similar to those for the initial hepatectomy, however, there is an increased risk of bleeding after repeat resection (Fernandeztrigo et al, 1995).

The majority of patients who develop recurrence following hepatic resection of colorectal metastases relapse within 2 years of surgery. Therefore, aggressive and regular surveillance may improve the detection of recurrent disease (Wanebo et al, 1996a). Patients who develop recurrence after hepatic resection should be considered

in the same way as for the initial resection and offered repeat surgery based on operative risk and probable survival (Nordlinger et al, 1994).

Alternative Treatment Strategies

Cryotherapy

Cryoablation of hepatic metastases using insulated probes containing liquid nitrogen has been used by several groups (Weaver et al, 1995). Focal *in-situ* destruction of hepatic metastases by cryotherapy is technically possible and can be performed safely when monitored by intraoperative ultrasound. Initially, hepatic cryotherapy was used as a cytoreductive procedure in patients who had unresectable metastases. However, with further experience it has also developed a role in managing residual inaccessible lesions in conjunction with liver resection (Adam et al, 1997).

The technique may be complicated by haemorrhage, thrombocytopenia, hypothermia, 'cracking' of the liver surface leading to problematic bleeding, disseminated intravascular coagulation and subsequent multisystem organ failure. Recurrence following cryotherapy of unresectable liver metastases is frequent and may be due to residual disease at the treatment site or to growth of micrometastatic disease elsewhere in the liver.

Other forms of cytoreductive therapy

Other cytoreductive techniques used to palliate patients with unresectable hepatic metastases include ethanol injection, thermotherapy, and interstitial radiotherapy (Thomas et al, 1993; Vogl et al, 1998). These techniques may be performed percutaneously but have the disadvantage that the scale of tissue destruction is not imaged (as it is by intraoperative ultrasound in cryotherapy) and is therefore relatively uncontrolled.

Hepatic artery chemoembolisation

Hepatic artery chemoembolisation was developed as a treatment for irresectable non-disseminated liver tumours. It involves selective injection into the hepatic artery of a combination of cytotoxic agents and an occluding agent such as gelfoam or starch

microspheres (Lang & Brown, 1993). The aim is to provide acute ischaemia with localised chemotherapy within the liver. There is little evidence that it produces any survival benefit and its main use is likely to be in the palliative treatment of localised but irresectable lesions.

Portal vein embolisation

Preoperative portal vein embolisation can decrease the likelihood of liver insufficiency occurring after extensive liver resection by inducing hypertrophy in the future remnant liver (Soyer et al, 1992). In patients with non-cirrhotic livers, preoperative portal vein embolisation can be expected to induce a 40-60 per cent increase in the size of the non-embolised portion (Kawasaki et al, 1994); a similar degree of compensatory hypertrophy is not seen after arterial embolisation. This technique is most likely to be useful as part of a multimodality treatment strategy including neoadjuvant chemotherapy. Two studies focusing primarily on the down-staging of metastatic liver disease with chemotherapy, also included patients who additionally underwent portal vein embolisation to increase the size of the future remnant liver (Elias et al, 1995; Bismuth et al, 1996).

Immunotherapy

Treatments aimed at reversing the depression of host defences in the perioperative period may reduce the dissemination of disease that invariably occurs following liver resection (Panis et al, 1992) and thereby improve the long-term results of surgery. Interleukin-2 is an immunostimulant and has been used as neoadjuvant therapy in patients with resectable colorectal liver metastases. In a Phase II randomised study, preoperative interleukin-2 increased the mean lymphocyte count in the postoperative period and was associated with an acceptably low level of toxicity (Elias et al, 1995). Long-term results are awaited to determine whether prevention of perioperative immunodepression can reduce the potential for perioperative tumour cell dissemination and metastasis implantation, leading to improved long-term survival.

Clinical trials have shown that the monoclonal antibody that binds a tumour specific cell surface glycoprotein, 17-1A, is effective in increasing survival following

resection of Dukes C primary colorectal tumours (Riethmuller et al, 1994). Targeting of liver metastases with monoclonal antibodies that recognise specific antigens within tumour cells has also been investigated (Welt et al, 1994). This work has shown that the monoclonal antibody could identify previously undetected metastases, which could aid decisions on surgical treatment. In Phase III clinical trials monoclonal antibodies to 17-1A were compared with standard adjuvant chemotherapy. The results have shown that treatment with antibodies to 17-1A was less effective than adjuvant chemotherapy alone and added no further effect when given in combination with chemotherapy (unpublished data from Glaxo-Wellcome). This work suggests that there is no therapeutic role for anti-17-1A therapy.

Another interesting application of immunotherapy for the treatment of liver metastases involves the use of active specific immunisation with vaccines. In a Phase II clinical trial, 23 patients were immunised with autologous, irradiated, metastases-derived tumour cells following resection of their colorectal liver metastases. After a follow-up of 18 months only 61 per cent of the immunised group developed recurrence compared with 87 per cent in the group treated by resection alone (Schlag et al, 1992).

Gene therapy

The advances made in the understanding of the genetic events underlying the progression to malignancy have allowed the development of new therapies for the treatment of patients with advanced tumours. Experimental approaches to cancer gene therapy have involved the suppression of oncogene expression, the restoration of defective tumour suppressor genes, e.g. p53 (Bookstein et al, 1996), and the introduction of cytokine genes or MHC genes into tumour cells in order to enhance anti-tumour activity (Fearon et al, 1990). The introduction of pro-drug activating genes, or suicide genes, provides a further strategy for treating advanced tumours. Suicide gene therapy involves the insertion of a gene coding for an enzyme that converts a non-toxic pro-drug into a potent cytotoxic agent. Using this approach in an animal model regression of established hepatic metastases was demonstrated following *in-situ* transduction of the herpes simplex thymidine kinase gene promoted by treatment with ganciclovir (Caruso et al, 1993).

The Role of Matrix Metalloproteinases in Metastasis

Metastatic spread of cancer continues to be the greatest barrier to cure. Understanding the molecular mechanisms is vital, for unlike tumourigenesis, metastasis does not appear to require the presence of altered or mutated genes. Instead, metastasis is mediated mostly by altered expression of genes coding for a variety of different proteins such as angiogenic factors, proteases, adhesion proteins and motility factors. One class of the proteases that has been repeatedly implicated in metastasis is the matrix metalloproteinases.

The matrix metalloproteinases (MMP's) are a family of zinc-dependent endopeptidases that collectively have the capacity to degrade all the major components of the extracellular matrix. These enzymes are present in normal healthy individuals and have been shown to have an important role in physiological processes associated with tissue remodelling, such as wound healing (Wolf et al, 1992), angiogenesis (Mignatti et al, 1989), bone resorption (Delaiise & Vaes, 1992), trophoblast-decidua invasion (Cross et al, 1994), and lung development (Ganser et al, 1991). The main interest in matrix metalloproteinases is their role in certain disease processes in which breakdown of the extracellular matrix is a key feature. Such diseases include emphysema (D'Armineto et al, 1992), periodontitis (Overall et al, 1991), and rheumatoid arthritis (Hayakawa et al, 1991), as well as cancer.

Classification of Matrix Metalloproteinases

Currently, at least 20 family members have been identified (Table 2). The main characteristics of these proteinases are:

1. The catalytic mechanism depends on a metal ion i.e. zinc, at the active site
2. All matrix metalloproteinases possess certain domains that are conserved between different members
3. Most are secreted in a zymogen form (Stromelysin 3 appears to be an exception and is secreted in an active form (Pei & Weiss, 1995), while some newly discovered matrix metalloproteinases are membrane bound)
4. The zymogen forms can be activated either by proteinases or organo-mercurials
5. Activation is usually accompanied by a loss of Mr of approximately 10000
6. The enzymes cleave at least one component of the extracellular matrix
7. Activity is inhibited by tissue inhibitors of matrix metalloproteinases

There are four major subgroups of matrix metalloproteinases, identified by their substrate preferences: the collagenases, stromelysins, gelatinases and membrane type matrix metalloproteinases.

The collagenases comprise interstitial collagenase (MMP-1), polymorphonuclear neutrophil collagenase (MMP-8) and collagenase 3 (MMP-13). These matrix metalloproteinases catalyse degradation of fibrillar forms of collagen i.e. types I, II and III. MMP-1 shows a preference for type III, while MMP-8 preferentially degrades type I collagen. The preferred form of collagen degraded by MMP-13 is currently unknown.

Table 2. List of matrix metalloproteinases with some of their main substrates

MMP	Protein name	Main substrates
MMP-1	Interstitial collagenase	Fibrillar collagens
MMP-2	Gelatinase A (72kDa)	Gelatins, collagen IV, collagen I
MMP-3	Stromelysin 1	Proteoglycans, collagen IV, extracellular matrix glycoproteins
MMP-7	Matrilysin	Proteoglycans, collagen IV, extracellular matrix glycoproteins
MMP-8	Polymorphonuclear neutrophil collagenase	Fibrillar collagens
MMP-9	Gelatinase B (92kDa)	Gelatins, collagen IV
MMP-10	Stromelysin 2	Proteoglycans, collagen IV, extracellular matrix glycoproteins
MMP-11	Stromelysin 3	α-1 proteinase inhibitor
MMP-12	Metalloelastase	Elastin
MMP-13	Collagenase 3	Fibrillar collagens
MMP-14	Membrane type MMP-1	ProMMP-2, fibrillar collagens, proteoglycans
MMP-15	Membrane type MMP-2	Not determined
MMP-16	Membrane type MMP-3	ProMMP-2
MMP-17	Membrane type MMP-4	Not determined
MMP-18	Putative MMP	Not determined
MMP-19	Rheumatoid arthritis associated MMP	Not determined
MMP-20	Enamelysin	
MMP-21,22,23	Recently cloned MMP's	Not determined
MMP-24	Membrane type MMP-5	Not determined
MMP-25	Membrane type MMP-6	Not determined

The gelatinases, which are also known as type IV collagenases, degrade gelatin (denatured collagen) and types IV, V, VII, IX and X collagen. Type IV collagen is particularly abundant in basement membranes, the membranes that separate organ parenchyma from the underlying stroma. Degradation of type IV collagen by the gelatinases occurs within the triple helical regions. This subgroup has two distinct members, known as gelatinase A (MMP-2) and gelatinase B (MMP-9). Generally, these two gelatinases are thought to have similar substrate specificity. However, recently MMP-2, but not MMP-9, was shown to cleave the ectodomain of fibroblast growth factor (FGF) receptor 1 (Levi et al, 1996). Thus, MMP-2 may in addition to degrading certain collagens, modulate the mitogenic and angiogenic activities of FGF.

The third subgroup of matrix metalloproteinases are the stromelysins, i.e. stromelysin 1 (MMP-3), stromelysin 2 (MMP-10), stromelysin 3 (MMP-11) and matrilysin (MMP-7). Stromelysin 1, 2 and matrilysin have relatively broad substrate specificity, catalysing degradation of many different substrates in the extracellular matrix (Chambers & Matrisian, 1997). The substrates include proteoglycans (core protein), non-collagenous proteins such as laminin, fibronectin and the non-helical regions of collagen IV. Stromelysin 3 has not yet been found to degrade any matrix protein, but has been shown to hydrolyse α-1 proteinase inhibitor (Pei et al, 1994), a member of the serpin family (serine proteinase inhibitor). Stromelysin 3 is also different because it is processed intracellularly by furin (Chambers & Matrisian, 1997). Furin is a transmembrane serine protease found in the trans-Golgi network. Stromelysin 3 can therefore be secreted in a predominantly active form, whereas, most matrix metalloproteinases are secreted as latent proenzymes that are activated extracellularly.

The fourth subgroup consists of the membrane type matrix metalloproteinases (MT-MMP's), as these proteinases possess a transmembrane domain. Four members of this group have been described, the best characterised being MT1-MMP. This matrix metalloproteinase has been shown to catalyse activation of progelatinase A (Sato et al, 1994) and to degrade a variety of extracellular matrix substrates (Ohuchi et al, 1997).

Site of production

Expression of matrix metalloproteinases has been demonstrated in normal tissues by immunohistochemical methods, and more recently by *in situ* hybridisation. MMP-2 is the most commonly expressed enzyme in normal adult tissues and expression is confined to the stromal cells (Matrisian, 1993). MMP-7 has been shown in glandular epithelial cells of the gastrointestinal tract and endometrium (Rodgers et al, 1993), and MMP-9 in haematopoietic cells (Hibbs et al, 1987). However, most studies have concentrated on expression in tumour tissue. A few have demonstrated staining for the gelatinases to be localised in tumour cells (D'Errico et al, 1991; Hoyhtya et al, 1994), but the majority have shown that expression is primarily in stromal cells (Newell et al, 1994; Zeng & Guillem, 1995; Gallegos et al, 1995). The stromelysins are also produced by stromal cells (Gallegos et al, 1995), whereas matrilysin seems to produced by neoplastic cells (Newell et al, 1994) and MT1-MMP is expressed on the tumour cell membrane (Sato et al, 1994).

Regulation of Activity

Gene Expression

Normal gene expression of matrix metalloproteinases is characterised by tightly controlled regulation to maintain normal tissue function. In malignancy, this tight control, which limits enzyme expression in normal tissues, is lost. High levels of MMP mRNA in cancer tissues may be induced specifically by growth factors such as epidermal growth factor, transforming growth factor β, tumour necrosis factor α and interleukin 1 (Matrisian, 1993) and, possibly, by oncogene activation (Matrisian, 1990).

Secretion of matrix metalloproteinases in latent form requiring activation

One of the characteristics of matrix metalloproteinases is that they are all secreted as inactive proenzymes, with the exception of MMP-8 and the membrane bound matrix metalloproteinases. This allows an important mechanism in the control of matrix metalloproteinases. All matrix metalloproteinases can be activated *in vitro* with organomercurial compounds, but the agents responsible for the physiological activation of matrix metalloproteinases have not been clearly defined. MMP-1 and MMP-9 have been shown to be activated by certain serine proteinases, although this does not appear to be the case for MMP-2 (Okada et al, 1992).

Numerous studies indicate that matrix metalloproteinases have the ability to activate one another (Crabbe et al, 1994; Sang et al, 1995). This is well illustrated by MT1-MMP, which is produced on the cell membrane of tumour cells. MT1-MMP activates MMP-2 (Sato et al, 1994), which is produced primarily by stromal fibroblasts, so that interactions take place between factors produced by the tumour cells and those from the surrounding stroma.

Inhibition by tissue inhibitors of matrix metalloproteinases

The third regulatory mechanism is the binding of the active matrix metalloproteinases to tissue inhibitors of matrix metalloproteinases. Four naturally occurring specific inhibitors have been described, namely TIMP-1, -2, -3 and -4. These inhibitors differ in a number of ways including sites of expression, molecular weight, ability to interact with proenzymes and the extent of glycosylation (Chambers & Matrisian, 1997). The tissue inhibitors of matrix metalloproteinases inhibit active matrix metalloproteinases by forming strong 1:1 stoichiometric non-covalent complexes. In addition to binding to the active forms, TIMP-1 complexes with proMMP-9 while TIMP-2 binds to proMMP-2 (Parsons et al, 1997; Duffy & McCarthy, 1998). Surprisingly, some of the tissue inhibitors of matrix metalloproteinases can stimulate cell growth, at least *in vitro*. Thus, both TIMP-1 and TIMP-2 have been shown to stimulate proliferation of a wide variety of cells (Hayakawa et al, 1992; Nemeth & Goolsby, 1993). These actions of TIMP-1 and TIMP-2 appear to be independent of their inhibitory MMP capacity. TIMP-1 and TIMP-2 can therefore be regarded as multifunctional proteins.

The Role of Matrix Metalloproteinases in Cancer

During metastasis, there are a series of collagen containing structural barriers that cells must pass. Extracellular matrix and basement membrane barriers must be breached for cells to intravasate and extravasate. The basement membrane underlying endothelial cells presents, in many organs, a continuous collagen containing structural barrier to completion of the metastatic process. Within tissue, at either primary or secondary tumour sites, extracellular matrices appear to require degradation to permit tumour cell invasion and spread. Therefore, metastatic cells require sufficient degradative enzymatic capacity to break down these proteinaceous structural barriers. Alternatively, some of the required proteolytic activity may be derived from tumour associated host tissues, including adjacent stromal tissue and tumour infiltrating immune cells. Support for a requirement for enhanced proteolytic function associated with cancer comes from pathological studies of tumours, in which defects in the basement membranes adjacent to the tumours are commonly associated with malignant but not benign tumours (Barsky et al, 1983).

Matrix metalloproteinases have been associated with the malignant phenotype for several decades (Liotta et al, 1982; Duffy, 1987). Several studies have presented evidence that malignant tumours contain proteolytic activity capable of degrading collagen *in vitro* (Liotta et al, 1980; Salo et al, 1982). With the advent of more sophisticated biochemical and molecular biological techniques, it became possible to identify individual proteases responsible for the activities detected in tumour cells. Proteases of all five major classes (serine, aspartic, cysteine, threonine and metalloproteinases) have been linked with the malignant phenotype (Sloane et al, 1994). The first member of the matrix metalloproteinase family to be cloned was transin, the rat homologue of stromelysin 1 (Matrisian et al, 1985). The protein product of this complementary DNA (cDNA) was identified as a protease that was over-expressed in malignant mouse skin tumours, and was related to interstitial collagenase (MMP-1) (Goldberg et al, 1986). Since this early work, extensive literature demonstrating the association of matrix metalloproteinase family members and tumour progression has developed [reviewed in (Guillem et al, 1996)]. Several generalisations can be made:

1. The number of different matrix metalloproteinase family members that can be detected tends to increase with progression of the tumour.

2. The relative level of any individual matrix metalloproteinase tends to increase with advancing tumour stage.

3. Matrix metalloproteinases can be made by either tumour cells themselves or, quite commonly, as a host response to the tumour.

The expression pattern of matrix metalloproteinases, therefore, supports a role for these enzymes in later stages of tumour progression. Matrix metalloproteinases are found most abundantly in tumours in which the basement membrane is breached, and there is evidence for local invasion and distant metastases.

Further evidence for the involvement of matrix metalloproteinases in invasion and metastasis comes from studies inhibiting *in vitro* and *in vivo* tumour-cell invasion via the addition of synthetic or endogenous matrix metalloproteinase inhibitors, such as the tissue inhibitors of matrix metalloproteinases (Alvarez et al, 1990). This suggests that the invasive potential of cancer cells may be, in part, caused by a local imbalance of matrix metalloproteinase over tissue inhibitors of matrix metalloproteinase activity. In support of this hypothesis is the precise regulation of MMP/TIMP balance that seems to be essential in regulating many physiological processes associated with tissue remodelling, such as trophoblast-decidua invasion (Cross et al, 1994), angiogenesis (Mignatti et al, 1989), and lung development (Ganser et al, 1991). Furthermore, disruption of the MMP/TIMP balance leads to profound changes in extracellular matrix integrity observed not only during neoplastic invasion, but in other disease processes, such as emphysema (D'Armineto et al, 1992), periodontitis (Overall et al, 1991), and rheumatoid arthritis (Hayakawa et al, 1991).

However, the simplistic expectation that malignant tumours would have increased matrix metalloproteinase expression accompanied by decreased tissue inhibitors of matrix metalloproteinase levels is often not met. In several cases, malignant tumours have been shown to have increased rather than decreased levels of tissue inhibitors of

matrix metalloproteinases (Grignon et al, 1996; McCarthy et al, 1999). The tissue localisation of both specific matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in and around a tumour can be complex, with variable expression within the tumour versus adjacent stromal cells. Tumour localisation studies can give only a snapshot at one point in time, and there is difficulty in interpreting these results. For example, is over-expression of a particular enzyme or inhibitor an indication of a functional role for it in the malignant process or is it a sign of the host response (effective or ineffective)? The localisation and interplay between matrix metalloproteinases and their inhibitors *in vivo* is complex and as yet poorly understood.

Matrix Metalloproteinases and Prognosis in Cancer

The early data implicating matrix metalloproteinases in metastasis was based on correlation between levels of the proteinases and metastatic potential in model systems. In 1987, the original proposal was made that proteinases involved in experimental metastasis might be markers of metastatic potential or prognosis in human cancers (Duffy, 1987). It was not until 1991 that a study correlating levels of a matrix metalloproteinase in a primary cancer with patient outcome was first published (Daidone et al, 1991). Using immunohistochemistry the authors reported that high levels of 'collagenase IV' correlated with locoregional recurrences in node negative breast cancer patients. However, no relationship was found between 'collagenase IV' staining levels and either relapse free or overall survival. Since then however, two further studies using immunohistochemistry with specific antibodies against both MMP-2 and MMP-9, and gelatin zymography, have failed to demonstrate any significant correlation between activity levels of either MMP-2 or MMP-9 and prognosis in breast cancer (Visscher et al, 1994; Remacle et al, 1998). In contrast to MMP-2 and MMP-9, high levels of MMP-11 have predicted poor outcome in breast cancer, although not in node negative patients, the subgroup where new prognostic indicators are most urgently required (Chenard et al, 1996).

While MMP-2 and MMP-9 may not be prognostic in breast cancer, both these matrix metalloproteinases are independent predictors of outcome in patients with gastric cancer. Using gelatin zymography, it was found that for MMP-2, total as well as the pro and active forms were prognostic, while for MMP-9, both total and proforms predicted outcome (Sier et al, 1996). MT1-MMP also appears to be prognostic in gastric cancer. Patients with a high tumour/normal mucosa ratio for this matrix metalloproteinase have a significantly shorter overall survival than patients with a low ratio (Mori et al, 1997). In this study however, MT1-MMP was not an independent prognostic marker while lymph node status and depth of tumour invasion were.

Preoperative levels of both MMP-2 and MMP-3 in blood have been shown to correlate with disease recurrence in patients with advanced urothelial cancers (Gohi et al, 1996).

More recently, in-situ hybridization has been used to investigate the expression of MMP-2 and MMP-9 in pancreatic cancer (Kuniyasu et al, 1999). Up-regulation of MMP-2 and MMP-9 and down-regulation of E cadherin (epithelial cell adhesion protein) at the periphery of neoplasms has significant prognostic value. The ratio of type IV collagenase expression (mean of the expression of MMP-2 and MMP-9) to E-cadherin expression (MMP:E-cadherin ratio) at the periphery of tumours was significantly higher in patients with recurrent disease, than in patients who were disease free. Multivariate analysis of overall survival showed that the MMP:E-cadherin ratio was a significant independent prognostic factor, whereas stage, nodal metastasis, and histological type were not.

Role of Matrix Metalloproteinases in Colorectal Cancer

Primary site

Matrix metalloproteinase over-expression has been detected in human colorectal cancer specimens, and various matrix metalloproteinase proteins have been identified in the serum of patients with colorectal cancer (Zucker et al, 1993). Immunohistochemical studies have localised MMP-1, MMP-2, and MMP-9 protein expression to the extracellular matrix surrounding colorectal neoplasms (Gallegos et al, 1995). Furthermore, in-situ hybridization studies have shown that, MMP-2 mRNA is expressed primarily by peritumour fibroblast-like cells, and MMP-9 mRNA is detected primarily in macrophages (Pyke et al, 1993), and not in the colorectal cancer cells themselves. Similarly, in-situ hybridization studies have localised MMP-1 in the colorectal cancer stroma, particularly within eosinophils (Gray et al, 1993). MT1-MMP expression has also been detected by in-situ hybridization in the stromal cells of colorectal cancer, with the pattern of expression closely resembling that of MMP-2 (Okada et al, 1995). This is consistent with the finding that MT1-MMP is involved in activation of MMP-2.

Thus far, the only matrix metalloproteinase that has been localised to colorectal cancer tumour cells and not to surrounding stroma, both at the protein and mRNA level, has been MMP-7. Increased expression of the stromelysins (MMP-3, -10, & -11) has also been demonstrated in the stromal compartments of colorectal cancer. Whilst expression has been high for MMP-11 (Urbanski et al, 1993), the expression of MMP-3 and MMP-10 has not been universal (Newell et al, 1994). Immunohistochemical and in-situ hybridization studies show that TIMP-1 and TIMP-2 are also expressed primarily in surrounding stromal cells (Hewitt et al, 1991; Zeng et al, 1995).

Liver metastases

There is little published work relating to matrix metalloproteinases and colorectal cancer liver metastases. The expression of MMP-9 and TIMP-1 mRNA has been

studied by *in situ* hybridization (Zeng & Guillem, 1995). This work demonstrated a distinct pattern of expression in colorectal cancer liver metastases. MMP-9 localised within peritumour stroma or at the interface between the tumour stroma and normal liver, whereas TIMP-1 mRNA was located throughout the malignant tumour stroma. Neither MMP-9 nor TIMP-1 mRNA was observed in normal liver. MMP-9 positive cells were identified as macrophages, while TIMP-1 was detected in fibroblast-like stromal cells. The focal expression of MMP-9 at the interface between liver metastases and normal liver and its apparent macrophage origin, suggest an important role for macrophages in degrading the extracellular matrix of colorectal cancer liver metastases.

The distinct pattern of cellular TIMP-1 and MMP-9 expression noted by *in situ* hybridization suggests that elevated TIMP-1 expression may not simply be a response to local increases in MMP-9 expression. This notion is supported by the fact that TIMP-1 has growth promoting properties. TIMP-1 accounts for a significant portion of the growth factor activity of serum and is capable of stimulating a wide range of human and bovine cell lines, including those derived from tumour (Hayakawa et al, 1992). Therefore, in addition to its role as a metalloproteinase inhibitor, TIMP-1 may also function as a growth factor in the pathogenesis of a variety of diseases. The mechanism of this multiple function of the tissue inhibitors of matrix metalloproteinases is currently unknown.

Further work on MMP-9 expression in colorectal cancer liver metastases has been carried out by the same workers using enzyme-linked immunosorbent assay (ELISA) and zymography. In contrast to their previous work, they found that the latent form of MMP-9 was expressed in both liver metastasis and paired adjacent normal liver tissue (Zeng & Guillem, 1998). However, the active form of MMP-9 was only present in the liver metastasis and not normal liver tissue, suggesting that proMMP-9 activation may be a pivotal event during colorectal cancer liver metastasis formation.

More recently, work by this group has used reverse transcription-polymerase chain reaction to investigate the expression of various matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (Kelly et al, 1999). The work examined the

mRNA expression of MMP-1, -2, -3, -7, -8, -9, and -13, and TIMP-1 and -2 in 30 patients. Three distinct areas of each liver sample were examined:

1. within liver metastases
2. immediately adjacent normal liver (within 1 cm)
3. distant normal liver (>5cm)

The results show that MMP-2, MMP-3 and MMP-8 were expressed almost universally throughout each of the three areas examined, whilst MMP-13 was expressed weakly by only three patients (Table 3).

MMP-1 was expressed in all 30 metastases, in 18 samples of adjacent liver (weaker in 14), and in 11 samples of distant liver (weaker in 8).

MMP-7 was expressed by 28 metastases, in 25 samples of adjacent liver (weaker in 10), and 20 samples of distant liver (weaker in 7).

MMP-9 was expressed by 25 metastases, in 12 samples of adjacent liver (weaker in 5), and 11 samples of distant liver (weaker in 8).

TIMP-1 and TIMP-2 were expressed at equivalent levels in all 30 metastases and distant liver samples. However, in the adjacent liver TIMP-1 and TIMP-2 levels were lower in 18 and 24 samples respectively.

These results show upregulation of MMP-1, MMP-7 and MMP-9 in the colorectal cancer liver metastases and downregulation of TIMP-1 and TIMP-2 in the immediately adjacent liver. This suggests that the more classical notion of enhanced levels of matrix metalloproteinases accompanied by decreased levels of tissue inhibitors of matrix metalloproteinases may facilitate local growth of liver metastases.

Table 3. Numbers of patients (out of 30) expressing MMP's and TIMP's in liver metastases and normal liver. Numbers in parentheses represent weaker expression.

MMP or TIMP	Liver metastases	Adjacent liver	Distant liver
MMP-1	30	18 (14)	11 (8)
MMP-2	30 (2)	29 (3)	30 (3)
MMP-3	30	27 (10)	28 (8)
MMP-7	28	25 (10)	20 (7)
MMP-8	30	30	30
MMP-9	25	12 (5)	11 (5)
MMP-13	(3)	(2)	0
TIMP-1	30	30 (18)	30
TIMP-2	30	30 (24)	30

Matrix Metalloproteinases and Prognosis in Colorectal Cancer

The matrix metalloproteinases have long been thought of as potential markers of prognosis in cancer because of their intimate involvement in metastasis. Various studies have examined matrix metalloproteinase expression in colorectal cancer with the aim of correlating the levels with relapse, metastases and survival.

MMP-1 expression has been investigated in 64 primary colorectal tumours using immunohistochemistry (Murray et al, 1996). Positive immunoreactivity for MMP-1 was identified in 10 of the tumours, whereas 54 tumours showed no MMP-1. In the tumours that showed immunoreactivity, more than 90 per cent of tumour cells were positive. Statistical analysis showed that survival of patients with MMP-1 positive tumours was significantly less than that of patients whose tumours were MMP-1 negative. The occurrence of MMP-1 remained significant after multivariate analysis for Dukes' stage and patient age, indicating that MMP-1 is an independent prognostic factor in colorectal cancer.

The expression of MMP-7 has been shown to have a significant correlation with Dukes' stage, with the highest expression being found in liver metastases (Mori et al, 1995; Adachi et al, 1999). Furthermore, MMP-7 has been demonstrated in colorectal adenomas, suggesting that MMP-7 expression may be an early event in colorectal tumourigenesis (Newell et al, 1994).

The expression level of MMP-9 mRNA was assessed in both tumour and paired normal mucosa from 71 patients with primary colorectal cancer (Zeng et al, 1996). Over-expression of MMP-9 mRNA correlated significantly with status of synchronous distant metastases and Dukes' stage. High tumour/normal mucosa ratios were also associated with a significantly shorter disease free and overall survival. In univariate and multivariate analyses, tumour/normal mucosa MMP-9 mRNA level was found to be an independent prognostic factor for disease free survival. With overall survival as the end point, MMP-9 mRNA was prognostic in univariate but not multivariate analysis.

Further work, using immunohistochemistry, has been carried out to investigate patterns of expression of MMP-2, MMP-9 and TIMP-2 (Ring et al, 1997). This work showed that TIMP-2 expression was valuable for the discrimination between macroscopically localised and metastatic colorectal cancer, but it could not predict which of the potentially cured patients were likely to have micrometastases. In addition, they concluded that MMP-2 and MMP-9 staining was of minor value in staging and prognostic prediction. This clearly contradicts the previous work showing a prognostic role for MMP-9 (Zeng et al, 1996).

No work has been published on matrix metalloproteinases as predictors of outcome in patients with colorectal liver metastases.

Matrix Metalloproteinases as Therapeutic Targets in Cancer

The different molecules involved in metastasis as well as being potential prognostic indicators are also potential targets for anti-cancer therapies. As originally designed, the strategy of inhibiting matrix metalloproteinase activity was directed at preventing metastasis formation. The initial presentation of most cancer patients occurs at a later stage in the disease when tumour cell invasion and dissemination have already occurred. The majority of cancer patients already have metastases at the time of diagnosis of their primary tumour.

Several factors now indicate that the matrix metalloproteinases may be used as therapeutic targets in cancer therapy. The most compelling of these is the possible use of the tissue inhibitors of matrix metalloproteinases as cytostatic agents, which would prevent the growth both of primary tumour and metastatic foci. This may be as a result of direct action of these inhibitors on the tumour cells, or may be the secondary effects of inhibiting a matrix metalloproteinase activity that either release growth factors sequestered in the extracellular matrix or inhibits tumour angiogenesis (Stetler-Stevenson et al, 1996).

The aim of using selective inhibitors is to halt tumour progression without inducing significant toxicity. The potential utility of this cytostatic approach to cancer chemotherapy is best exemplified by the use of tamoxifen to treat invasive breast cancer (Falkson et al, 1994).

Cytostatic strategies could be used as an adjuvant to conventional cytotoxic therapy. By alternating cytostatic therapy with cytotoxic agents tumour growth could be prevented. Enhancement of cytotoxic therapies, which rely on active tumour cell replication, might be achieved by initiating cytotoxic therapy immediately prior to release from cytostatic therapy. This could reduce the tumour burden which could then be amenable to continued cytostatic therapy. Limiting the size of the tumour cell population exposed to the cytotoxic agents could also help prevent the development of drug resistance. However, all of these possibilities depend on the identification of suitable matrix metalloproteinase inhibitors.

Naturally occurring tissue inhibitors of matrix metalloproteinases

TIMP-1 and TIMP-2 have been shown to inhibit chemoinvasion (Albini et al, 1991; Tsuchiya et al, 1993), and to reduce tumour growth and metastasis in animal models (Alvarez et al, 1990; Tsuchiya et al, 1993). Furthermore, monoclonal antibodies against gelatinases have been shown to reduce invasion of tumour cells through reconstituted basement membrane (Hoyhtya et al, 1990). This evidence suggests that altering the balance between matrix metalloproteinases and their naturally occurring inhibitors does modify the behaviour of invasive tumours. However, because of their protein nature (expensive to produce, susceptibility to digestive proteolytic degradation and antigenicity), and their multiplicity of actions (ability to stimulate cell growth as well as inhibit matrix metalloproteinases), it is unlikely that tissue inhibitors of matrix metalloproteinases will be widely used as anti-cancer agents. Interest has, therefore, focused on the development of synthetic matrix metalloproteinase inhibitors.

Synthetic inhibitors of metalloproteinases

Many of the synthetic inhibitors are peptides similar to the cleavage site in collagen (Talbot & Brown, 1996). Inhibition is effected by a zinc-binding group, which inhibits matrix metalloproteinases at their active site. Some of the zinc-binding groups currently under investigation include hydroxamates, carboxylates, amino carboxylates and sulphydrals. Two of the hydroxamate inhibitors have been widely evaluated in model systems and have entered clinical trials, i.e. batimastat and marimastat.

Batimastat

Batimastat (BB-94) is a low molecular weight synthetic inhibitor of matrix metalloproteinase activity. It possesses a collagen-like structure that enables binding to the active site of matrix metalloproteinases, and a hydroxamate group, which chelates zinc at the active site (Talbot & Brown, 1996). Batimastat is a broad-spectrum matrix metalloproteinase inhibitor with concentrations causing 50 per cent inhibition of enzyme (IC_{50}) in the low nanomolar range for all the matrix metalloproteinases. *In vitro*, batimastat was found to inhibit extracellular matrix

degradation by melanoma cells and prevent endothelial cell invasion through an artificial basement membrane (Talbot & Brown, 1996).

Batimastat has been shown to significantly reduce the number and size of lung metastases when administered intraperitoneally to mice given B16-BL6 murine melanoma cells (Chirivi et al, 1994). It has also been used in two colonic cancer animal models. In the first, a significant reduction in primary tumour growth, locoregional invasion and metastasis was seen in the batimastat treated animals, with a modest improvement in survival (Wang et al, 1994). Inhibition of primary tumour growth was somewhat unexpected, and histological examination of the primary tumour tissues suggested that batimastat treatment resulted in formation of fibrous tissue encapsulation and production of an avascular stroma. In the second study, which evaluated liver invasion in a human colorectal tumour line, invasive growth was inhibited in the batimastat treated animals and marked tumour necrosis was seen histologically in the tumours of these animals (Watson et al, 1995).

Batimastat has also been studied in a human ovarian carcinoma xenograft. It was shown to decrease tumour burden, resolve ascites and improve survival in mice. Post-mortem examination of these animals showed the tumour to be encapsulated in dense tissue stroma with necrosis of some of the tumour cells (Davies et al, 1993).

More recently, further *in vivo* work has provided more evidence for the potential role of matrix metalloproteinase inhibitors in the treatment of advanced cancers. The first study examined the ability of batimastat to inhibit liver metastases of murine B16F1 melanoma cells, following injection of the cells into mice via a mesenteric vein to target the liver (Wylie et al, 1999). Treated mice were found to have a reduction in the mean volume of liver metastases, although not a reduction in number of metastases. The treated mice were also found to have a significantly reduced percentage vascular volume within the liver metastases, indicating inhibition of angiogenesis. In the second study, the effect of batimastat was assessed on the growth of an aggressive model of peritoneal carcinomatosis producing haemorrhagic ascites and metastases, obtained in the rat by intra-peritoneal injection of DHD/K12 cells (Aparicio et al, 1999). The results showed that batimastat had the ability to

significantly prolong survival, and reduce both peritoneal carcinomatosis and liver metastases number, when compared with controls.

The low solubility and poor oral bioavailability are severe limitations for the formulation and delivery of batimastat. It has, therefore, been administered intraperitoneally as a suspension. Systemic toxicities were reported to be minimal but local side effects included peritoneal irritation, abdominal pain and vaso-vagal reactions (Wojtowicz-Praga et al, 1997).

Marimastat

Marimastat (BB-2516), like batimastat has a collagen mimicking hydroxamate structure, however, marimastat has relatively good oral bioavailability (Talbot & Brown, 1996). In animal studies, marimastat inhibited tumour progression of both breast and lung cancers (Wojtowicz-Praga et al, 1997).

Marimastat has been studied in both healthy volunteers and patients with cancer. The phase I study confirmed high plasma levels in volunteers receiving single oral doses of between 25 and 800 mg (Drummond et al, 1995). A series of pilot phase I-II studies are underway, including trials in patients with gastric, colorectal and pancreatic cancer. In the study of marimastat in gastric cancer, the tumour was examined endoscopically and biopsied before the drug dose and again after 28 days of treatment. Preliminary findings included evidence of fibroblastic matrix development in the tumours of some patients, similar to the histological changes observed in some animal cancer models (Parsons et al, 1996). A more recent multicentre randomised trial studied marimastat as maintenance therapy in 369 patients with inoperable gastric cancer. Survival at 1 year was 20 per cent in the group treated with marimastat compared with 14 per cent in the group receiving the placebo (Fielding et al, 2000).

In trials of marimastat in patients with colorectal and pancreatic cancer, the cancer antigens carcinoembryonic antigen and carbohydrate antigen 19-9 have been used to detect biological activity at different doses of the drug (Gore et al, 1996). The effect on the rate of rise of cancer antigen was found to be dose dependent, and several patients have shown serological and radiological evidence of disease stabilisation

(Rasmussen & McCann, 1997; Primrose et al, 1999). A further multicentre randomised trial has compared marimastat with gemcitabine in 414 patients with pancreatic cancer. Survival analysis showed the highest dose of marimastat to be equally effective as gemcitabine (Rosemurgy et al, 1999).

The principal adverse event with the use of marimastat was musculoskeletal pain, which was dose and time dependent, but also reversible.

Other inhibitors

Inhibition of matrix metalloproteinase activity by low molecular weight compounds, such as batimastat and marimastat, is not the only mechanism for blocking activity of the proteinases. Other approaches include prevention of expression (e.g. by antisense molecules or ribozymes) or inhibition of zymogen activation (e.g. by D-penicillamine) (Opendakker et al, 1997). A recent study examined the effects of a matrilysin-specific antisense oligonucleotide on *in vitro* invasion and liver metastasis in nude mice of two human colon carcinoma cell lines (Miyazaki et al, 1999). *In vitro* the antisense oligonucleotide effectively inhibited both the secretion of matrilysin (MMP-7) and invasion through a reconstituted membrane. Also in the nude mouse model, experimental liver metastasis was potently suppressed. These results suggest that matrilysin has an important role in the liver metastasis of human colon cancer and that antisense oligonucleotides may have a therapeutic potential for the prevention of metastasis.

Chapter Three

Patients and Methods

Introduction

Previous research by this group has looked at the expression of several matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in liver metastases from colorectal cancer (Kelly et al, 1999). Fresh tissue was obtained from 30 patients undergoing hepatic resection of metastases from colorectal cancer. Total cellular RNA was extracted from the metastases, the immediately adjacent liver (within 1 cm), and the distal liver (>5cm), and subjected to reverse transcription. An oligo dT primer was used for the reverse transcriptase reaction, followed by target-specific primer pairs for the polymerase chain reaction. The reverse transcription polymerase chain reaction products were analysed by non-denaturing polyacrylamide gel electrophoresis, and specificity confirmed by sequencing. The results showed high expression of MMP-1, MMP-7 & MMP-9 in the colorectal cancer metastases and reduced expression of TIMP-1 & TIMP-2 in the immediately adjacent liver. Immunohistochemistry was then carried out on specimens from the 30 patients and confirmed the findings of the polymerase chain reaction work showing that the differences in expression for MMP-1, MMP-7, MMP-9, TIMP-1 & TIMP-2 were not just restricted to the mRNA but were carried through to the protein level.

The natural progression of this work was to investigate the expression of these 3 matrix metalloproteinases and 2 tissue inhibitors of matrix metalloproteinases and to see if there was any correlation with patient outcome. In collaboration with Mr M Rees at North Hampshire Hospital, Basingstoke, access was allowed to a series of patients who had undergone liver resection for colorectal cancer metastases going back as far as 1987. Patients were referred from many areas in England and Wales, although predominantly Southern England, and also as far afield as Portugal, Greece and Jordan. Data was collected prospectively on every patient undergoing liver surgery. A single research nurse has been responsible for the dedicated collection of all patient data according to four patient record proformas. The data set for each patient is complete up to his or her last appointment (or telephone consultation if appropriate) or his or her death.

The formalin fixed wax embedded specimens could be subjected to immunohistochemistry and expression of MMP-1, MMP-7, MMP-9, TIMP-1 & TIMP-2 then compared with patient outcome.

Clinical Review of Patients

Primary disease

There were 105 consecutive patients (58 male and 47 female) with a mean age of 58 years (range 28 to 83 years) at the time of primary surgery for colorectal cancer (Appendix I). The primary resections were performed between 1983 and 1996. The primary tumour was situated in the colon in 65 patients and the rectum in 40 patients (Table 5). Following referral for liver surgery 100 patients were considered to have undergone a curative primary resection although 44 of these patients were known to have synchronous liver metastases. The 5 patients who did not to have curative primary surgery included 3 with local tumour infiltration, one with suspicious mesenteric lymph nodes and one who was considered to have undergone a palliative procedure.

The histology according to Dukes' classification (as described in the Introduction) showed that 4 (4 per cent) patients were stage A, 22 (21 per cent) stage B, 24 (23 per cent) stage C_1, 5 (5 per cent) stage C_2, and 50 (48 per cent) stage D.

Table 5. Site of primary tumour in all 105 patients

Site of Tumour	Total
Caecum	3
Ascending colon	8
Transverse colon	6
Descending colon	14
Sigmoid colon	27
Rectosigmoid	7
Rectum	40

Adjuvant therapy was used in 27 patients: 1 patient had pre-operative radiotherapy with post-operative chemotherapy, 2 patients had post-operative radiotherapy alone, 3 patients had post-operative radiotherapy and chemotherapy, 1 patient had pre- and post-operative chemotherapy and 20 patients had post-operative chemotherapy alone.

Secondary disease

The 105 patients had a mean age of 60 years (range 29-86 years) at the time of liver surgery and all resections were performed between 1988 and 1996 (Appendix II). The average length of time between primary surgery and diagnosis of liver metastases was 13.2 months (range –1 to 119 months), with a mean time of 5.8 months (range 0 to 42 months) between diagnosis and resection of the liver metastases. A single consultant liver surgeon performed all liver resections.

Disease was confined to the liver in 97 patients, with 4 patients having peritoneal deposits, 3 patients with colorectal tumour, and 1 patient with diaphragmatic deposits. The liver metastases were confined to the right lobe in 56 patients, the left lobe in 20 patients, and both lobes in 29 patients. Eighty-eight patients had 1-3 metastases, 16 patients had more than 3 metastases but less than 50 per cent liver involvement and only one patient had both more than 3 metastases and greater than 50 per cent liver involvement. The diameter of the largest deposit ranged from 1 to 23 cm.

The types of liver resection performed were: right hemihepatectomy in 66 patients, left hemihepatectomy in 14 patients, segmentectomy in 15 patients, left lateral segmentectomy in 5 patients, extended right hemihepatectomy in 3 patients, and large wedge resection in 2 patients. The liver resection was considered to be curative in 98 patients with the remaining 7 patients undergoing a palliative procedure (3 patients had disease confined to the liver and 4 patients had peritoneal deposits).

Adjuvant therapy was given to 29 patients: 14 patients had pre-operative chemotherapy, 4 patients had pre- and post-operative chemotherapy, and 7 patients had post-operative chemotherapy, 3 patients had radiotherapy and one patient had pre-operative chemotherapy and radiotherapy.

Recurrent disease

Recurrent disease was seen in 81/105 (77 per cent) patients. The time to recurrence ranged from 2.5 to 65.8 months, but in 58/105 (72 per cent) patients recurrent disease occurred within 2 years of liver surgery. Surgery for recurrent disease was performed in 7 patients. This re-resection occurred between 8.9 and 52.7 months after the original liver resection.

Additional therapy for recurrent disease was given to 51 patients: 44 had systemic chemotherapy, 5 had radiotherapy and 2 had regional chemotherapy. Systemic chemotherapy was given to 4 of the 7 patients who had surgery for recurrent disease with the other 3 patients receiving no additional therapy.

Outcome

One patient died in the peri-operative period: this occurred on day 4 postoperatively and was the result of a cerebrovascular accident. In total 79 patients have died with 26 still alive at their last outpatient visit. Of the 79 that have died the majority did so because of recurrent disease (Table 6). The median survival of the 79 patients who died was 1.95 years (95% confidence interval: 1.62-2.57 years). The median survival for the 26 patients still alive is 5.40 years (95% confidence interval: 4.97-7.25 years) and 20 patients remain disease free.

Table 6. Cause of death with recurrent disease status

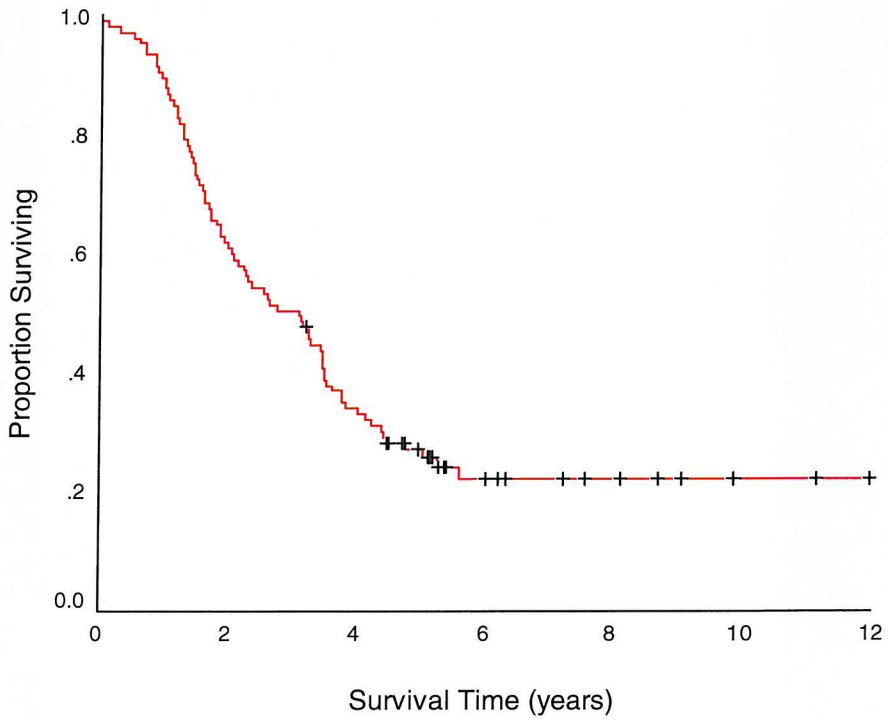
Cause of Death	Number of Patients
With and because of recurrent disease	69
Other cause but without recurrent disease	2
Cause unknown but with recurrent disease	5
Cause unknown, recurrent disease status unknown	3
Total	79

Of the 7 patients who had surgery for recurrent disease 4 have died. The mean survival of the 4 who died was 39.3 months (range 31.3 to 44.3 months). The mean survival of the 3 patients still alive is 64.4 months (range 42.1 to 103.5 months) and 2 patients remain disease free.

The overall 5-year actuarial survival of the 105 patients was 27.0 per cent (95% confidence interval: 18.8-35.8 per cent) with a median survival of 3.13 years (95% confidence interval: 2.25 - 4.02 years). The Kaplan-Meier survival curve for the 105 patients is shown in Figure 2.

Seventeen patients had post-operative complications although all of these were treated conservatively and resulted in complete recovery. These included one pulmonary embolism, 4 cardiac arrhythmias, 2 patients with hepatic insufficiency, 5 chest infections, 2 patients with urinary retention, one sacral sore, and 2 patients with partial small bowel obstruction.

Figure 2. Kaplan Meier survival curve for all 105 patients (+ censored values)



Immunohistochemistry

Introduction

The history of immunostaining methods date back to the early 1940's when Albert H. Coons at Harvard Medical School demonstrated that it was possible to localise antigens in tissue sections by means of fluorescein labelled antibodies (Coons et al, 1941). Immunohistochemistry is a powerful research tool that allows the localisation of protein expression within tissue sections (Mighell et al, 1998). Specialist equipment is not required, reagents including primary antibodies are commercially available, and techniques are relatively easy to master. The basic principle of immunohistochemistry is that a specific antibody will combine with its specific antigen to give an antibody-antigen complex.

Antigens

The majority of antigens are macromolecules, usually proteins or polysaccharides. Within these macromolecules short regions known as epitopes provide the binding site for each of the antibodies raised to any particular antigen. The vast majority of epitopes are peptides, short sequences of amino acids on the surface of the antigen. The epitope recognised by an antibody may be dependent upon the specific three-dimensional antigenic conformation (e.g. a unique site formed by the interaction of two native protein sub-units), or the epitope may correspond to a simple primary sequence region. Such epitopes are described as conformational and linear respectively.

Complex mixtures of antigens exist *in vivo*. A single gene can generate several different protein isoforms via two principal mechanisms. First, alternative splicing of the primary gene transcript may produce multiple different mature transcripts, each of which codes for a slightly different protein (Sharp, 1994). Second, many proteins undergo post-translational modifications, such as glycosylation, phosphorylation, and proteolytic processing which add a further level of complexity to proteins derived from a single gene. Disease states can also be associated with an alteration in

expression pattern or generation of new isoforms (Mighell et al, 1997). New isoforms can also be a consequence of gene disruptions such as either nucleotide point mutations or rearrangements that alter the primary sequence of expressed protein (Baas et al, 1994).

The range of possible binding sites is enormous, with each potential binding site having its own structural properties derived from covalent bonds, ionic bonds and hydrophilic and hydrophobic interactions. For efficient interaction to occur between the antigen and the antibody, the epitope must be readily available for binding. If the target molecule is denatured, e.g. through fixation, the epitope may be altered and this may change (improve or decrease) its ability to interact with an antibody (Cattoretti et al, 1993). Changes in pH may also affect antigen conformation.

Antibodies

The antibody molecule, despite its exquisite functional diversity, is highly conserved structurally (Swanson, 1988). Antibodies are produced in response to the invasion of foreign molecules in the body, and exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains. Each antibody contains two identical copies of a heavy chain, and two identical copies of a light chain, named as such by their relative molecular weights. Antibodies can be divided into five classes: IgG, IgM, IgA, IgD, and IgE, based on the number of Y units and the type of heavy chain. The light chains of any antibody can be classified as either kappa (κ) or lambda (λ) type (a description of the molecular characteristics of the polypeptide).

The most commonly used antibody is IgG, which can be cleaved into three parts, two F(ab) regions and one Fc, by the proteolytic enzyme papain, or into two parts, one $F(ab')_2$ and one Fc by the proteolytic enzyme pepsin. The F(ab) regions comprise the 'arms' of the antibody, which are critical for antigen binding. The Fc region comprises the 'tail' of the antibody and plays a role in immune response, as well as serving as a useful handle for manipulating the antibody during some immunological procedures. The number of F(ab) regions on the antibody, corresponds with its subclass, and determines the valency of the antibody (the number of arms with which the antibody may bind its antigen).

Most primary antibodies are commercially prepared and many different factors may influence this preparation. The source and preparation of the immunogen used to stimulate production of antibodies is of fundamental importance. For most antibodies two broad groups of immunogen exist: synthetic peptides and purified protein preparation (Mighell et al, 1998). Synthetic peptides have the significant advantage that the amino acid sequence is known. This can be of crucial importance in the interpretation of immunohistochemistry studies, both with respect to the isoforms of the target protein that can be detected and any cross-reactivity with similar peptide sequences in other proteins. However, disadvantages potentially occur. First, an isolated synthetic peptide sequence may lack the normal three-dimensional structure of the native protein. Secondly, *in vivo* other proteins can be intimately associated with the protein of interest. Both of these reasons may mask the target epitopes, prevent detection *in vivo* by antibodies raised to synthetic peptides and so yield false negative results. Thirdly, post-translational modifications can be crucial for specific recognition (Mandel et al, 1992).

Use of purified proteins as immunogens eliminates many of the difficulties associated with synthetic peptides, but presents a different set of problems. Purification of a protein to homogeneity from either cells or tissues can be technically difficult. Contaminating proteins may be more antigenic than the protein of interest so that the immunisation with even small amounts may yield a disproportionate and unwanted immunogenic response. A different problem can arise when the targeted antigen includes highly immunogenic epitopes that are not specific to the antigen of interest, e.g. post-translational modifications may be similar between proteins that are otherwise very different (Mighell et al, 1998).

Many of the antibodies used in immunohistochemistry are raised by repeated immunisation of a suitable animal, e.g. rabbit, sheep, goat or donkey, with a suspension of the appropriate antigen. Serum is then harvested at the peak of antibody production. One characteristic of large antigen molecules is that they induce the activation of many antibody producing B cell clones in the immunised animal. This polyclonal mixture of resulting antibodies may then recognise a variety of epitopes on the antigen. Because these polyclonal mixtures of antibodies react with multiple epitopes on the surface of the antigen, they will be more tolerant of minor changes in

the antigen, e.g. polymorphism, heterogeneity of glycosylation, or slight denaturation, than will monoclonal antibodies. However, the greater the number of different antibodies to the target protein in a single preparation, the greater the likelihood of cross-reactivity with similar epitopes in other proteins. False positive staining will then occur (Mighell et al, 1998). A homogeneous population of antibodies (monoclonal antibodies) can be raised by the fusion of B lymphocytes with immortal cell cultures to produce hybridomas (Milstein, 1981). Because monoclonal antibody preparations include only a single antibody they avoid many of the problems associated with polyclonal antibody preparations, e.g. background staining. However, because monoclonal antibodies react with one epitope on the antigen, they are more vulnerable to the loss of epitope through chemical treatment of the antigen than are polyclonal antibodies (Chemicon, 1998).

Antibody-antigen interactions

The bonding between antigens and antibodies is dependent on hydrogen bonds, hydrophobic bonds, electrostatic forces and van der Waals forces. These are all bonds of a weak, non-covalent nature, yet some of the associations between antigen and antibody can be quite strong. Like antibodies, antigens can be multivalent, either through multiple copies of the same epitope, or through the presence of multiple epitopes, which are recognised by multiple antibodies. Interactions involving multivalency can produce more stable complexes, however multivalency can also result in steric difficulties, thus reducing the possibility for binding. All antigen-antibody binding is reversible, however, and follows the basic thermodynamic principles of any reversible bimolecular interaction:

K_A= [Ab-Ag]/[Ab][Ag]

K_A is the affinity constant,
[Ab] and [Ag] are the molar concentrations of unoccupied binding sites on the antibody and antigen respectively,
Ab-Ag is the molar concentration of the antibody-antigen complex

The time taken to reach equilibrium is dependent on both the rate of diffusion and the affinity of the antibody for the antigen, and can therefore vary widely. The affinity constant for the antibody-antigen binding can span a wide range, extending from below 10^5 mol^{-1} to above 10^{12} mol^{-1}. Temperature, pH and solvent can affect affinity constants. Affinity constants can be determined for monoclonal antibodies, but not for polyclonal antibodies, as multiple bonds take place between polyclonal antibodies and their antigens (Chemicon, 1998).

Avidity is a measure of the overall stability of the antibody-antigen complex. It is controlled by three major factors: the affinity of the antibody for the epitope, the valency of the antigen and the antibody, and the structural arrangement of the interacting parts. Three aspects of this binding process are particularly important in immunohistochemistry. First, most antigenic determinants may be recognised by several different clonal products, each of which will bind the determinant in competitive fashion. Second, antibodies may be polyfunctional with spatially separated microdomains in the variable regions that can recognise different and not necessarily related determinants (Richards et al, 1981). This level of diversity, however, may not affect the overall avidity or specificity of polyvalent sera, because the chance that a significant number of antibodies in the heteroantiserum will recognise the same non-target determinants is quite small. Together with the presence of multiple determinants on most cellular antigens, this diversity of antibody response to a single immunogen determines the relative specificity of heteroantisera *in vivo* and in immunohistochemical applications. In contrast, because a unique combining region defines monoclonal antibodies, non-target and target recognition in tissue depends on the relative affinity of the antibody for each reactive epitope. In addition, monoclonal antibodies may specifically recognise target epitopes that are present in functionally or structurally unrelated antigens (Richards et al, 1981). Finally, the antibody must be considered as an antigen, because most immunohistochemical methods rely on the ability of an antibody bridge to specifically recognise another antibody that is bound to tissue antigens. Despite conservation of both structural and functional domains between species, immunoglobulin is an effective immunogen. In particular, Fc domains are highly antigenic (Richards et al, 1981).

Tissue manipulation and antigen retrieval

The ability of antibodies to recognise antigens in tissue depends on which determinants are recognised and how well they survive tissue processing. The tissue specimens that are most commonly used in immunohistochemical studies have been fixed in formalin and then embedded in paraffin wax in preparation for archival storage. The mechanisms of tissue fixation remain poorly understood, but this process may mask epitopes exposed *in vivo*. This masking probably occurs via either intra- and/or intermolecular cross-linking of proteins, or by alteration of target protein tertiary structure (Cattoretti et al, 1993). Type, temperature and duration of fixation can be important variables and can markedly influence patterns of immunoreactivity for some antibody-antigen combinations. Occasionally, tissue fixation can enhance immunoreactivity (Hall et al, 1990).

Some epitopes masked either *in vivo*, or more frequently by tissue fixation and processing, can be exposed via antigen retrieval techniques. The mechanisms of antigen retrieval are also poorly understood (Shi et al, 1997), and indeed may mask or even destroy some epitopes. Antigen retrieval may augment the visualisation of conformational epitopes in part by unmasking amino acid sequences (Battifora & Kopinski, 1986). Partially masked sequences may still bind to antibody, but antibody affinity generally decreases with diminishing epitope size (Richards et al, 1981). Linear epitopes are more likely to be restored by antigen retrieval methods provided the sequence is not specifically cleaved. Because of these considerations, it is likely that immunoreactivity for any antigen will be broader when polyclonal antibodies are used for detection, whereas immunoreactivity with monoclonal antibodies may be exquisitely dependent on tissue preservation (Swanson, 1988). Two broad groups of antigen retrieval have been described for archival tissue immunohistochemistry; enzyme mediated and heat mediated. Both methods are thought to unmask hidden epitopes via different mechanisms (Cattoretti et al, 1993; Mighell et al, 1995).

Enzyme mediated antigen retrieval

Enzyme mediated antigen retrieval techniques are only effective for some antibody-epitope combinations. The diversity of proteolytic enzymes used, such as trypsin,

pepsin and pronase, suggests a non-specific mechanism. However, enzyme concentration and duration of digestion can be important in obtaining optimal immunoreactivity for different antigens (Cattoretti et al, 1993; Shi et al, 1997). It has been suggested that enzymes have limited effectiveness in paraffin sections and possibly only act on the section surface (Cattoretti et al, 1993).

Heat mediated antigen retrieval

Heat mediated antigen retrieval techniques are a more recent advance in immunohistochemistry. As with enzyme mediated antigen retrieval techniques they are not useful for all antibody-epitope combinations. High temperatures possibly expose masked epitopes by several inter-linked mechanisms including peptide cleavage, disruption of fixation cross-links and alteration of protein tertiary structure.

Several variations of heat mediated antigen retrieval technique have been described, but all involve heating tissue sections in solution. It is the temperature achieved and the length of incubation that is critical rather than the method by which the heat is delivered (von Waisielewski et al, 1994; Taylor et al, 1996; Shi et al, 1997). Successful methods of heat delivery include microwave ovens, pressure cookers, steamers, hot plates and thermal cyclers (Taylor et al, 1996). Each of these methods has advantages and disadvantages, and these must be considered in experimental design and subsequent interpretation of staining patterns (Mighell et al, 1995; Taylor et al, 1996).

A large number of different solutions have been tested for their usefulness in heat mediated antigen retrieval (Cattoretti et al, 1993; von Waisielewski et al, 1994). Buffer pH, molarity, and metal ion content can have a marked influence on staining intensity for some antibody-epitope combinations (Cattoretti et al, 1993; Taylor et al, 1996; Shi et al, 1997). Investigation of the influence of solution composition has been mostly restricted to cellular antigens (nuclear, cytoplasmic or cell membrane), and little is known about the influence of solution composition on immunoreactivity of extracellular matrix proteins.

Antigen retrieval of archival tissue has many advantages, but staining patterns must be interpreted within the limitations of the techniques (Mighell et al, 1998). It is possible that the absence of immunoreactivity after antigen retrieval reflects that either the target epitope has been destroyed, remains masked or was never present. Conversely, antigen retrieval may expose cross-reactive epitopes on proteins distinct from the target protein. Apparent inappropriate immunoreactivity has been observed under extreme antigen retrieval conditions such as the high temperatures of autoclave antigen retrieval and after use of highly acidic buffer in microwave antigen retrieval (Mighell et al, 1995; Shi et al, 1995).

The Evolution of Immunohistochemistry

In order to exploit the ability of antibody to recognise antigen in tissue sections, the antibody to antigen interaction must be detectable. Coons and colleagues provided the first practical approach to this problem by conjugating fluorescein to antibodies, applying the conjugate to tissue sections, and revealing antigen-antibody complexes by fluorescent emission in blue light (Coons et al, 1941). However, diagnostic immunohistochemistry requires antigen detection systems that are applicable to routinely processed tissue under conditions in which morphological characteristics are adequately preserved. The methods require the production of an insoluble, stable, opaque reaction product that is visible by standard light microscopic techniques (Sternberger, 1986). These requirements led several investigators to apply enzyme histochemical techniques to immunohistochemical probes by combining highly active enzymes with antibodies (Nakane & Pierce, 1967). Several enzymes have been used successfully, including intestinal alkaline phosphatase, glucose oxidase, beta-glucuronidase, and acid phosphatase. Of these, alkaline phosphatase has proved particularly useful in immunohistochemistry (Cordell et al, 1984). Nonetheless, the immunohistochemical systems that appear to be the most reactive, and that have enjoyed the greatest popularity, are those that use horseradish peroxidase.

Horseradish peroxidase (HPO) is a haem-containing glycoprotein composed of carbohydrates disposed around a protein shell (Farr & Nakane, 1981). Using the electron donor 3,3´-diaminobenzidine tetrahydrochloride (DAB) or its congeners, in the presence of peroxide, an insoluble polymeric dark brown precipitate is generated by peroxidase (Sternberger, 1986). A variety of other electron donors have been used with HPO, but the resultant reaction products are rarely as dense or are soluble in alcohol and xylene, making aqueous mounting media necessary (Sternberger, 1986). Therefore, further description concentrates on the HPO-DAB system and discussion of the performance of HPO-linked immunohistochemical systems is based on the following criteria: efficiency (signal to noise – a measure of background staining), accuracy (resolution), precision (reproducibility), sensitivity (the smallest amount of antigen that the method can detect), and specificity (the contribution of the method to the unique identification of target antigen) (Swanson, 1988).

Direct immunohistochemistry

Conjugation of HPO to antibodies with known tissue specificity allows for direct visualisation of antigen-antibody complex. However, this technique is generally less sensitive than comparable fluorescent methods, because antigen loss is inherent in routine processing protocols. It is also less efficient, because tissue fixation may foster non-specific binding of labelled antibody to tissue (Farr & Nakane, 1981).

Indirect immunohistochemistry

The use of heteroantisera raised to immunoglobulins of differing species for the immunologic detection of tissue antigens was first described in 1954 (Weller & Coons, 1954). These secondary antibodies, when conjugated to fluorescein, HPO, or other histochemical labels, are the basis of universal indirect labelling methods. Such procedures are substantially more sensitive than direct methods but are no less accurate or precise (Farr & Nakane, 1981). Increased sensitivity results principally from the ability of the polyvalent secondary antibody to recognise multiple sites on the Fc and Fab portions of the primary antibody. Problems with efficiency and specificity remain with this technique, because the use of polyvalent secondary antibodies carries the risk of non-specific binding to irrelevant tissue antigens (Farr & Nakane, 1981; Sternberger, 1986), and increase the likelihood of non-immunologic reactivity (Gosselin et al, 1986).

Unlabelled antibody-bridge techniques

These problems were largely overcome in 1959, by a method in which the fluorescein or enzyme label is removed to a third layer (tertiary) antibody that has been raised in the same animal as the primary antibody (Jankovic, 1959). This reagent can be linked to tissue antigens through an unlabelled secondary antibody, which serves as an immunologic bridge between primary and tertiary reactants (Jankovic, 1959; Farr & Nakane, 1981). Efficiency and specificity are improved because, in principle, non-specifically bound secondary antibody is unlikely to bind tertiary reagents with high affinity (Gosselin et al, 1986). Bridge techniques also enhance the sensitivity of the assay, because the ability of the secondary antibody to bind primary and labelled

moieties is not affected by the presence of a relatively large enzyme conjugate (Sternberger, 1986). In addition, an antigen-antibody interaction may be used as a label instead of an enzyme conjugate, by the use of tertiary heteroantisera that are generated by use of the enzyme as immunogen. However, the anti-HPO sera produced needs to be highly purified to ensure the sensitivity and precision of the method, and so in 1970 a method was devised that used a pre-formed peroxidase-antiperoxidase (PAP) complex, using crude anti-HPO heteroantisera, thus circumventing the need for purified antibodies (Sternberger et al, 1970). It was predicted that the use of PAP would give an average of 12.4 reactive HPO molecules associated with each antigen localised in tissue, thus substantially increasing the sensitivity of PAP compared with previous methods (Sternberger et al, 1970). Because a thermodynamically stable complex is formed regardless of the affinity of anti-HPO for HPO, linking of reactive enzyme to antigens in tissue is limited primarily by the ability of the secondary unlabelled antibody to bind PAP. Precision is thus augmented. Specificity and accuracy are also improved, because recognition of primary antibody is facilitated without loss of efficiency (Sternberger et al, 1970).

It was not until 1982 that stable and useful PAP complexes were produced for monoclonal anti-HPO antibodies (Mason et al, 1982). In contrast to PAP complexes made with heteroantisera, monoclonal PAP is composed of only one antibody and two HPO molecules, but even so, the use of monoclonal PAP results in comparable enhancement in sensitivity (Mason et al, 1982).

Because of the relatively small size of the complex, PAP methods are sensitive to differences in antigen density; however, a linear relationship between antigen density and the optical density of the immunohistochemical reaction product does not exist. In fact, with PAP and other bridge techniques, staining intensity may show a paradoxical reduction under conditions of high antigen intensity. This phenomenon results from saturation of the secondary antibodies. At high antigen density the bridge may become doubly affixed to primary antibodies in close approximation, leaving no binding sites for PAP or anti-HPO antibodies (Bigbee et al, 1977).

The avidin-biotin-peroxidase complex

An alternative to the immunologic binding of the HPO carrier has been developed based on the interaction of biotin and egg-white avidin (Guesdon et al, 1979; Hsu et al, 1981). Avidin is a basic glycoprotein with a tetrameric structure that allows the binding of four biotin molecules (Green, 1975). This interaction is essentially irreversible and has an affinity constant of approximately 1×10^{15}, which is as much as 1×10^{9} greater than most antigen-antibody interactions.

The small size of biotin allows several molecules to be attached to globulins or enzyme molecules without substantially altering antigen binding or enzymatic activity. The next step was to apply biotinylated secondary antibodies to antibodies bound to antigen and expose the resulting complex to avidin (Guesdon et al, 1979; Hsu et al, 1981). Subsequent application of biotinylated HPO yielded a complex containing as many as three HPO molecules bound to each avidin molecule. The sensitivity of this indirect bridged avidin-biotin-peroxidase (IBABC) complex is theoretically limited only by the number of secondary antibodies bound to primary antibody and the number of reactive biotin molecules on each secondary antibody. However, IBABC performs less well than expected in tissue, perhaps because of steric constraints limiting the number of biotin sites available for avidin binding (Hsu et al, 1981).

These practical problems were addressed by applying pre-formed avidin-biotin-peroxidase complexes (ABC) to biotinylated secondary antibodies (Hsu et al, 1981). Because ABC is generated in relative avidin excess to ensure that available biotin binding sites are not saturated, relatively large lattice-like complexes are formed that are composed of several avidin molecules linked by biotinyl-HPO bridges (Hsu et al, 1981). As a result, ABC delivers a large number of active HPO molecules to each available biotin binding site, potentially enhancing the sensitivity of the assay compared with PAP or other methods. However, the large size of ABC may lead to increased non-specific precipitation and relatively poor tissue penetration (Sternberger, 1986), and the glycoprotein content of avidin favours non-specific interaction with cell membranes and nucleic acids resulting in loss of efficiency and accuracy (Green, 1975). Furthermore, although the large ABC lattice ensures

visualisation of labelled antigen in conditions of low antigen density, the steric effects of such large complexes applied to high antigen density equate with relative insensitivity to differences in antigen concentration in tissue (Sternberger, 1986).

The ABC method has been improved by the use of a biotin binding protein isolated from *Streptomyces avidinii* (streptavidin). Like avidin, streptavidin is a tetrameric protein with four high avidity-binding sites for biotin, but unlike avidin, streptavidin is not glycosylated (Green, 1975). Streptavidin exhibits less non-specific binding at a neutral pH. As a result, streptavidin-linked immunohistochemical systems appear to perform with greater sensitivity and efficiency for a given antigen density or antibody dilution (Swanson, 1988).

Materials and Methods

The paraffin embedded tissue blocks for 105 patients, who had undergone hepatectomy for colorectal cancer liver metastases, were obtained from North Hampshire Hospital, Basingstoke. At this stage only the patients name, date of birth and operation date were known with all further clinical detail including outcome data being withheld until the experiments and analyses had been completed. Multiple blocks were often available for each patient and therefore a single block was selected for each patient. Ideally the chosen block would contain both liver metastasis and adjacent normal liver to allow comparison of expression of MMP-1, MMP-7, MMP-9, TIMP-1, and TIMP-2 in these two different areas.

Twelve sections of 4μm thickness were cut for each patient using a conventional rotary microtome. The sections were then mounted on 3-aminopropyltriethoxysilane (APES) coated slides, which aids adhesion of tissue sections, and dried for at least 72 hours at room temperature.

The immunostaining technique was based on the Southampton University Department of Pathology protocol: Streptavidin-Biotin Peroxidase Complex (StABCPx) immunostaining for fixed, paraffin embedded sections using monoclonal or polyclonal antibodies (protocol sheet SOP LE73/4). This technique had previously been successfully used to identify expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (Kelly et al, 1999).

Primary antibodies

All primary antibodies were titrated on positive control sections (tissues known to be positive for each antibody) using a number of different dilutions starting around the dilution recommended in the datasheet supplied with the antibody or if the antibody had been used before, starting around the previous working dilution. All new batches were titrated using 'double dilutions' e.g. 1/2, 1/4, 1/8, 1/16, 1/32 (doubling the dilution each time) to give the optimal antibody working dilution that would give the strongest specific antigen staining with the lowest non-specific background staining.

After titration all antibodies were aliquoted out and frozen immediately at -20°C as repeated freezing and thawing is detrimental to an antibody. Negative control sections were run with no primary antibody and also with matching isotype antibodies (IgG_1).

Endogenous avidin binding sites

Endogenous avidin binding sites (EABS) are widely distributed throughout a number of tissues particularly liver, kidney and mast cells. If not blocked these sites will bind non-specifically to the enzyme-labelled Streptavidin biotin complex used for the final stage in the StABCPx technique. Reacting the tissue with unconjugated avidin, which is then saturated with unlabelled biotin, can block EABS. This procedure effectively blocks further non-specific attachment of the enzyme-labelled avidin biotin complex.

Antigen retrieval

Antigen retrieval was by heat mediation using a conventional microwave. Several experimental runs were performed using different settings for both the time and the microwave power. The 3 highest microwave power settings were tested using run times between 10 and 60 minutes. The optimum retrieval was thus established that maximised antigen-antibody interaction but did not destroy the tissue section.

Procedure

The paraffin was removed from each section by immersing in xylene (2x5 minutes) and rehydrated by immersing in 100% alcohol (2x1 minute) and 70% alcohol (1x1 minute).

Endogenous peroxidase was inhibited by treating each slide with 200µl of freshly prepared inhibitor (Appendix I) for 10 minutes.

The slides were then washed well in running tap water for 3 minutes.

Antigen retrieval was performed using a microwave oven (Tecnolec T200M; 750 Watts). Three plastic staining racks were filled with 24 slides and placed in a polythene box with a perforated lid (to allow steam to escape). To maintain a constant load, blank slides without sections, were used to make up the total of 72 slides. The box was filled with 1500 ml of 0.01M citrate buffer (Appendix I). The box was placed in the centre of the microwave oven, set to full power and allowed to run for 25 minutes. When the time had elapsed the box was removed and quickly filled with cold running tap water. All racks were left in the running water for 3 minutes. The slides were then washed in TRIS buffered saline (TBS) for 2x5 minutes (Appendix III).

The EABS were blocked using Avidin/Biotin Blocking Kit (Vector Laboratories; SP-2001). One drop of the undiluted avidin solution was added to each slide and left for 20 minutes. The slides are then washed in TBS for 3x2 minutes. One drop of the undiluted biotin solution was then added to each slide and also left for 20 minutes followed by a wash in TBS for 3x2 minutes. 200µl of culture medium was then added to each slide and left for 20 minutes. The culture medium was then drained from each slide but not washed off.

200µl of primary antibody, correctly diluted in TBS, was then applied to each slide and incubated at 4°C for 18-24 hours (overnight).

The slides were allowed to warm to room temperature for 15 minutes and then washed in TBS for 3x5 minutes.

The secondary antibodies were then added at a dilution of 1:200 (1µl antibody to 199µl TBS). 200µl per slide of biotinylated sheep anti-mouse immunoglobulin (Amersham; RPN 1001) was added for monoclonal antibodies and 200µl per slide of biotinylated swine anti-rabbit immunoglobulin (Dako; E 0353) was added for polyclonal antibodies.

The streptavidin-biotin horseradish peroxidase (StABC/HRP) complex (Dako; K 0377) was prepared by adding the equivalent of 1µl of reagent A (streptavidin), 1µl of reagent B (biotinylated horseradish peroxidase) to 198µl of TBS making a 1:200 dilution. The solution was left for at least 30 minutes to complex.

The slides were washed in TBS for 3x5 minutes.
The pre-prepared StABC/HRP was applied to each slide (200µl) and left for 30 minutes. The slides were then washed in TBS for 3x5 minutes.

The horseradish peroxidase label is demonstrated using 3,3´-diaminobenzidine (DAB)(Biogenix; HK 153-5K). 160µl of working solution (Appendix III) were applied to each slide and allowed to develop for 8 minutes.

The slides were then rinsed in TBS, followed by a wash in running tap water for 2 minutes.

The slides were dipped in 70% alcohol.

The slides were counterstained with Harris' haematoxylin (see below) for 2 minutes and then rinsed in tap water for 1 minute. The slides were differentiated in 1% acid alcohol (Appendix III) for 5 seconds before running in tap water for at least 5 minutes to blue the sections.

The sections were dehydrated by taking through 70% alcohol (1x1 minute), 100% alcohol (2x1 minute) and xylene (2x5 minutes), and finally mounted in DPX.

Haematoxylin

Haematoxylin is extracted from the heartwood of the logwood tree (*Haematoxylon campechianum*). Pure haematoxylin is colourless but it can be readily oxidised to the reddish dye haematein, which is the active dyestuff in all so-called haematoxylin solutions. This change occurs as soon as the logwood is exposed to air so that pure colourless haematoxylin is never used. When first stained with an aluminium (or alum) haematoxylin, nuclei are a dark red colour. In order to change this to blue and to stabilise the dye, the sections must be treated with a weak alkali. In most regions the tap water is alkaline and may be used for this purpose, which is referred to as blueing (Dibrey & Rack, 1970). The solution used in these experiments was Harris'

haematoxylin (Appendix III), which was first described in 1900 (Harris, 1900). This haematoxylin does not need to ripen and gives fairly consistent staining results.

The same procedure was carried out for each of the 5 antibodies used i.e. MMP-1, MMP-7, MMP-9, TIMP-1, and TIMP-2, at their optimum dilutions as determined by titration (Table 7). However MMP-7, which was the only polyclonal antibody used, gave significantly more background staining. In an effort to reduce this background staining 1% bovine serum albumin (BSA) was applied to the slides for 30 minutes following antigen retrieval, and before blocking of the endogenous avidin binding sites. Additionally, because MMP-1 was required at a high concentration, an immunohistochemistry pen (Vector image pen) was used. Drawing around the sections with the pen prevents the antibody solution from running over the whole slide, thus reducing the amount of antibody solution that is required. This step was carried out just prior to the application of the primary antibody and reduced the amount of primary antibody required from 200µl to 50µl.

Table 7. Characteristics of antibodies

Antibody	Isotype	Form of MMP	Type	Dilution	Positive control
MMP-1	IgG_1	Pro & Active	Mouse monoclonal	1:33	Rheumatoid arthritis
MMP-7	-	Pro & Active	Rabbit polyclonal	1:400	Rheumatoid arthritis
MMP-9	IgG_1	Pro & Active	Mouse monoclonal	1:200	Tonsil
TIMP-1	IgG_1	Pro & Active	Mouse monoclonal	1:50	Foreskin
TIMP-2	IgG_1	Pro & Active	Mouse monoclonal	1:1600	Melanoma

MMP-1, MMP-7, and MMP-9 were supplied by British Biotech

TIMP-1 and TIMP-2 were supplied by Chemicon International Inc.

Specificity and species reactivity:

MMP-1, MMP-7 and MMP-9: specifically react with human MMP-1, MMP-7 and human and mouse MMP-9 and show no cross-reactivity between antibodies (R&D Systems Inc, 2000).

TIMP-1: the antibody specifically reacts with human TIMP-1 and shows no cross-reactivity between antibodies (Chemicon International Inc, 1999a).

TIMP-2: the antibody specifically reacts with human TIMP-2 but shows no cross-reactivity between antibodies (Chemicon International Inc, 1999b).

Slide Analysis

All of the slides were constantly checked for the quality of the specific and non-specific staining and if necessary re-runs were performed. The staining with each antibody was constantly monitored to maintain specificity, and then before the final analysis, all antibody staining was assessed to establish the pattern and likely nature of the cellular staining.

Immunohistochemistry is only semi-quantitative as an analytical tool. Slides are visually assessed using light microscopy. The accepted method (though not always used) for published work that uses immunohistochemistry quantitatively has 2 independent observers analysing the slides according to a predefined arbitrary scale (e.g. 0-4 or a 0,1+,2+,3+ etc.). This method allows for the estimation of inter-observer variability.

An alternative method for slide analysis uses digital image analysis, which allows the quantification of many variables e.g., cell counts, area of staining, total area etc. Image analysis has been shown to correlate well with visual analysis (Dahlen et al, 1999) and therefore can act as the 'second observer'. The analysis of the slides in our work uses digital image analysis to verify the visual analysis of a single observer.

Visual analysis

All slides were analysed using light microscopy at X250 magnification. The patterns of staining were described and then quantified using the following scale:

0: No staining
1: Less than 10 per cent staining
2: Approximately 10 to 40 per cent staining
3: Approximately 40 to 60 per cent staining
4: Approximately 60 to 90 per cent staining
5: Greater than 90 per cent staining

For the purposes of analysis each slide was divided into four separate areas and quantification was performed by examining each area completely:

Adjacent tumour: tumour within one visual field of the tumour margin
Distant tumour: rest of the tumour
Adjacent liver: liver within one visual field of the tumour margin
Distant liver: rest of the liver

This separation allowed unique assessment of the tumour/liver interface where potentially the most important biological events are occurring.

Digital image analysis

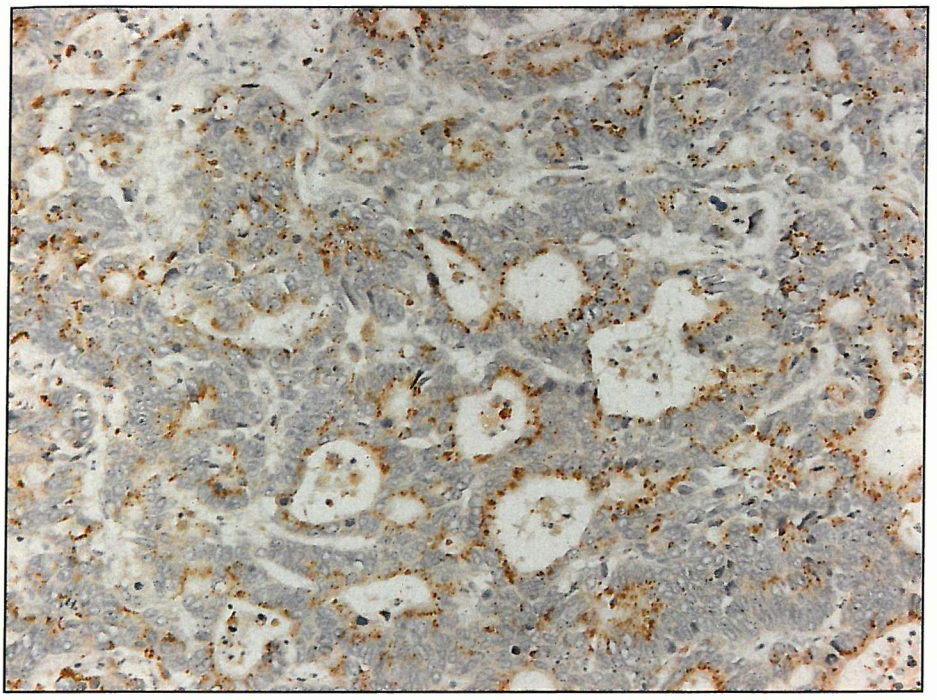
Image analysis was performed using Carl Zeiss KS400 software. Images were acquired digitally at X200 magnification and then analysed using a specific macro written by the Biomedical Imaging Unit, Southampton University Department of Pathology. The analysis performed allowed quantification of the positive staining identified in the visual analysis. It was not used to identify whether an area was positively or negatively stained. The results of the digital analysis were then correlated with the visual analysis to ensure reproducibility.

The analysis macro initially enhanced the contrast of positively stained cells (Figure 3b). The decision on positive staining was based on colour alone and not on intensity. Once positive staining was clearly identifiable to the macro a binary image was produced where all positive staining appears white against the black background of negative staining (Figure 3c). To reduce non-specific staining all positive staining below 30 pixels was discarded and considered as negative staining (Figure 3d). A binary mask was then made by drawing around the whole area that was to be used for the analysis whilst also drawing around any 'holes' (areas devoid of tissue) greater than approximately 600 μm^2 (Figure 3e). The resultant mask allowed calculation of the total area of tissue subjected to the staining process. The remaining positive staining within the mask (Figure 3f) was then calibrated according to the initial magnification used and measurements were calculated for the total number of

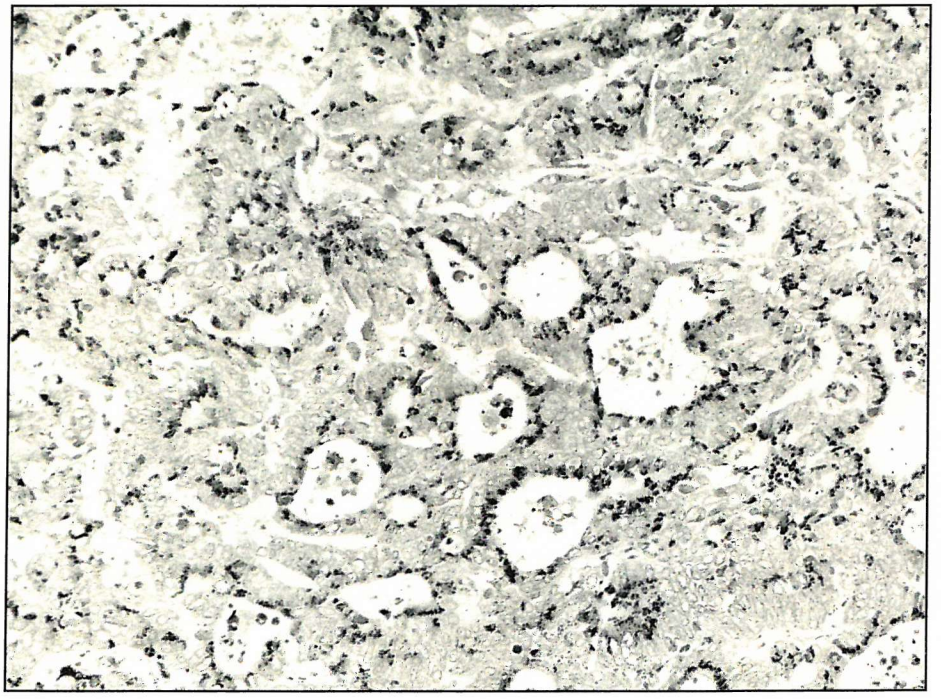
positively stained areas (count number), the total area of positive staining (count area) and the total area of the image that was analysed (field area).

Digital image analysis was performed to verify the visual scores, which, once validated, could then be used for all further statistical analysis. The areas chosen encompassed the various patterns of staining and were representative both in type and quantity. The objective was to ensure that each enzyme was assessed and that the various cellular patterns of expression (e.g. hepatocyte, fibroblast and macrophage staining) were all examined. The areas used were adjacent normal liver parenchyma for MMP-1, MMP-9 and TIMP-1, and adjacent tumour for MMP-7 and TIMP-2 (Appendix IV).

Figure 3. Digital image analysis: the various steps involved in data acquisition

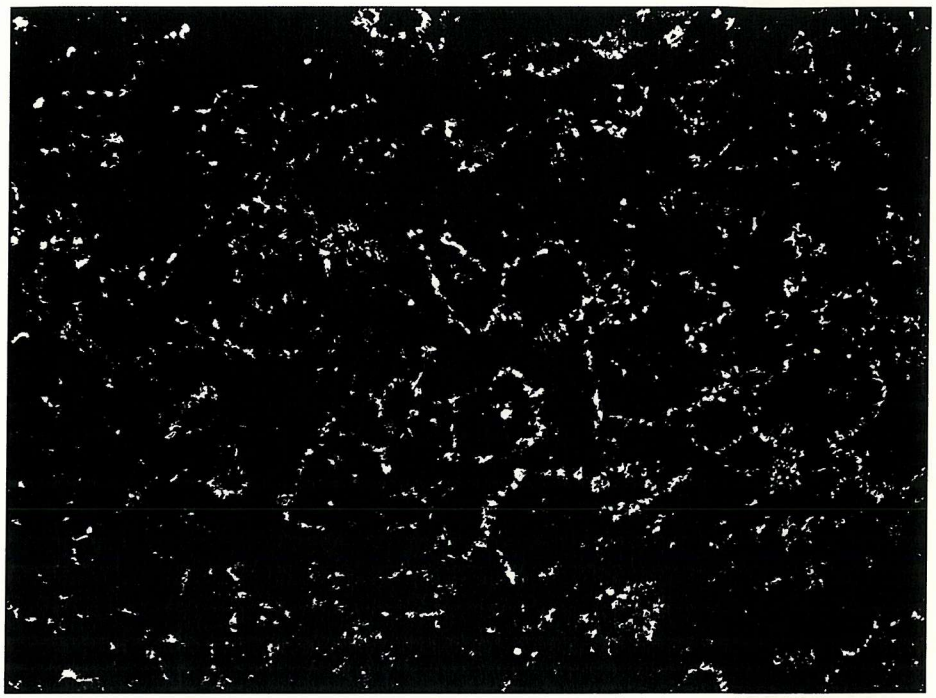


(a) An image showing staining of tumour cells for MMP-7 with typical brown cytoplasmic staining.

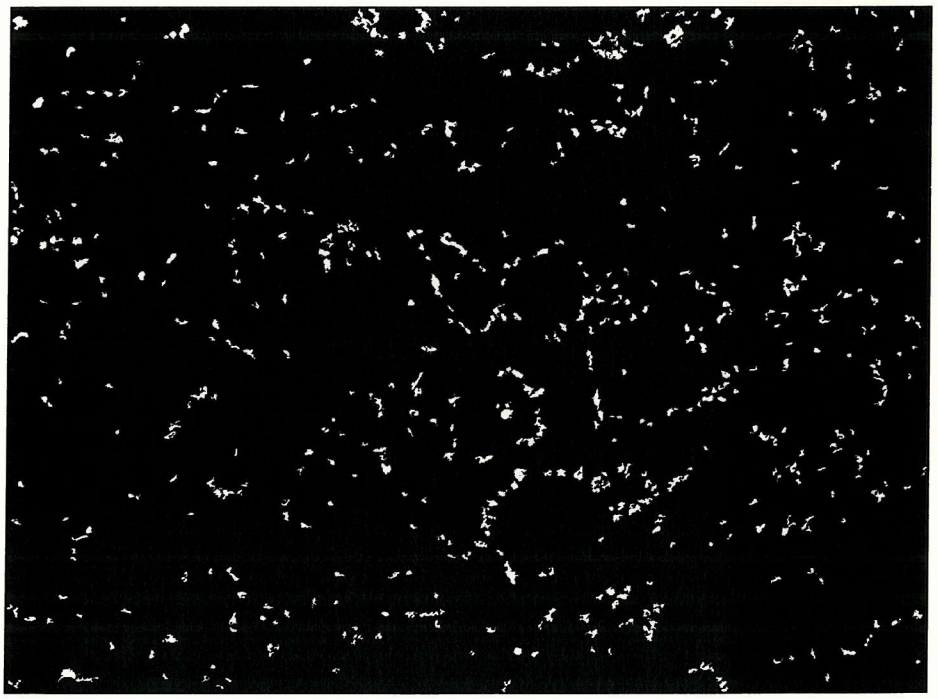


(b) The positive staining is identified and then enhanced. The image is converted to monochrome and the positive staining now appears black.

Figure 3. Digital image analysis: the various steps involved in data acquisition

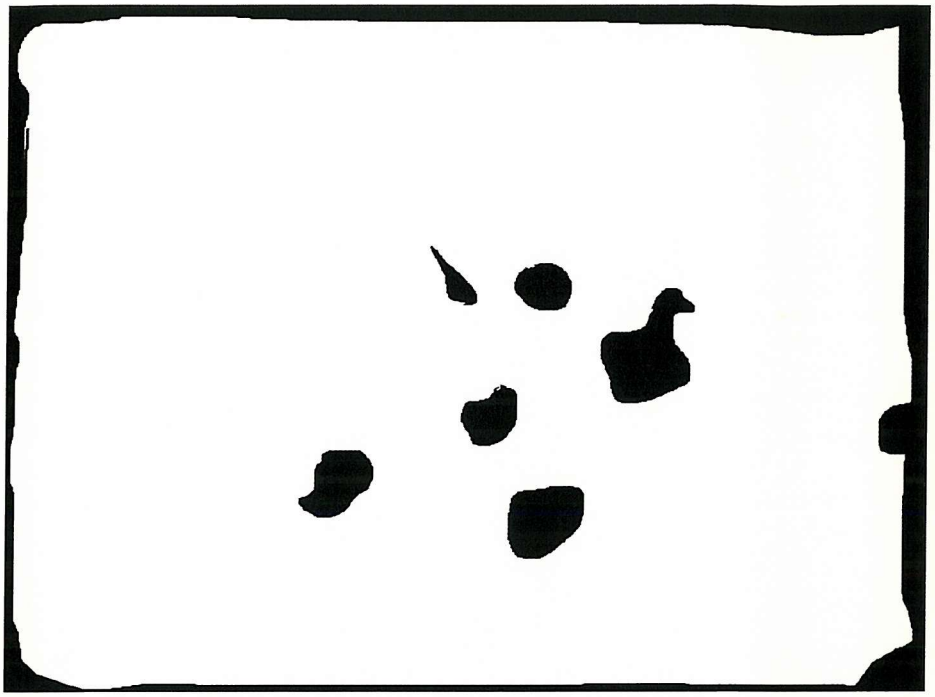


(c) A binary image is produced where positive staining appears white against a black background.

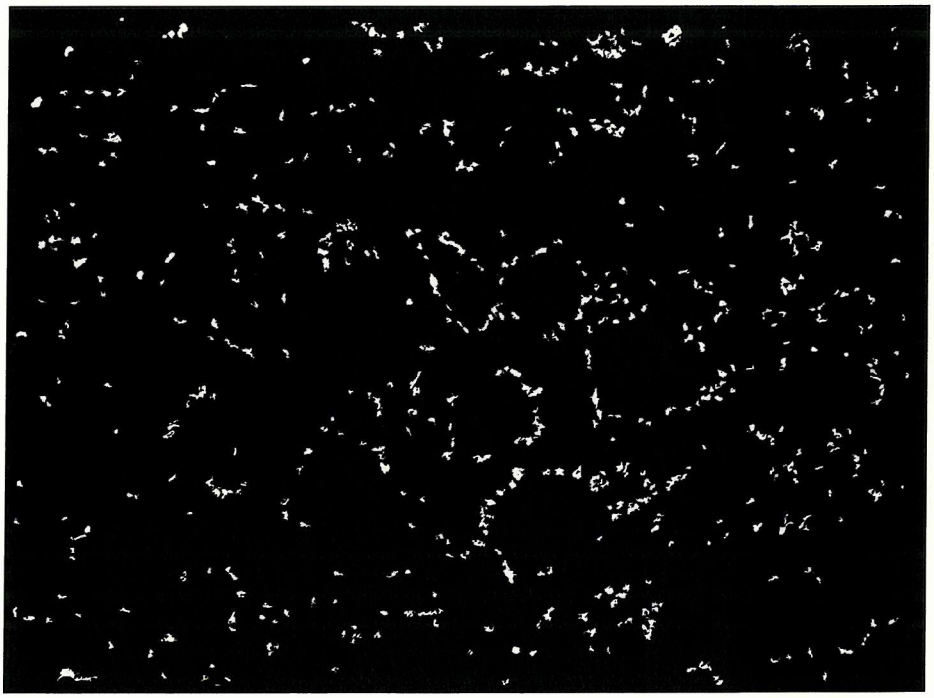


(d) To reduce non-specific positive staining any area less than 30 pixels in size is discarded.

Figure 3. Digital image analysis: the various steps involved in data acquisition



(e) A binary mask drawn to define the total area for analysis (field area) whilst excluding 'holes' which are devoid of tissue.



(f) The staining that remains is then used to calculate the number of positively stained areas (count number) and the total area of positive staining (count area).

Statistical Analysis

A log-rank test was performed to formally test for the equality of survivor functions. This is a non-parametric test, which makes use of the full survival data, without making any assumption about the shape of the survival curve. A significant p-value indicates that there are statistically significant differences in the survival functions. The Cox proportional hazards model has been used for multivariate analysis.

Survival estimates have been calculated using the Kaplan-Meier method to give actuarial survival. To indicate precision, the 95 per cent confidence intervals are given where applicable.

Survival analysis is the term used to describe the analysis of data that measure the time from a well-defined time origin until the occurrence of some particular event or end-point. As the name suggests, the event of interest is often death and the dependent variable is a survival time. The variable analysed in this work is the time from hepatic surgery until death.

A number of features of survival data make standard statistical analysis procedures unsuitable. Firstly, survival data are not symmetrically distributed. Typically, the distributions of survival time data tend to be positively skewed, as the majority of events usually occur towards the beginning of the period of interest with a small number of long-term survivors. Therefore, it is not reasonable to assume that survival data follow a normal distribution and an alternative distributional model for the data must be used. Another feature that renders standard data analysis methods unsuitable is that not all of the patients will have experienced the event of interest (in this case death). Some of the patients may have left the study early or may be lost to follow-up. Thus the only information that we have about some patients is that they were still alive at their last follow-up. These are termed censored survival times.

The fact that survival data is generally skewed means that the mean is not a good summary statistic to use as it is unduly influenced by extreme observations. As a result, the median survival time is the summary statistic usually preferred for survival time data and will be the measure presented in this work.

The distribution of survival times can be described using two functions, namely the survivor function S(t) and the hazard function h(t). The survivor function S(t) is the probability that a patient from the population will have a survival time greater than time t. The hazard function h(t) is the probability of an individual dying at time t for those patients who have lived to time t. The hazard function therefore corresponds to the instantaneous death rate for a patient surviving to time t.

In the estimation of survival data, the survivor function and the hazard function are estimated form the observed survival times. There are a number of methods of estimating these functions including non-parametric methods, such as the Kaplan-Meier estimate; parametric methods, such as the Weibull model; and semi-parametric methods, such as the Cox Proportional Hazards Model.

There are two main methods of estimating the survivor function, namely the Kaplan-Meier and life-table methods. Both methods are non-parametric since they do not require assumptions about the underlying distribution of the survival times. The Kaplan-Meier estimator is the most widely used method in medical research and was the method used in this work.

The percentage of patients surviving 1, 3, and 5 years were calculated in this work. The presence of censored observations means that we cannot simply calculate this percentage using the total number of patients as the denominator, as this would underestimate the survival rate. To avoid this, the Kaplan-Meier estimate of the survivor function was used to calculate the survival rates with corresponding 95% confidence intervals.

The simplest way of comparing the survival times of two or more groups is to plot the estimates of the survivor functions for each group on the same axes. Hypothesis tests can also be performed to formally test whether any apparent differences observed on inspecting these plots could be due to a real difference between the survival times of the group of individuals or merely due to chance variation. The method used to test these hypotheses is the log-rank test. The log-rank test is a non-parametric test of the equality of two or more survivor functions, which makes use of the full survival data, without making any assumption about the distribution of the survivor functions. It

essentially compares the number of observed deaths in each group to the expected number of deaths.

If we have m groups i=1,...,m, the test statistic is

$$\chi^2_{\log rank} = \sum_{i=1}^{m} \frac{(O_i - E_i)^2}{E_i}$$

where O_i represents the observed deaths in group I and E_i represents the expected deaths in group i.

P-values are obtained by comparing the values of the test statistic to a chi-squared distribution on m-1 degrees of freedom, where m is the number of groups. If there are significant differences across the covariate groups then the covariate is significantly associated with survival.

The Cox proportional hazards model is a semi-parametric multiple regression model. It is said to be semi-parametric because, although it makes no assumption about the shape of the distribution of the survival time, it does require assumptions about the hazard ratio. In this approach, the hazard function, h(t), is an unknown function of time. We then assume that anything which affects the hazard ratio does so by the same ratio at all times i.e. something that doubles the ratio on day one will also double the ratio on days two, three and so on.

The hazard ratio (HR) is the parameter used to describe the relative risk between two groups with respect to survival data. If the hazard ratio is less than 1 the hazard of death is smaller for an individual compared to an individual in the reference group. On the other hand if the hazard ratio is greater than 1, the hazard of death for an individual is greater than that for an individual in the reference group.

The test used for assessing and comparing different models was the likelihood ratio test. The likelihood ratio test compares two nested models, one with p parameters and the other with q parameters, where p<q. The null hypothesis is that there is no difference between the two models. That is, when including the extra variables in the Cox model does not help explain the survival data any more satisfactorily than the reduced model. If the null hypothesis is true then the test statistic follows a χ^2

distribution with q-p degrees of freedom. For example if we were considering the impact of adding gender as an explanatory variable, then we would use a comparison with a χ^2 distribution with one degree of freedom. If we were considering the impact of adding Dukes' Stage to the model, on the other hand, we would use a comparison with a χ^2 distribution with 4 degrees of freedom, since Dukes' stage has 5 levels, so has 4 dummy parameters associated with it.

Chapter Four

Results

Visual Analysis

Throughout the experimental and analytical stages the clinicopathological details were unknown except for an identifying code number. Therefore all analysis was performed without the knowledge of patient outcome.

Expression patterns for MMP-1

Staining for MMP-1 was found in both tumour and normal liver parenchyma, with greater than 10 per cent staining (visual score >1) seen in the tumour of 21/105 (20%) patients and in the normal liver parenchyma of 90/105 (86%) patients (Table 8). The majority of staining was seen in the normal liver parenchyma and appeared as fine granular cytoplasmic staining of the hepatocytes (Figs 4a & 4b). Some specimens showed greater staining in hepatocytes that were further away from the portal triads. At the tumour/liver interface staining was seen in inflammatory cells, which morphologically appeared to be both neutrophils and macrophages. Within the tumour the staining pattern was predominantly in the cytoplasm of the tumour cells (Figs 4c & 4d).

Greater than 50 per cent staining (visual score >2) was seen in 36/105 adjacent normal liver parenchymal specimens and 21/105 distant normal liver parenchymal specimens. Greater than 50 per cent staining was seen in 15/105 adjacent tumour specimens and 12/105 distant tumour specimens (Figure 5).

Out of the 105 specimens 77 (73%) showed no staining at all in the tumour, whereas only 1/105 liver parenchymal specimens showed no staining.

Table 8. Tabulation of the number of patients and their visual analysis score for each antibody in each of the 4 defined areas.

	Visual Score	Adjacent Tumour	Distant Tumour	Adjacent Liver	Distant Liver
MMP-1	**0**	80	77	0	1
	1	4	9	15	18
	2	6	7	54	65
	3	7	8	31	21
	4	6	2	5	0
	5	2	2	0	0
MMP-7	**0**	63	51	0	0
	1	17	21	0	0
	2	7	8	104	104
	3	11	16	1	1
	4	6	8	0	0
	5	1	1	0	0
	0	67	73	27	59
	5	0	0	0	0
TIMP-1	**0**	80	79	30	55
	1	20	17	24	21
	2	4	8	23	21
	3	1	1	13	5
	4	0	0	15	3
	5	0	0	0	0
TIMP-2	**0**	3	7	82	101
	1	16	16	17	3
	2	24	22	3	0
	3	33	39	2	0
	4	26	19	1	1
	5	3	2	0	0
	5	3	2	0	0

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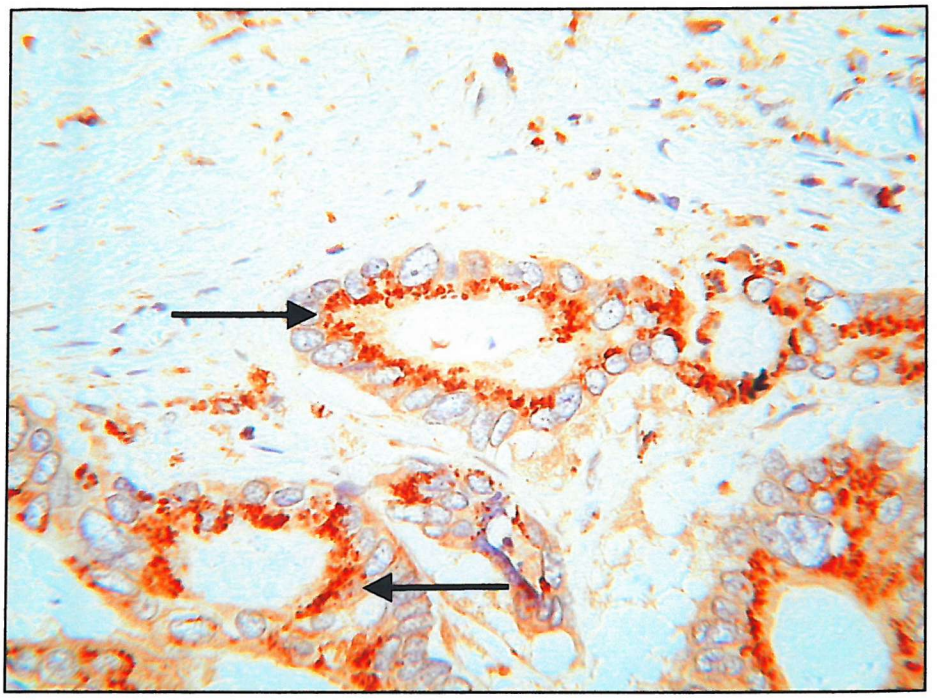
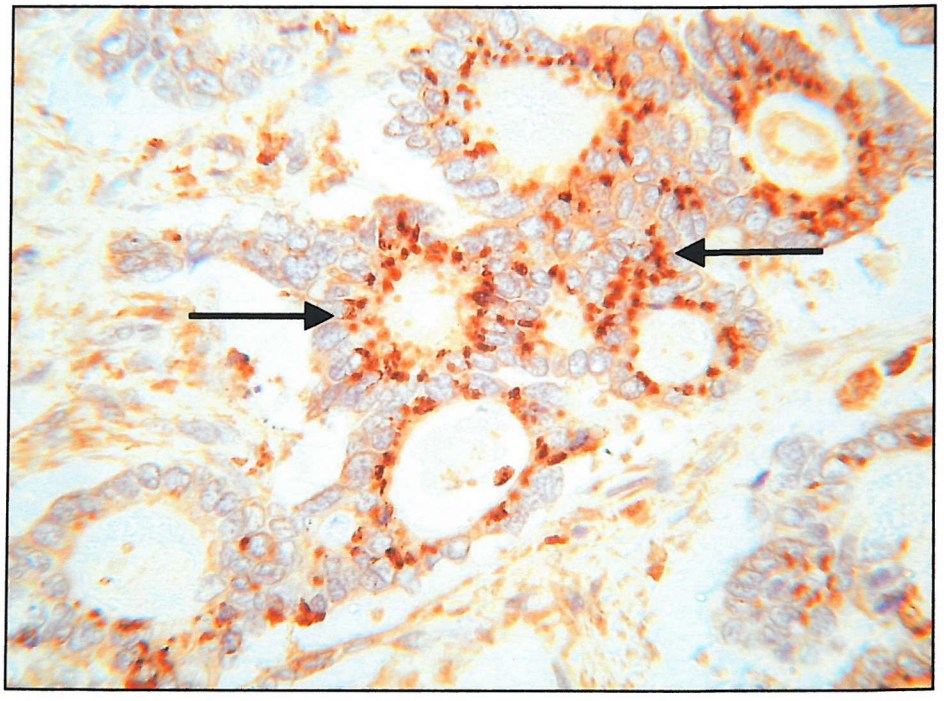
Figure 4. Staining patterns for MMP-1 within the tumour

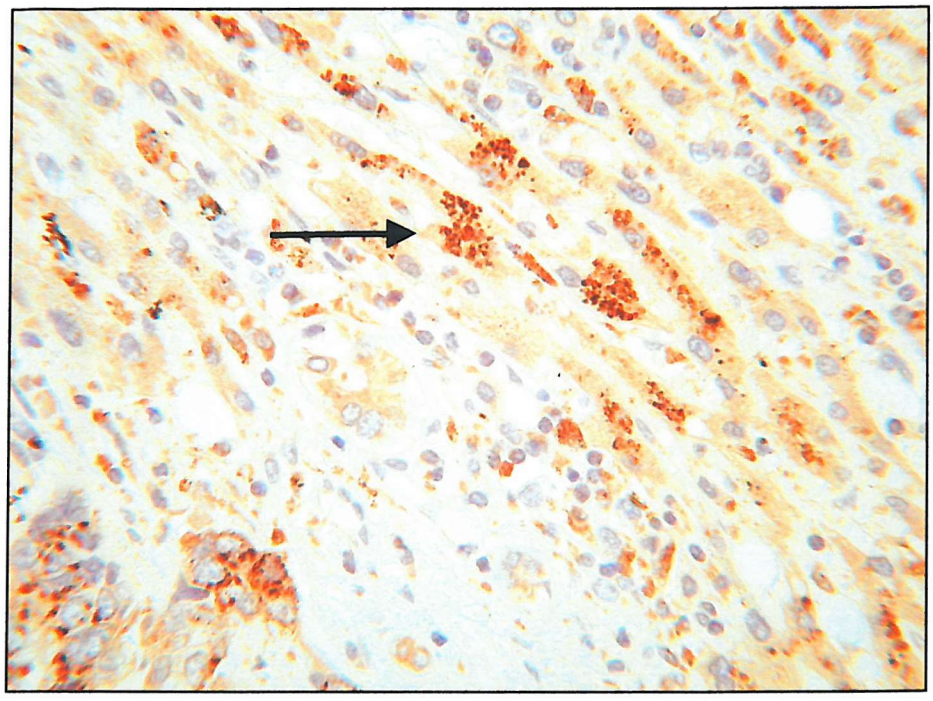


(a) MMP-1 (X400): showing the typical brown appearance of staining within tumour cell cytoplasm (arrows)

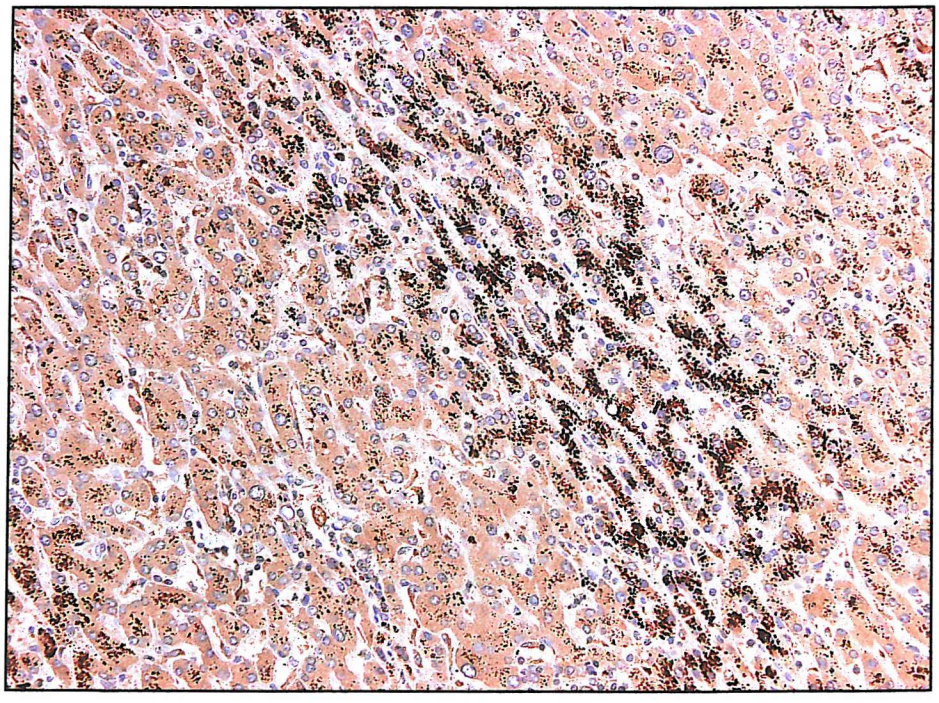


(b) MMP-1 (X400): staining of tumour cell cytoplasm (arrows)

Figure 4. Staining patterns for MMP-1 within the liver

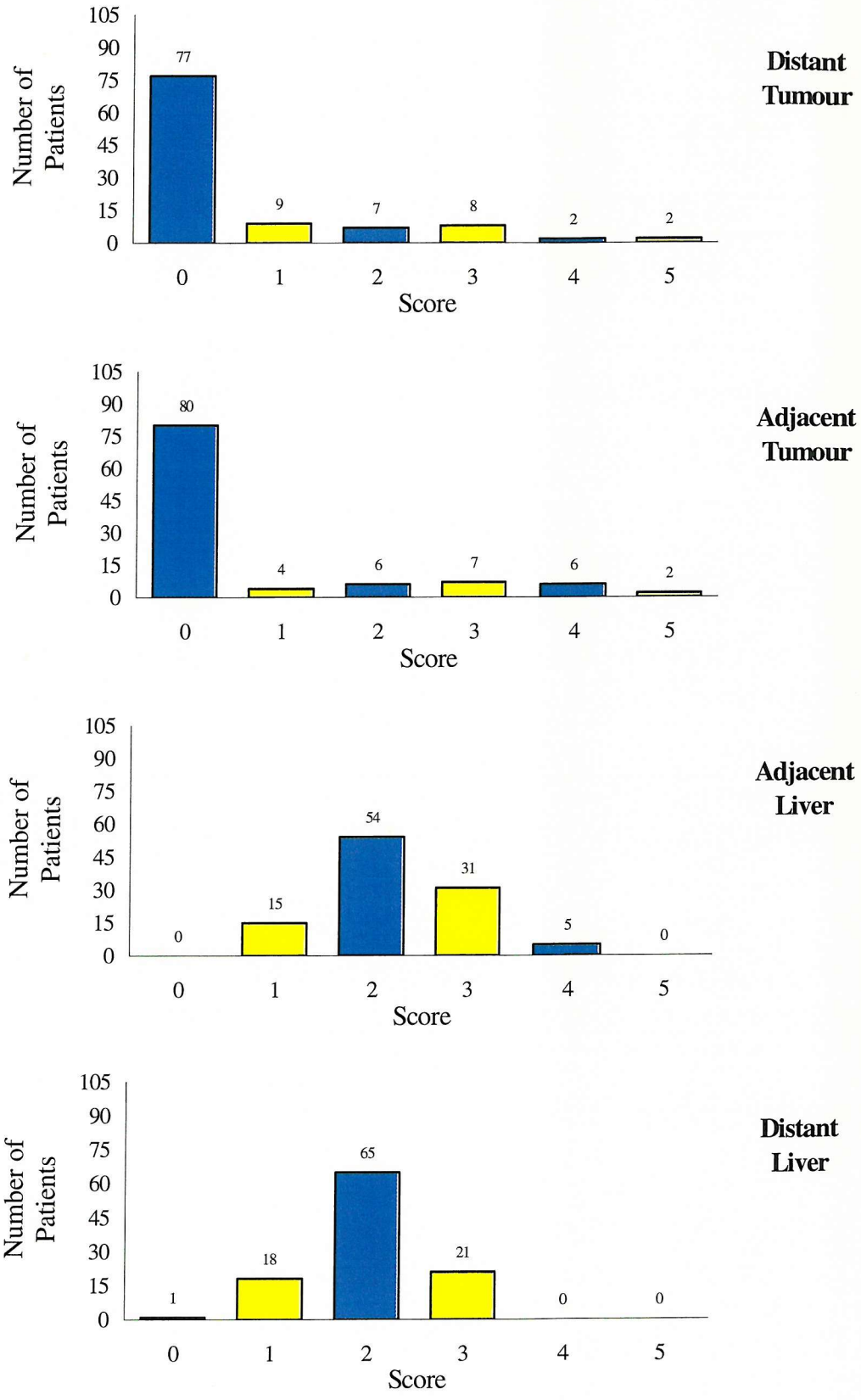


(c) MMP-1 (X400): showing staining in hepatocytes (arrow)



(d) MMP-1 (X200): lower magnification showing widespread hepatocellular staining

Figure 5. Bar charts showing visual analysis scores for MMP-1 in each of the 4 defined areas.



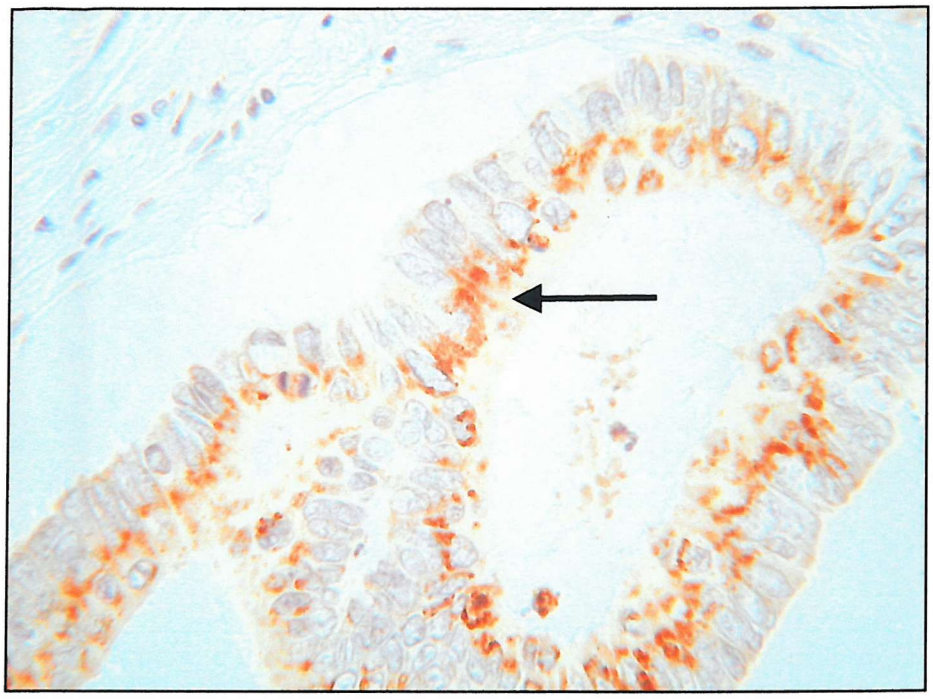
Expression patterns for MMP-7

Staining for MMP-7 was seen in both tumour and normal liver parenchyma, with greater than 10 per cent staining seen in the tumour of 33/105 (31%) patients and in the normal liver parenchyma of 105/105 (100%) patients (Table 8). The tumour staining was localised in the cytoplasm of the tumour cells (Figure 6a & 6b). The normal liver parenchyma showed fine granular cytoplasmic staining in the hepatocytes of all the specimens and was considered to be positive staining rather than non-specific background staining (Figure 6c). A control slide with no staining is shown for comparison (Figure 6d) Also a few positive inflammatory cells were identified in the stroma of the normal liver parenchyma.

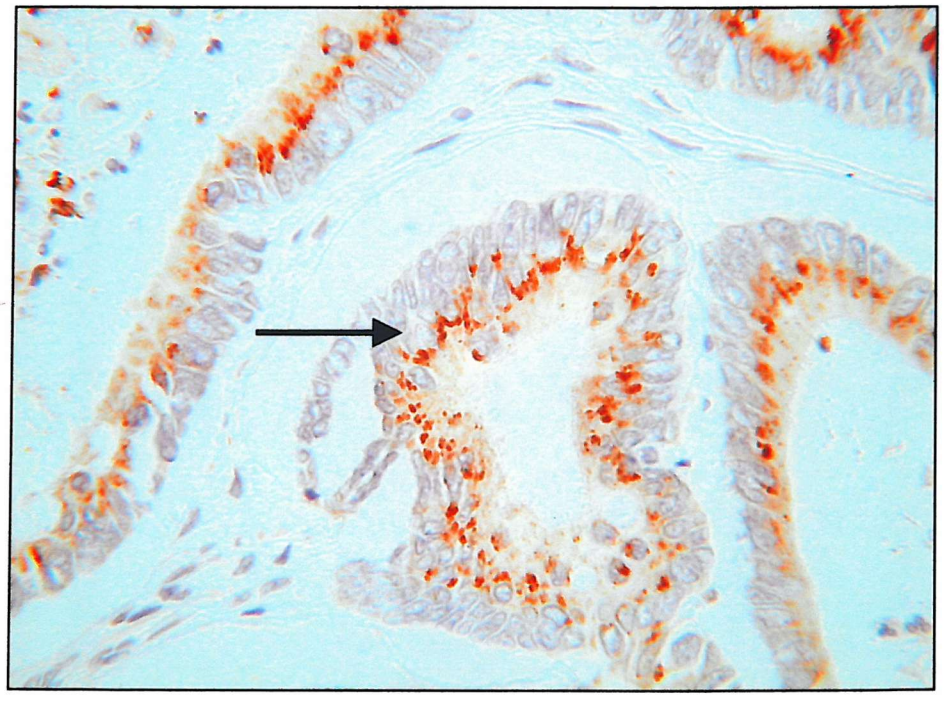
Greater than 50 per cent staining was seen in 18/105 adjacent tumour specimens and 25/105 distant tumour specimens (Figure 7). The normal liver parenchymal staining was uniform between both adjacent and distant areas with the majority scoring 2.

Out of the 105 specimens 51 (49%) showed no staining at all in the tumour, whereas all of the liver parenchymal specimens showed some staining.

Figure 6. Staining patterns for MMP-7 within the tumour

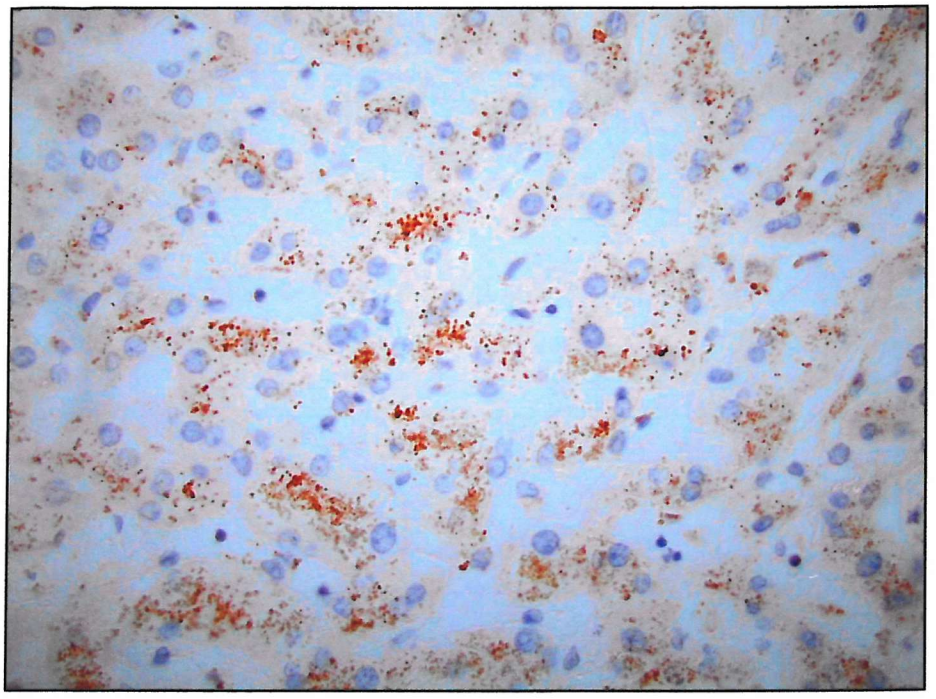


(a) MMP-7 (X400): showing staining of tumour cell cytoplasm

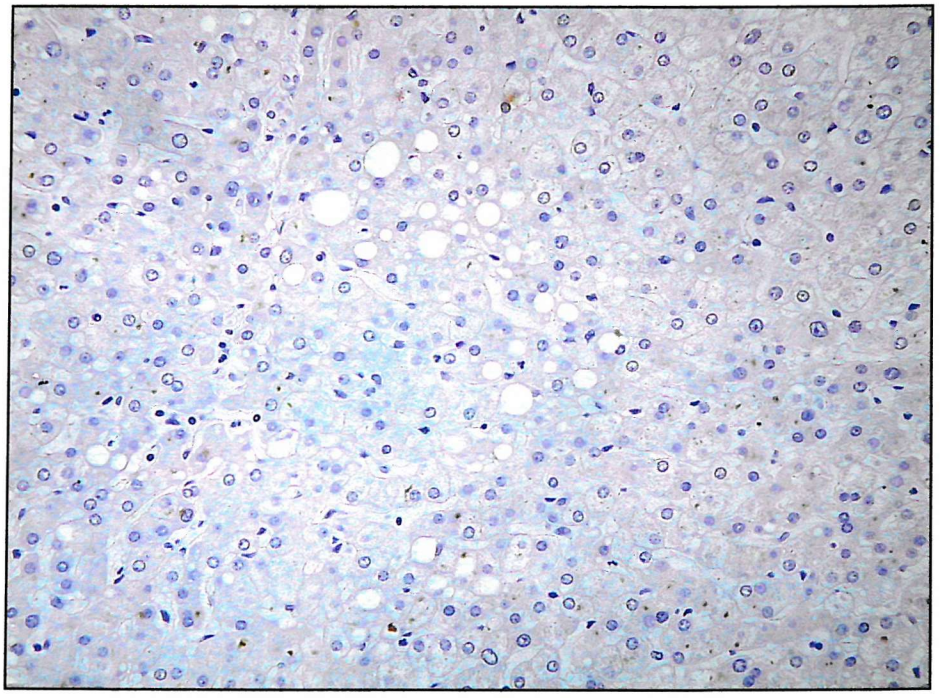


(b) MMP-7 (X400): staining of tumour cell cytoplasm (arrow)

Figure 6. Staining patterns for MMP-7 within the liver parenchyma and a control slide for comparison

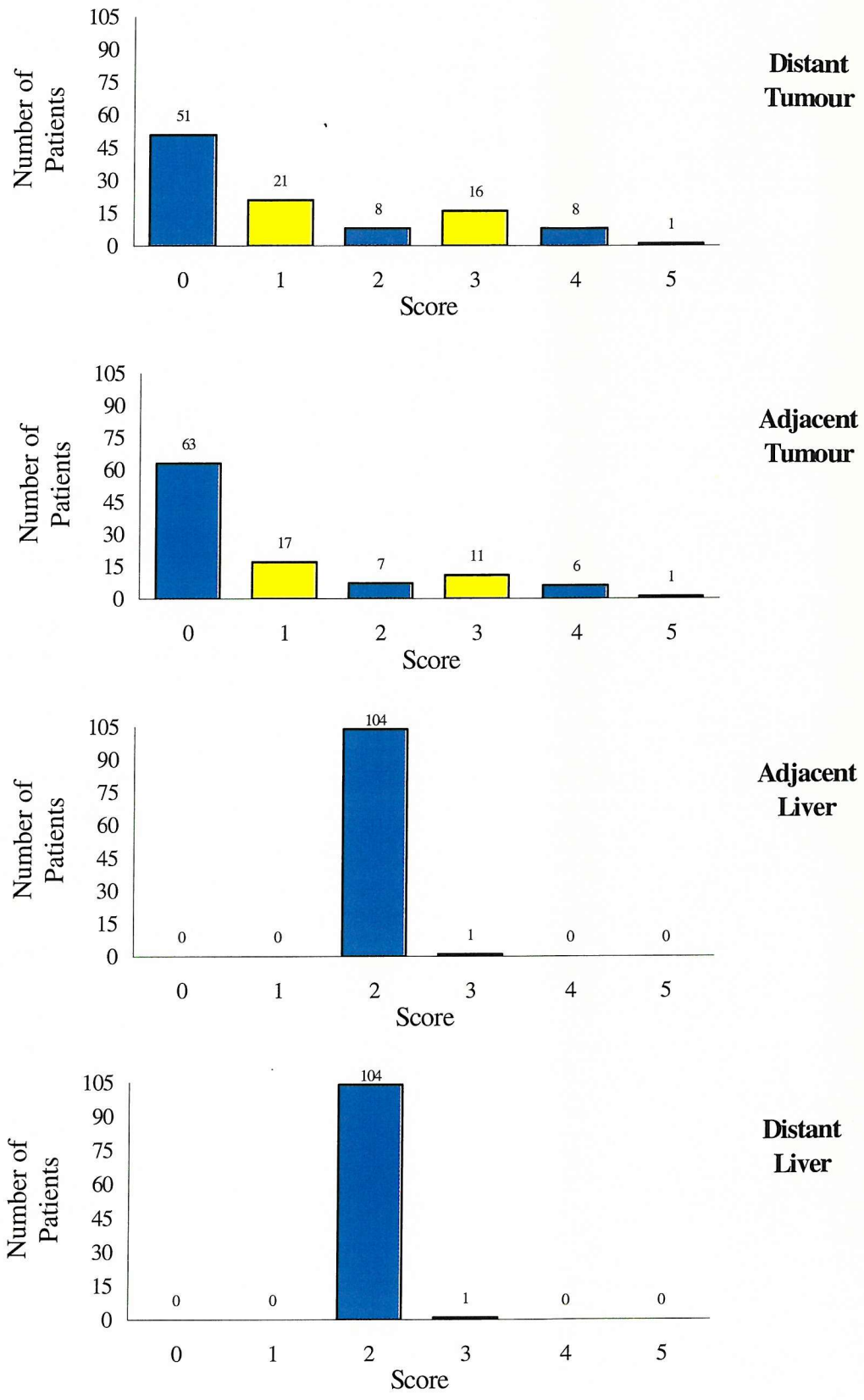


(c) MMP-7 (X400): showing staining of liver parenchyma



(d) Control slide (X200): showing no liver parenchymal staining

Figure 7. Bar charts showing visual analysis scores for MMP-7 in each of the 4 defined areas.



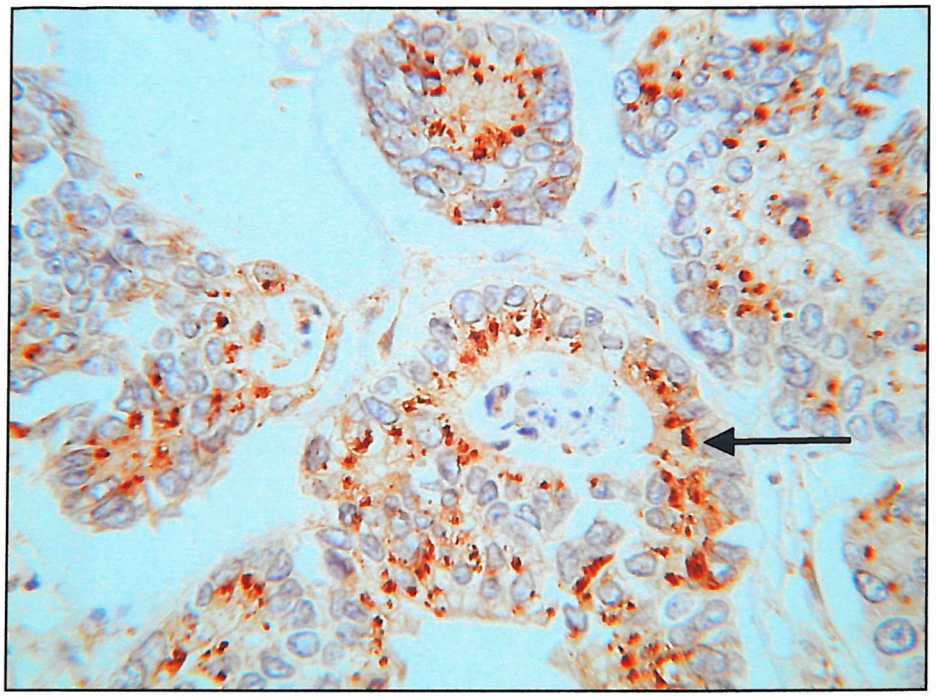
Expression patterns for MMP-9

Staining for MMP-9 was found in both tumour and normal liver parenchyma, with greater than 10 per cent staining seen in the tumour of 24/105 (23 %) patients and in the normal liver parenchyma of 56/105 (53%) patients (Table 8). The tumour staining was in tumour cell cytoplasm, inflammatory cells (morphologically both neutrophils and macrophages) and stromal fibroblasts (Figure 8a & 8b). The normal liver parenchymal staining was in inflammatory cells, again morphologically both neutrophils and macrophages, and in cells contained within the blood vessels (Figure 8c & 8d).

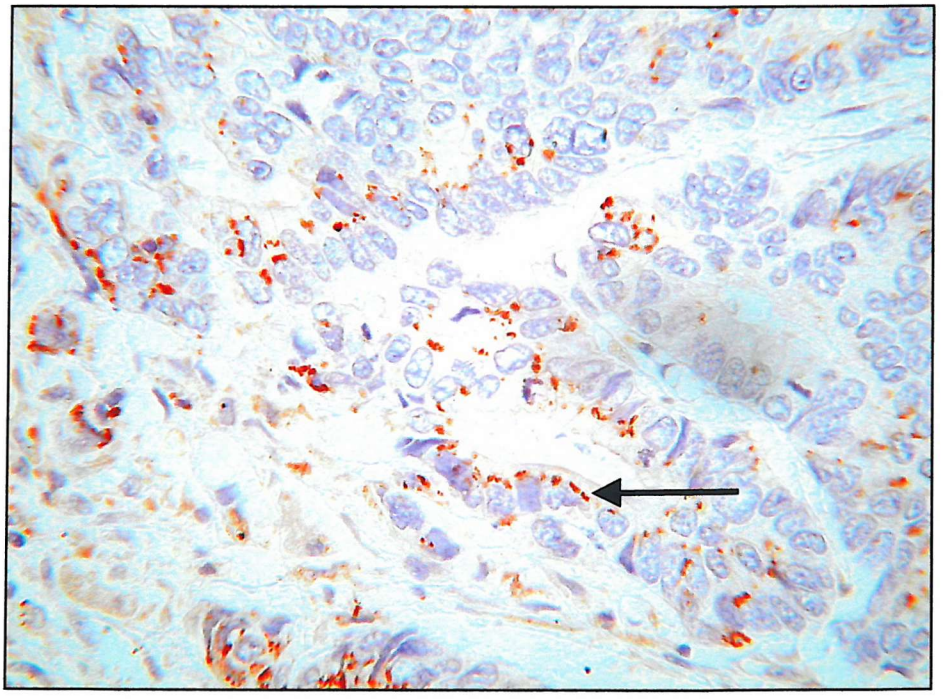
Greater than 50 per cent staining was seen in 9/105 tumour specimens and only 4/105 adjacent tumour specimens. Greater than 50 per cent staining was seen in 34/105 adjacent liver specimens and 11/105 distant liver specimens (Figure 9).

Out of the 105 tumour specimens 59 (56%) showed no staining at all in the tumour and 23/105 normal liver specimens showed no staining.

Figure 8. Staining patterns for MMP-9 within the tumour

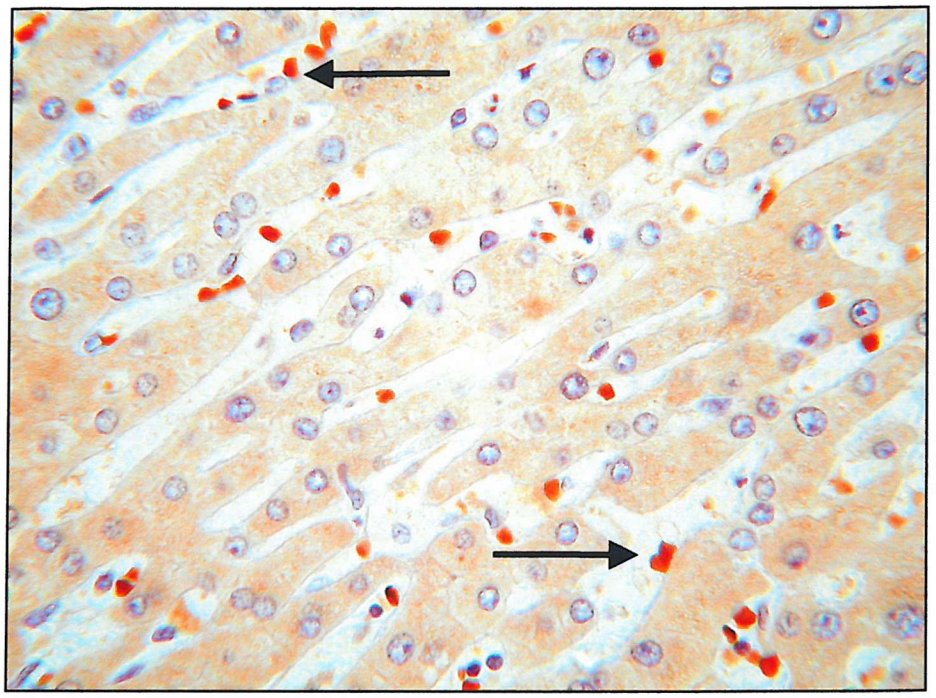


(a) MMP-9 (X400): staining of tumour cell cytoplasm (arrow)

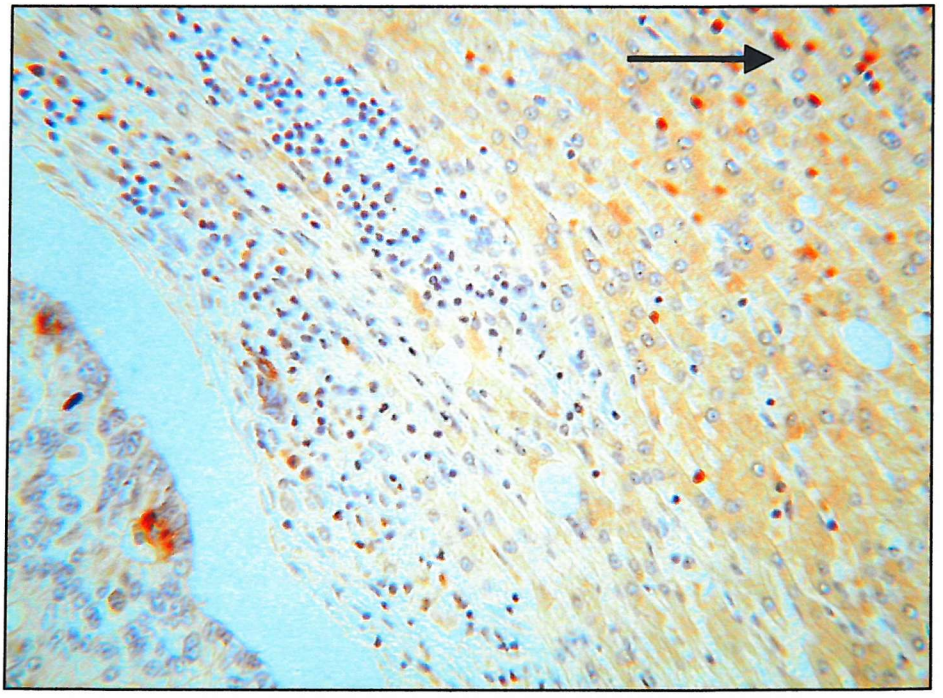


(b) MMP-9 (X400): staining of tumour cell cytoplasm (arrow)

Figure 8. Staining patterns for MMP-9 within the liver

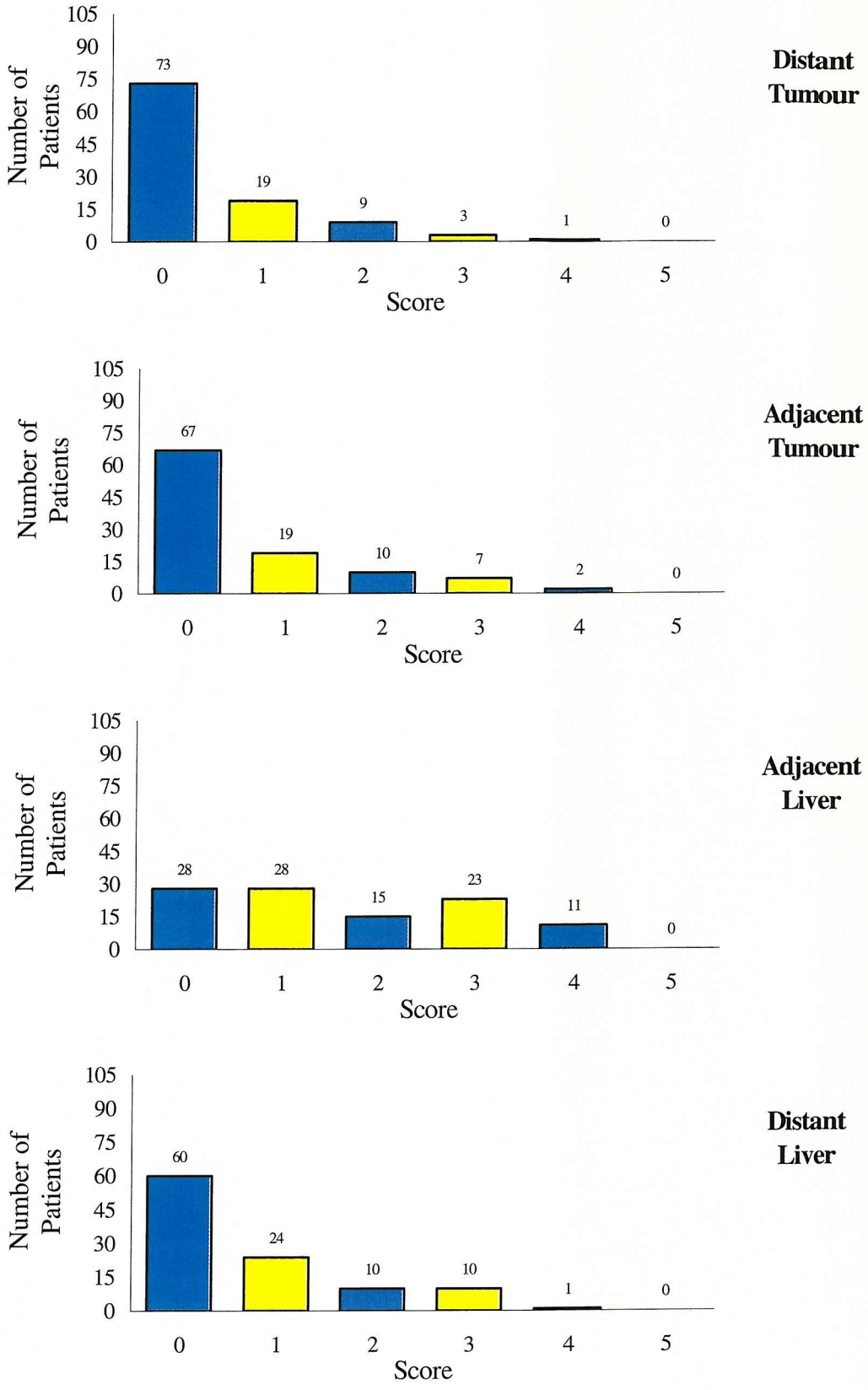


(c) MMP-9 (X400): showing inflammatory cell staining in normal liver parenchyma (arrows)



(d) MMP-9 (X250): showing inflammatory cell staining (arrow) in the liver parenchyma at the tumour/liver interface

Figure 9. Bar charts showing visual analysis scores for MMP-9 in each of the 4 defined areas.



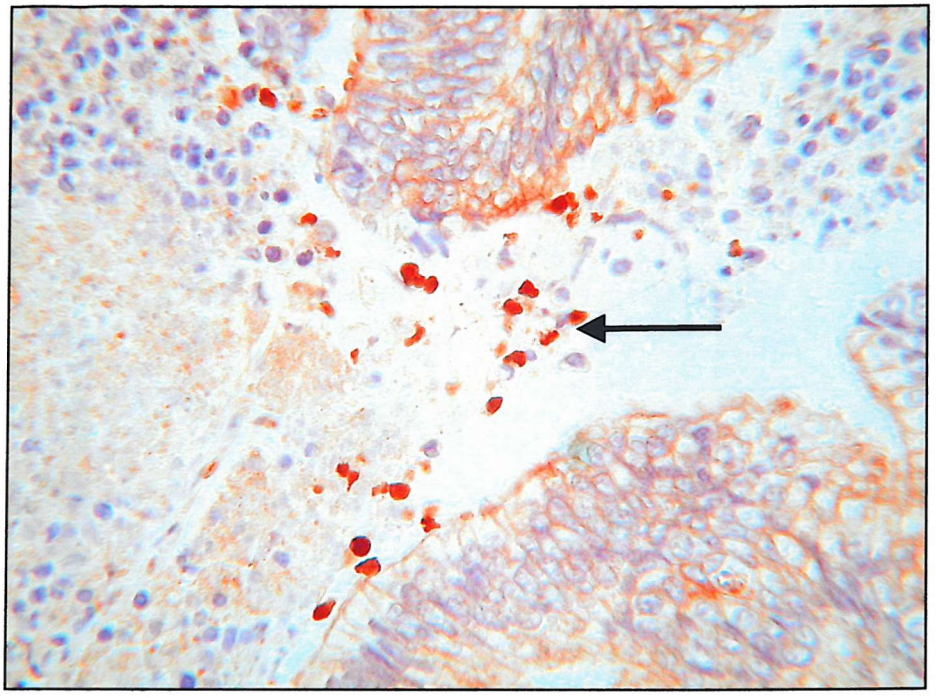
Expression patterns for TIMP-1

Staining for TIMP-1 was found in both tumour and normal liver parenchyma, with greater than 10 per cent staining seen in the tumour of 10/105 (10 %) patients and in the normal liver parenchyma of 57/105 (54%) patients (Table 8). The tumour staining was predominantly in inflammatory cells, morphologically macrophages, and also fibroblasts (Figure 10a). This staining pattern was particularly strong in necrotic areas of tumour. A few specimens also showed staining of the tumour cell cytoplasm (Figure 10b). The normal liver parenchymal staining was fine granular cytoplasmic staining of the hepatocytes. The tumour/liver interface also showed staining of cells that had the morphological appearances of neutrophils and erythrocytes (Figure 10c).

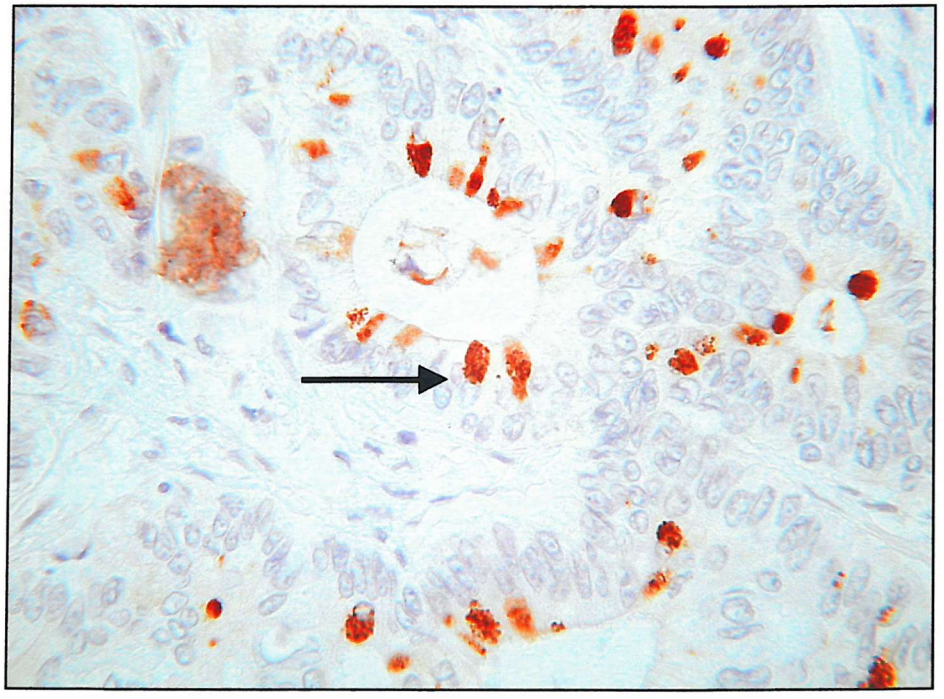
Greater than 50 per cent staining was seen in only 1 tumour specimen in both adjacent and distant areas. The normal liver parenchyma showed greater than 50 per cent staining in 28/105 adjacent normal liver specimens and 8/105 distant liver specimens (Figure 11).

Out of 105 tumour specimens 71 (68%) showed no staining at all in the tumour, and 28/105 normal liver parenchymal specimens showed no staining.

Figure 10. Staining patterns for TIMP-1 within the tumour

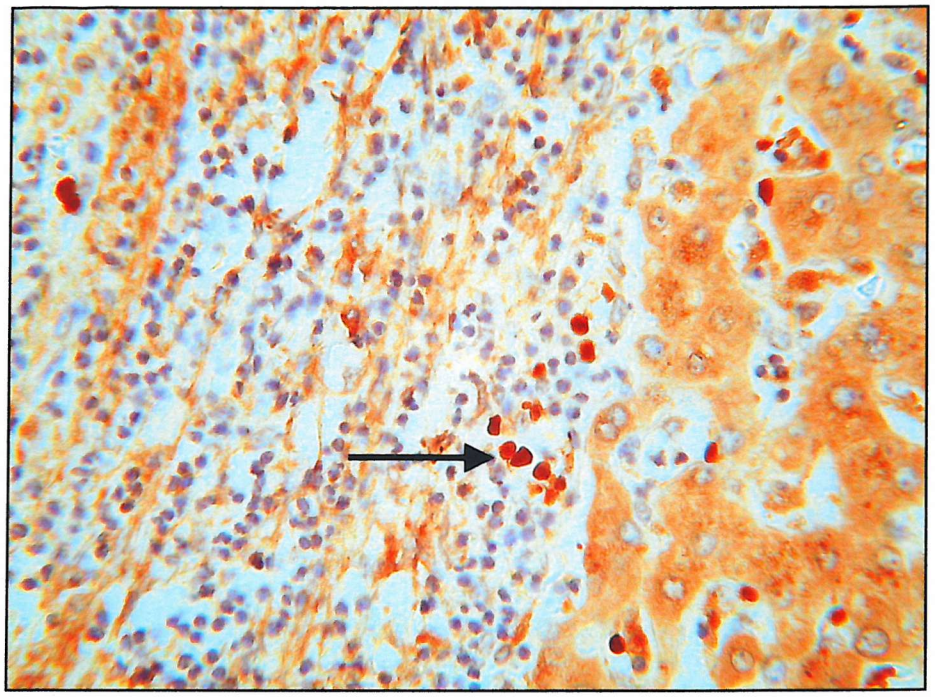


(a) TIMP-1 (X400): staining of inflammatory cells (arrow) within tumour

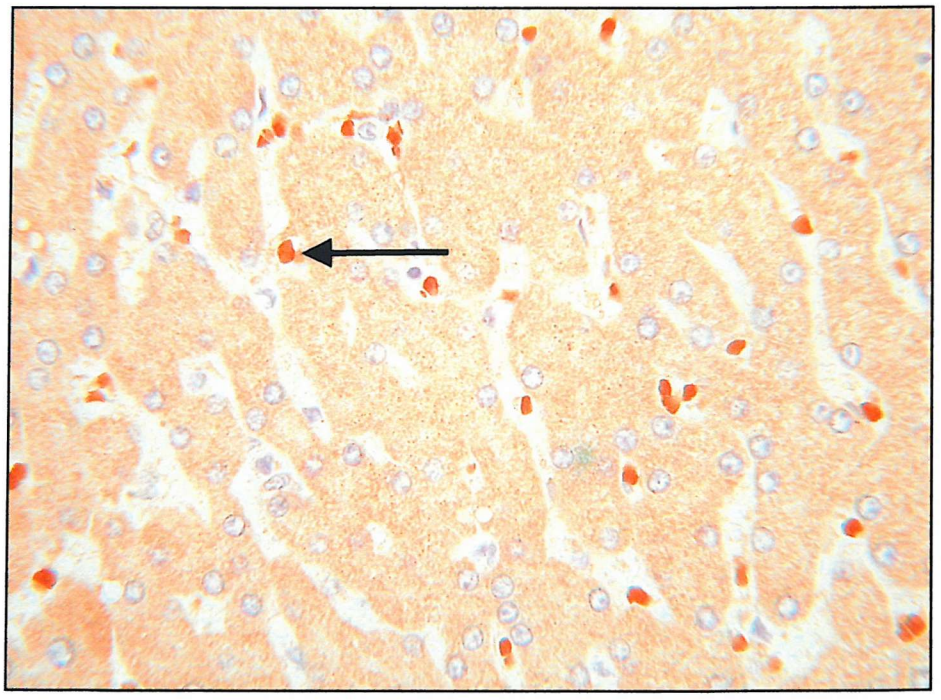


(b) TIMP-1 (X400): staining tumour cell cytoplasm (arrow)

Figure 10. Staining patterns for TIMP-1 within the liver

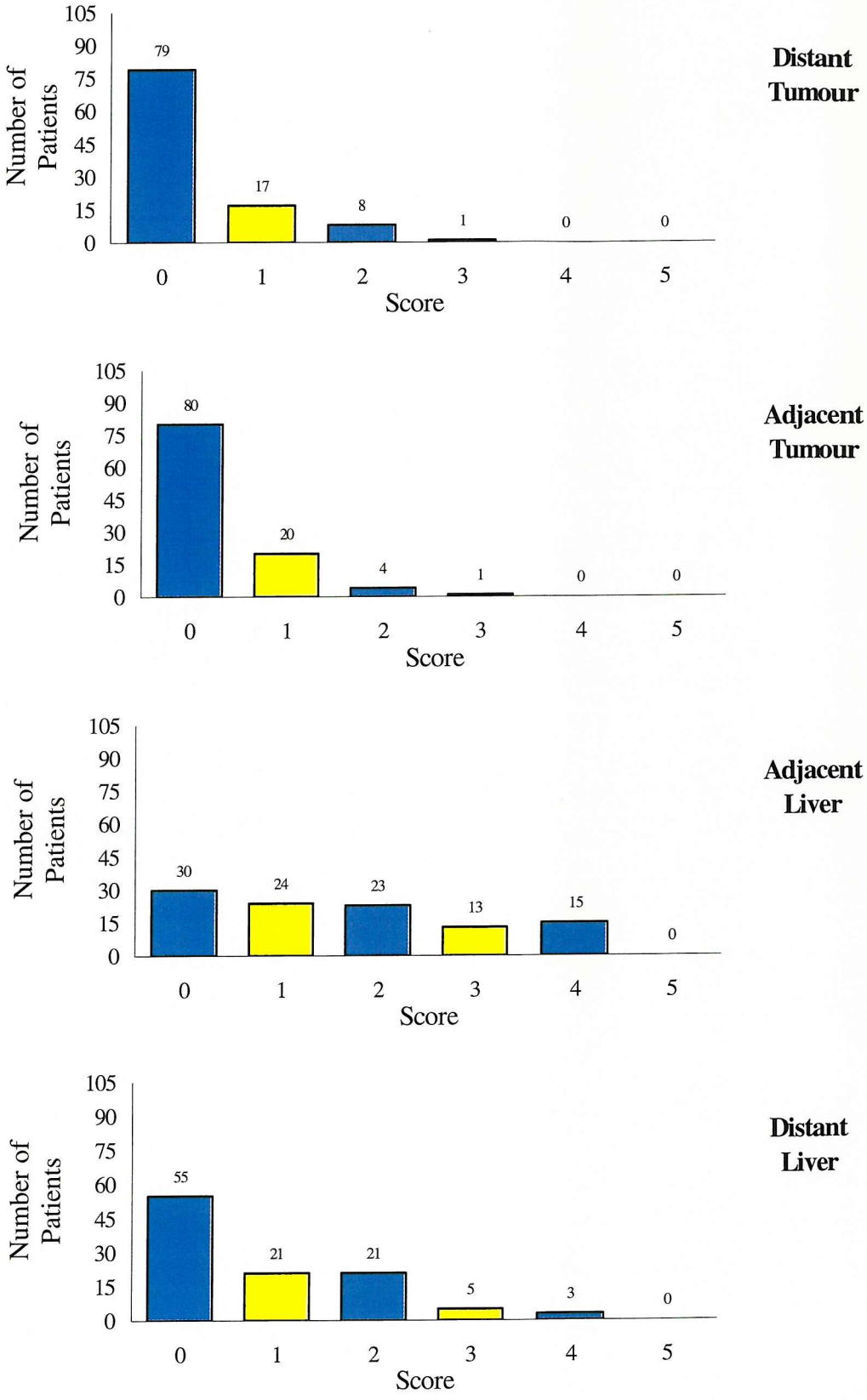


(c) TIMP-1 (X400): staining of inflammatory cells (arrow) at the tumour/liver interface



(d) TIMP-1 (X400): staining of inflammatory cells (arrow) within normal liver parenchyma

Figure 11. Bar charts showing visual analysis scores for TIMP-1 in each of the 4 defined areas.



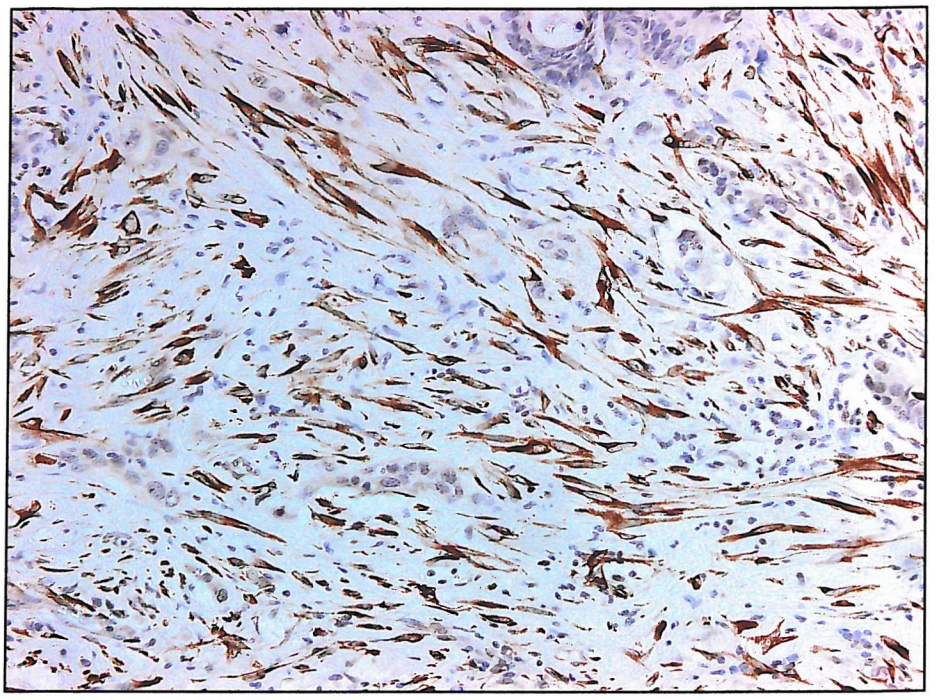
Expression patterns for TIMP-2

Staining for TIMP-2 was found in both tumour and normal liver parenchyma, with greater than 10 per cent staining seen in the tumour of 92/105 (88 %) patients and in the normal liver parenchyma of 6/105 (6%) patients (Table 8). The tumour staining was only of stromal fibroblasts (Figure 12). The normal liver parenchymal staining showed predominantly fine granular cytoplasmic staining of the hepatocytes but also some fibroblast staining.

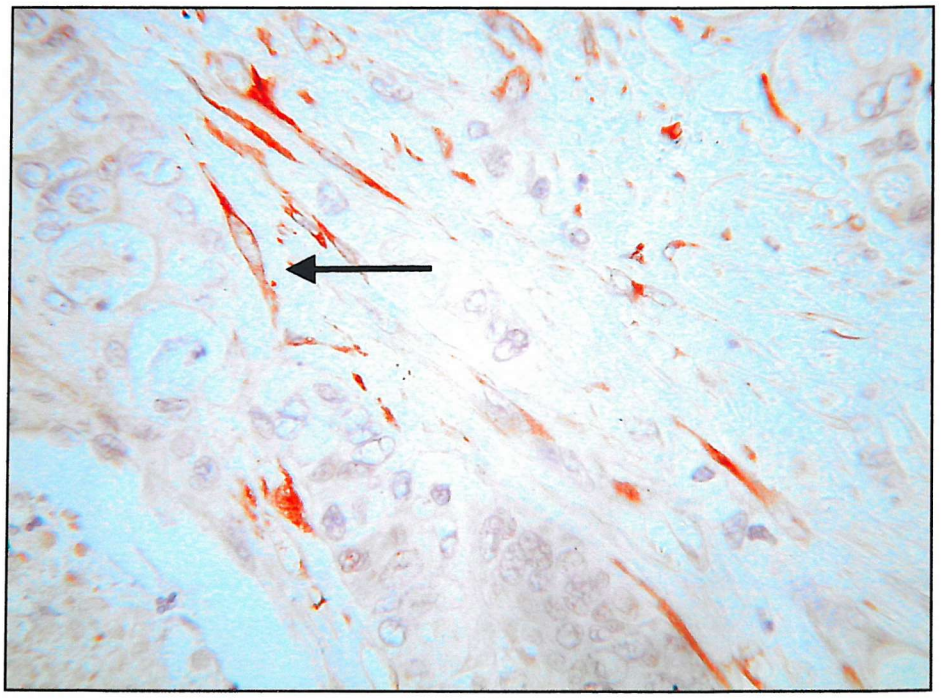
Greater than 50 per cent staining was seen in 62/105 adjacent tumour specimens 60/105 distant tumour specimens. Greater than 50 per cent staining was seen in only 3/105 adjacent liver specimens and 1/105 distant liver specimens (Figure 13).

Out of 105 tumour specimens only one showed no tumour staining at all, but 82/105 normal liver parenchymal specimens showed no staining.

Figure 11. Staining patterns for TIMP-2 within the tumour

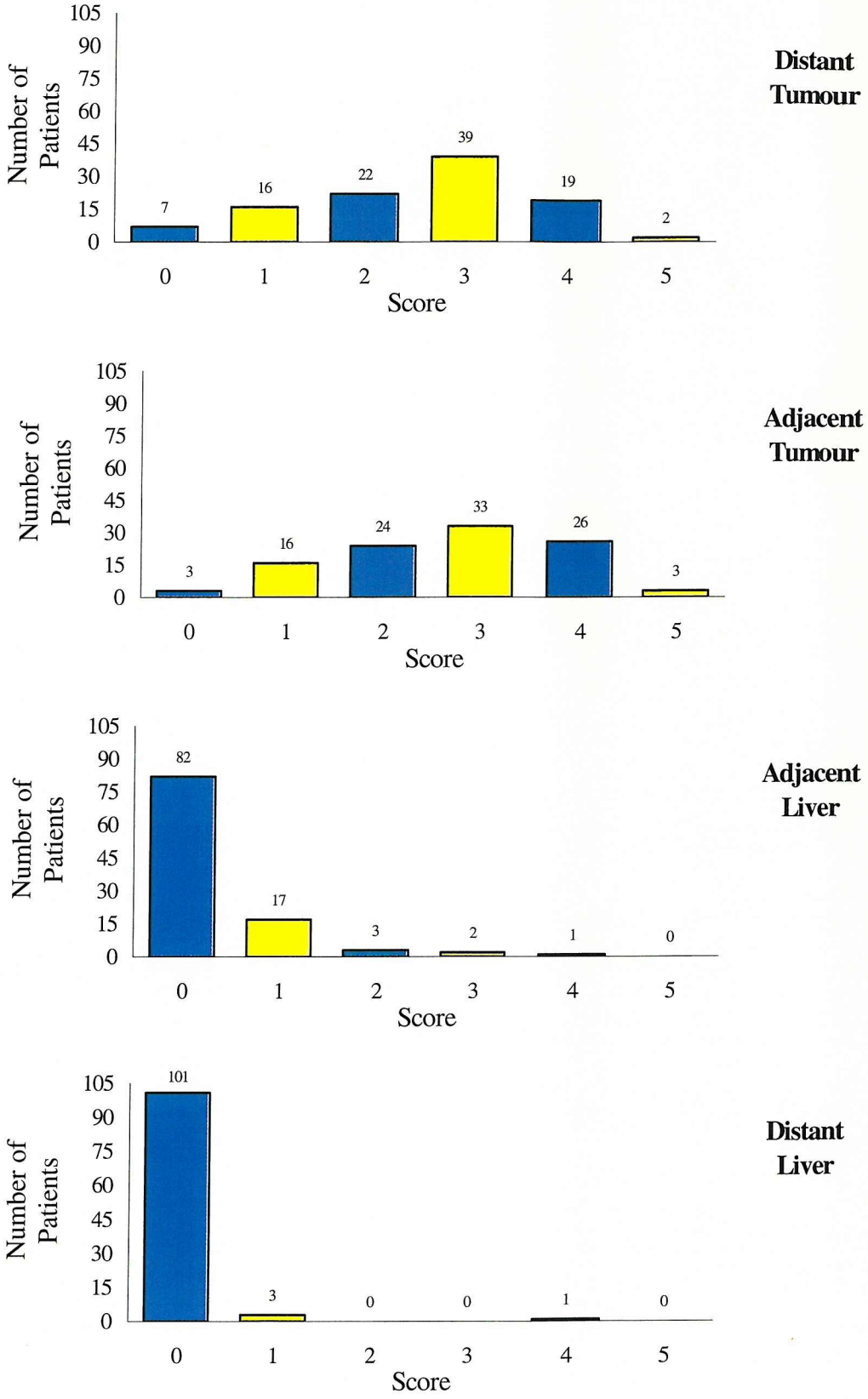


(a) TIMP-2 (X200): showing widespread fibroblast staining in tumour stroma



(b) TIMP-2 (X400): showing fibroblast staining in tumour stroma (arrow)

Figure 13. Bar charts showing visual analysis scores for TIMP-2 in each of the 4 defined areas.



Digital Image Analysis

The digital image analysis scores are shown with the visual scores for direct comparison (Appendix II). All five antibodies showed excellent correlation between the visual and digital image analysis scores with correlation values of 0.87 or greater (Figure 14). This correlation holds true when both the area of positive staining (count area) and number of positively stained areas (count number) are evaluated. All further statistical analysis was subsequently carried out on the visual analysis scores.

Figure 14. Scatter plots of digital image analysis scores versus visual scores for MMP-1. The image analysis scores are: count area (▲) which is the area of positive staining/total area x 100 and count number (▲) which is the number of positively stained areas/total area x 100.

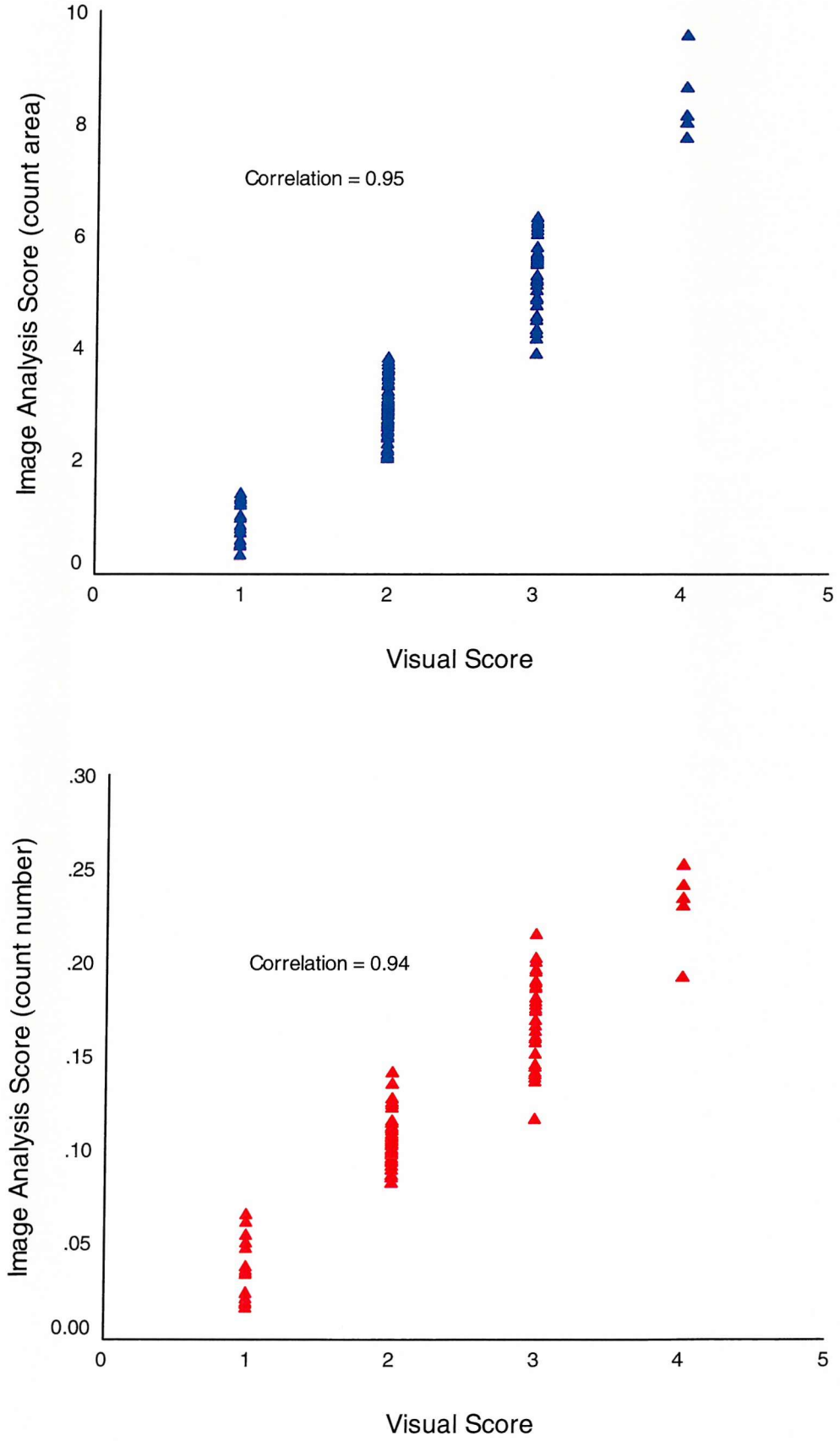
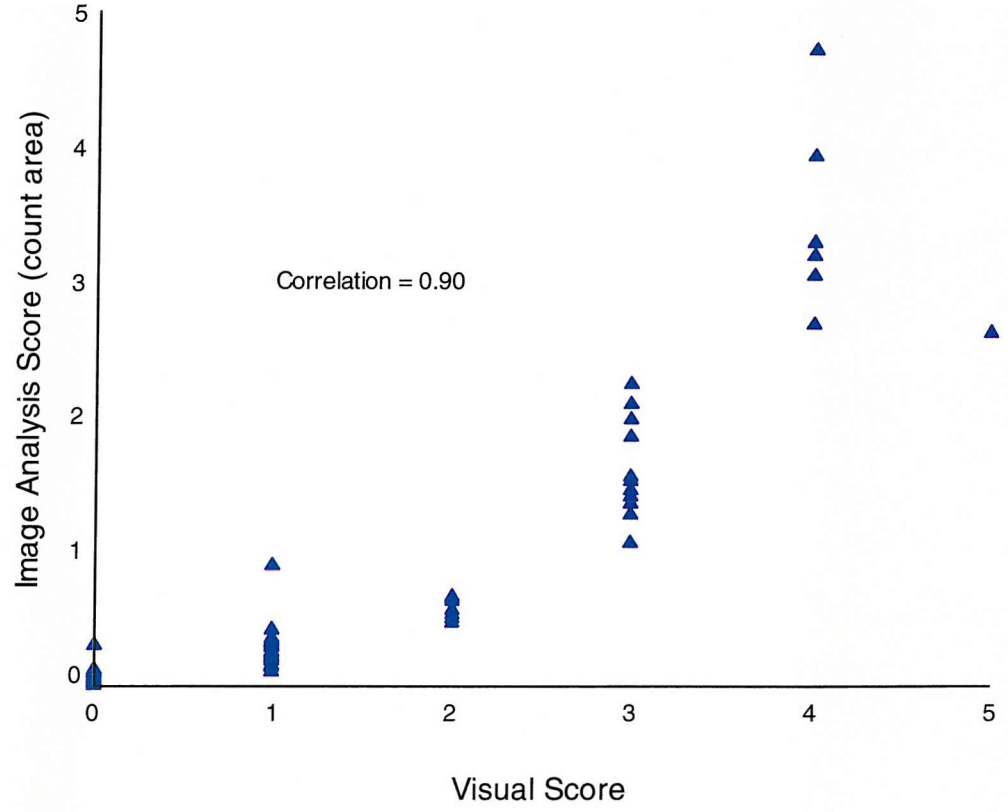


Figure 14. Scatter plots of digital image analysis scores versus visual scores for MMP-7. The image analysis scores are: count area (▲) which is the area of positive staining/total area x 100 and count number (▲) which is the number of positively stained areas/total area x 100.



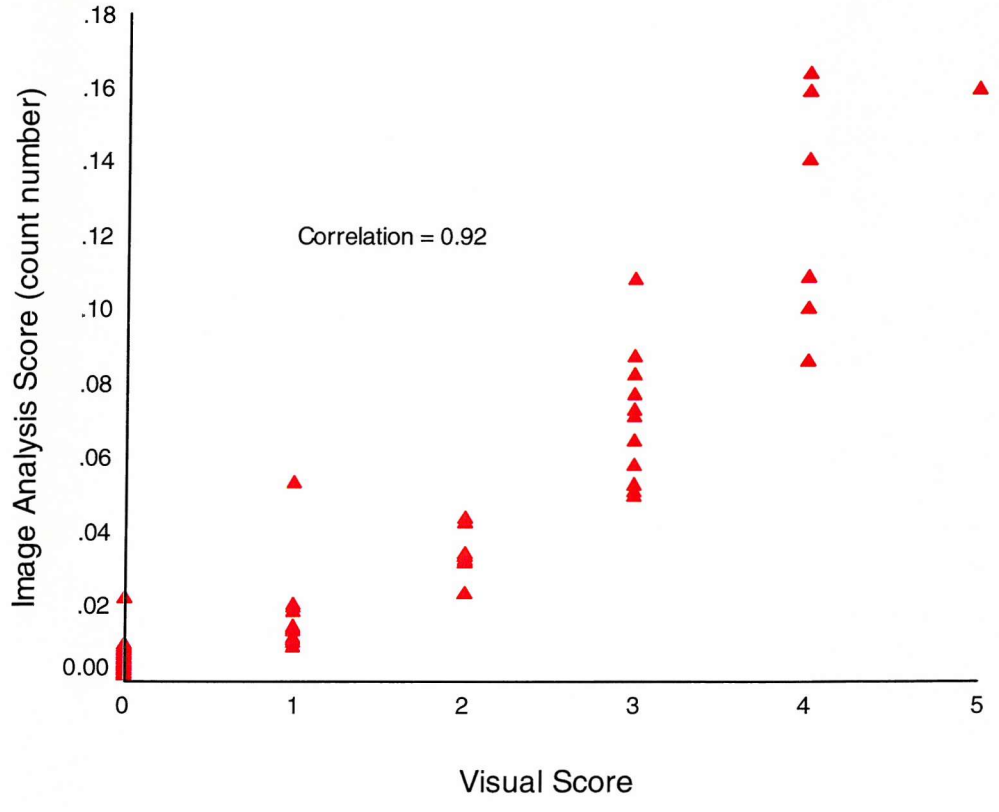
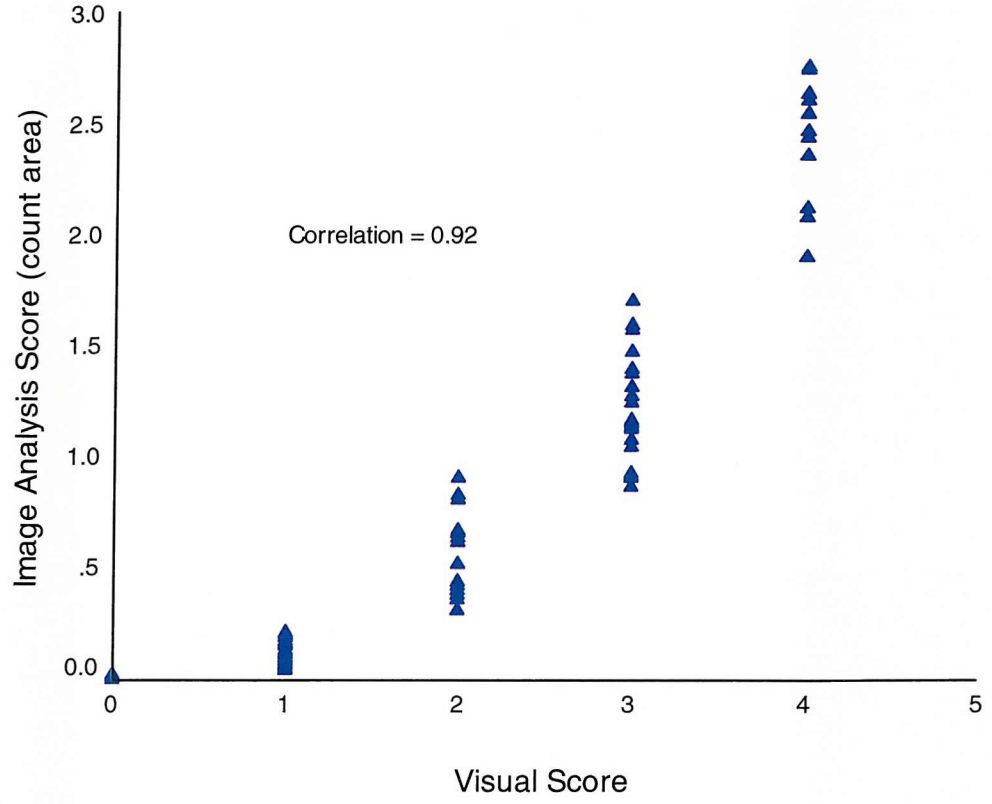


Figure 14. Scatter plots of digital image analysis scores versus visual scores for MMP-9. The image analysis scores are: count area (▲) which is the area of positive staining/total area x 100 and count number (▲) which is the number of positively stained areas/total area x 100.



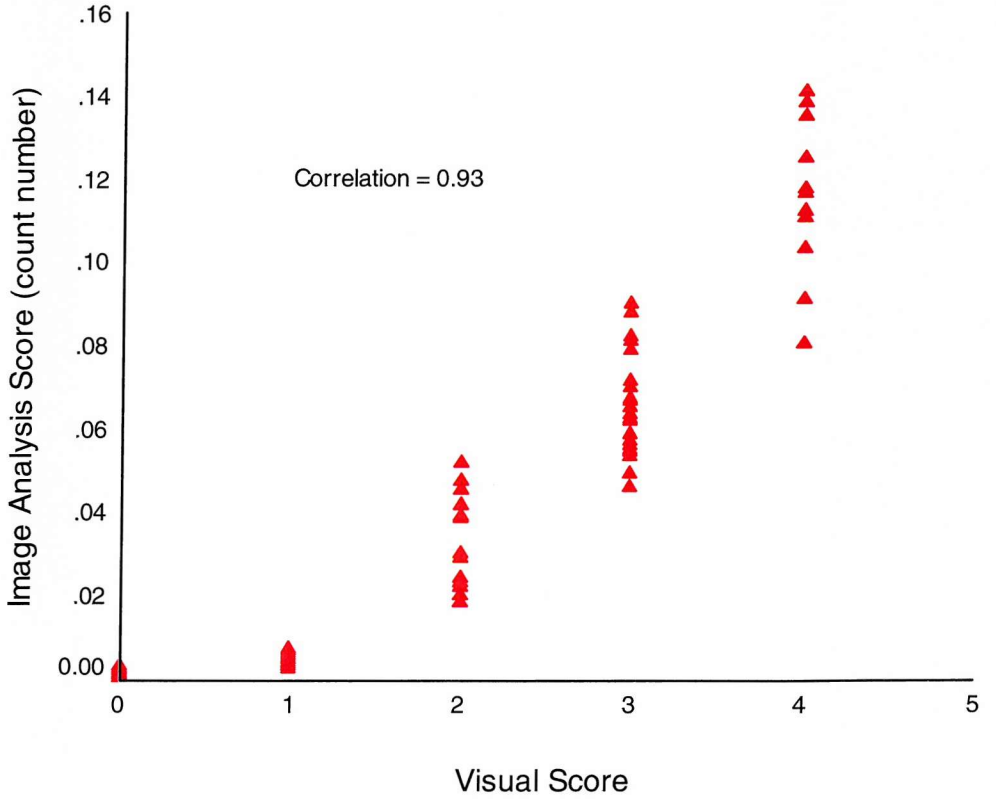


Figure 14. Scatter plots of digital image analysis scores versus visual scores for TIMP-1. The image analysis scores are: count area (▲) which is the area of positive staining/total area x 100 and count number (▲) which is the number of positively stained areas/total area x 100.

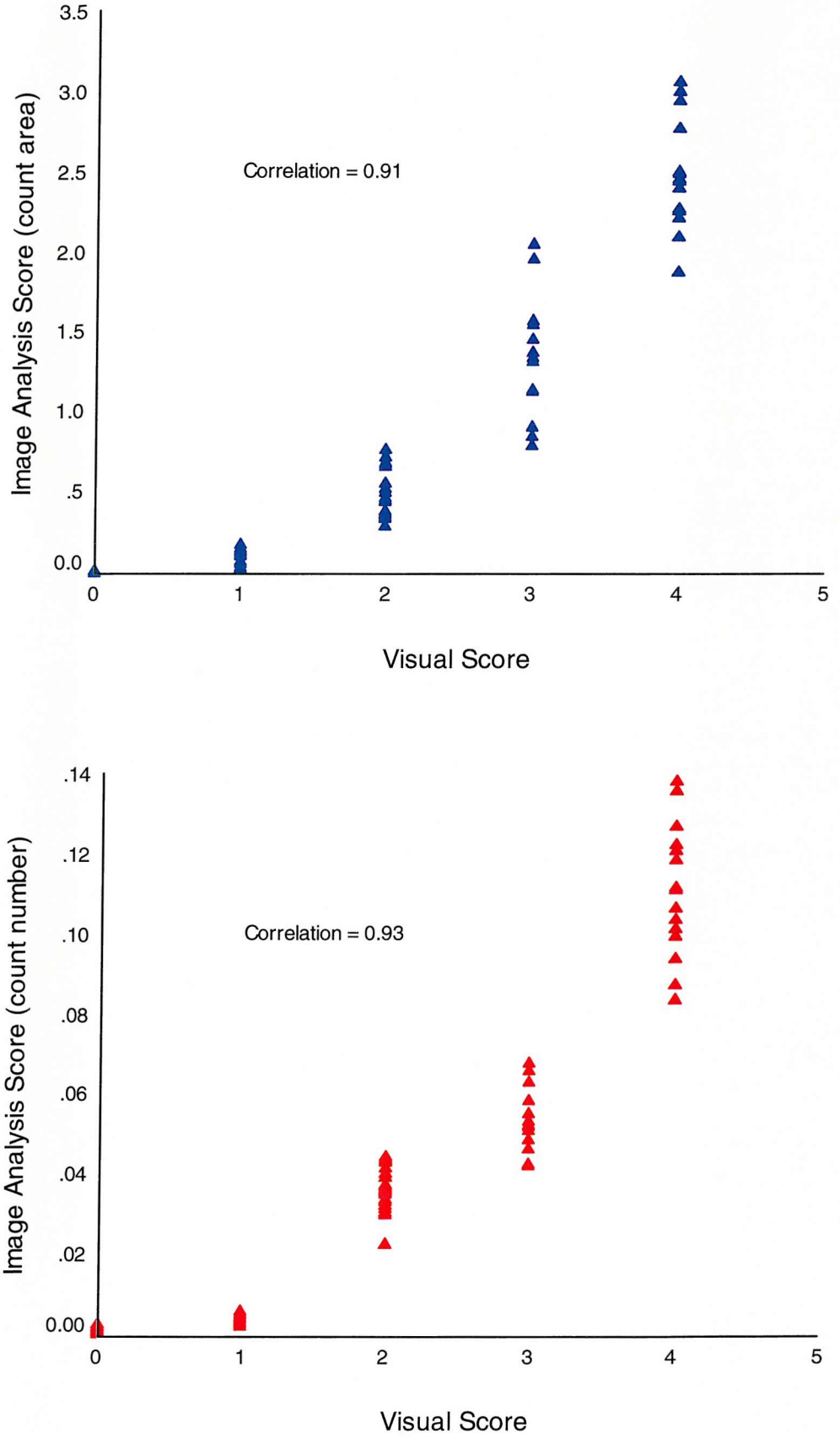
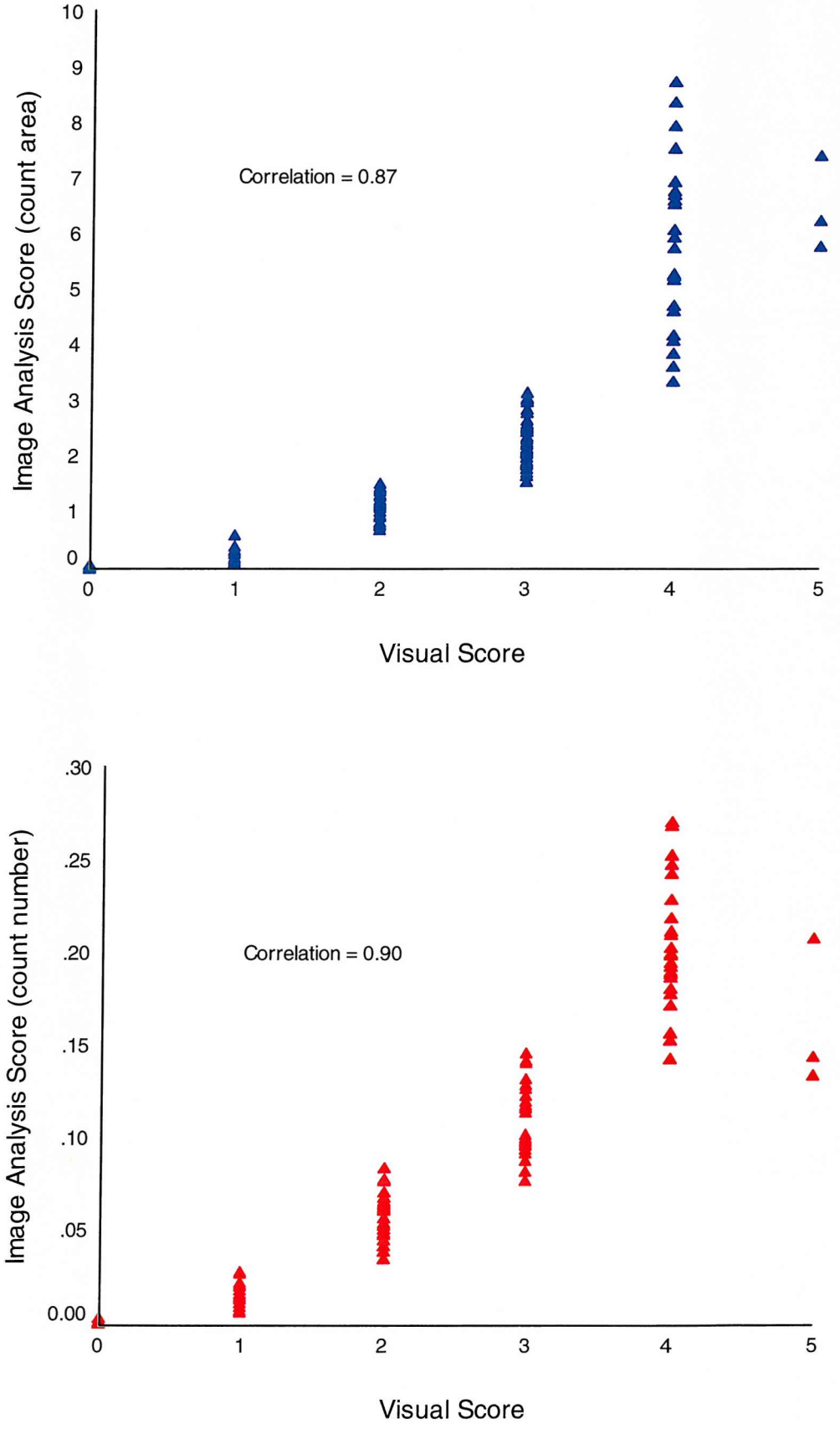


Figure 14. Scatter plots of digital image analysis scores versus visual scores for TIMP-2. The image analysis scores are: count area (▲) which is the area of positive staining/total area x 100 and count number (▲) which is the number of positively stained areas/total area x 100.



Statistical Analysis

Pattern of expression

The visual scores for all five antibodies were analysed to assess correlation between expression. Firstly, the visual scores for a single antibody were analysed to see if there was correlation in expression between the four areas of each slide e.g. did the values of MMP-1 expression in adjacent tumour for each patient correlate with the values of MMP-1 expression in adjacent liver for each patient (Table 9). Secondly the visual scores for each area of the slide were analysed to see if there was correlation between antibodies e.g. did the values of MMP-9 expression in adjacent tumour for each patient correlate with the values of TIMP-1 expression in adjacent tumour for each patient (Table 10).

When all four areas are considered for each antibody the only areas that correlate are adjacent tumour with distant tumour and adjacent liver with distant liver. This is true for each antibody with the exception of MMP-9 in the normal liver parenchyma where there is no correlation. The correlation values range from 0.55 to 1 with the strongest correlation being observed for MMP-1 (0.95 and 0.83) and MMP-7 (0.92 and 1.00).

The analysis between antibodies reveals very little correlation with only four values exceeding 0.50. The first of these is between the adjacent tumour of MMP-1 and MMP-9 that has a correlation coefficient of 0.78. Interestingly the other 3 values are all between MMP-9 and TIMP-1 in the distant tumour (0.50) and both normal liver parenchymal areas (0.74 and 0.56).

Table 9. Correlation figures between the four areas of each slide (adjacent & distant tumour and adjacent & distant liver) for each antibody

	MMP-1	MMP-7	MMP-9	TIMP-1	TIMP-2
	Adjacent Tumour				
Distant Tumour	0.95	0.92	0.63	0.55	0.69
Adjacent Liver	0.13	-0.07	0.14	0.33	0.40
Distant Liver	0.03	-0.07	-0.18	0.05	0.22
	Distant Tumour				
Adjacent Liver	0.17	-0.08	0.16	0.10	0.27
Distant Tumour	0.09	-0.08	-0.05	-0.04	0.18
	Adjacent Liver				
Distant Liver	0.83	1.00	0.25	0.57	0.60

Table 10. Correlation figures between the antibodies for each area of the slide

	Adjacent Tumour	Distant Tumour	Adjacent Liver	Distant Liver
	MMP-1			
MMP-7	-0.24	-0.21	0.10	-0.01
MMP-9	0.78	0.46	0.05	0.16
TIMP-1	-0.02	-0.03	0.05	0.02
TIMP-2	0.10	0.13	-0.07	0.10
	MMP-7			
MMP-9	-0.31	-0.21	-0.05	0.12
TIMP-1	0.03	-0.05	-0.04	-0.08
TIMP-2	0.08	0.12	-0.04	-0.02
	MMP-9			
TIMP-1	0.22	0.50	0.74	0.56
TIMP-2	0.12	0.12	0.00	-0.03
	TIMP-1			
TIMP-2	0.22	0.05	0.01	-0.13

Univariate analysis

The log-rank test was used to assess the significance of the clinicopathological variables involved in both the primary colorectal cancer and also the secondary liver metastases (Table 11).

Sex

The Kaplan-Meier survival curves for males and females demonstrate that up to 5 years the males have a slightly higher survival probability than the females, although after 5 years the survival curves cross and are very close together (Figure 15a). The associated log-rank test for differences in survival between males and females is not significant ($\chi^2 = 0.60$, df = 1, P = 0.437). The median survival time for males is 3.3 years (95% Confidence Interval (CI): 2.3 - 3.8 years) and the median survival time for females is 2.3 years (95% CI: 1.5 - 3.6 years).

Age

In order to produce survival plots and perform log-rank tests, the patients age at the liver surgery has been considered as a categorical variable split into approximately two equal groups: <60 years and ≥60 years. Kaplan-Meier survival curves for these age groups show that the curves are close together and cross after 5 years (Figure 15b). Up to 5 years those patients 60 years and over have a higher survival probability than those less than 60 years. The associated log-rank test for differences in the survival between the two age groups is not significant ($\chi^2 = 0.70$, df = 1, P = 0.401). The median survival for those patients under 60 years at the time of their liver surgery is 2.3 years (95% CI: 1.7 - 3.5 years). The median survival for those patients 60 years or over at the time of their liver surgery is 3.5 years (95% CI: 2.3 - 4.2 years).

Dukes' Stage

It might be expected that the relationship between Dukes' stage and survival would be ordered, with increasing Dukes' stage corresponding to decreasing probabilities of

survival. However, the Kaplan Meier curves show that this is not the case (Figure 15c). Up to two years, Dukes D corresponds to the highest survival curve, Dukes B the next highest, Dukes C1 the next and Dukes C2 is the lowest survival curve. After two years, Dukes A corresponds to the highest survival probabilities, Dukes B the next highest, followed by Dukes D, with Dukes C1 having the lowest survival probabilities. The curve corresponding to Dukes C2 starts as the lowest survival curve and crosses the other curves until it is the second highest survival curve. The associated log-rank test for differences in survival for the 5 Dukes' stages is significant at the 5% level (χ^2=9.82, df=4, P=0.044), however, because the results are not ordered this does not translate into a clinically significant result. This lack of an ordered pattern in the survival experience of each Dukes' stage might be because the survival times analysed here are from the time of surgery to remove liver metastases and so relate to the liver disease rather than the stage of the primary tumour at diagnosis. An alternative explanation is that patients undergoing hepatic resection are a highly selected group and not representative of the whole population of colorectal cancer patients. Moreover, this group of patients may be skewed by the use of adjuvant therapy for the primary disease.

Liver Tumour Diameter

The largest liver tumour nodule diameter has been categorized into two approximately equal groups: less or equal to 5cm and more than 5cm. The Kaplan Meier survival curves (Figure 15d) show a clear survival advantage for the smaller tumour diameter and this is significant at the 1% level (χ^2=6.64, df=1, P=0.010).

Adjuvant Therapy

The Kaplan Meier survival curves show that those patients who did not receive adjuvant therapy following liver resection have a higher chance of survival than those who did receive adjuvant therapy (Figure 15e). The associated log-rank test for differences in the survival functions was significant at the 5% level (χ^2=5.58, df=1, P=0.018). The median survival time is 1.7 years (95% CI: 1.4 - 2.8 years) for those patients who did have adjuvant therapy and 3.5 years (95% CI: 2.4 - 4.2 years) for those who did not. The most likely explanation is that adjuvant therapy would have

been given to the patients who were thought clinically to have the most advanced disease and therefore likely to have a poorer outcome.

Additional Therapy

The Kaplan Meier survival curves for additional therapy are very similar up to about 2 years, and then those patients who did not receive additional therapy have a better survival (Figure 15f). The associated log-rank test for differences in survival is highly significant (χ^2=7.02, df=1, P=0.008). The median survival is 2.3 years (95% CI: 1.7 - 3.3 years) for those patients who did receive additional therapy and 3.6 years (95% CI: 2.4 - 5.3 years) for those who did not. It is not surprising that additional therapy is associated with a significantly worse outcome, since the patients most likely to be given additional therapy have recurrent disease.

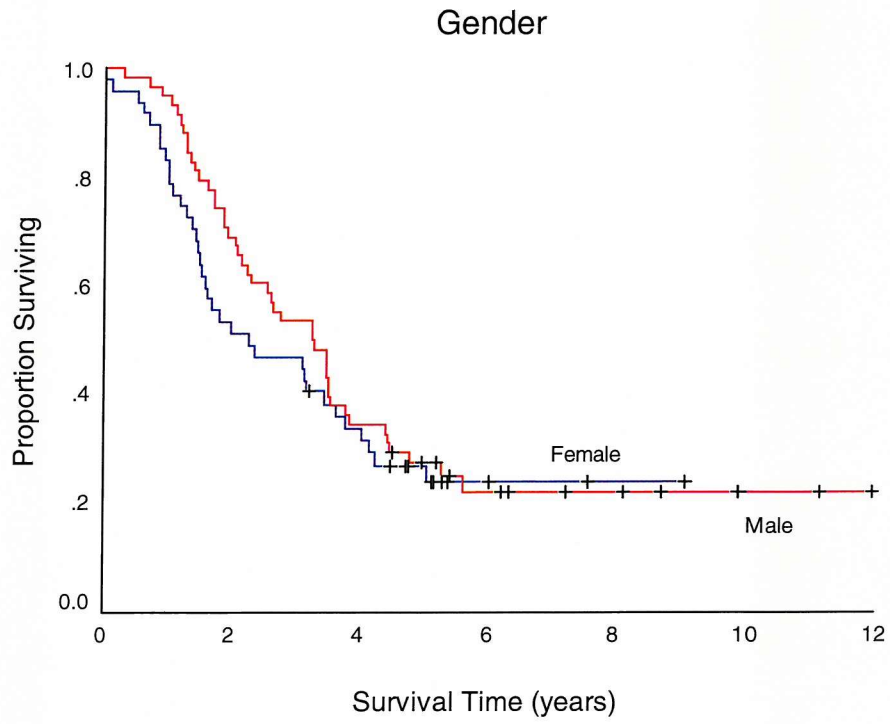
Blood Transfusion

The Kaplan Meier survival curves for those patients receiving a blood transfusion either during or after hepatic surgery show that those patients who did not have a blood transfusion have a much higher survival than those who did (Figure 15g). The associated log-rank test is significant at the 10% level but not significant at the 5% level (χ^2=3.60, df=1, P=0.058). The median survival for those patients that did receive a blood transfusion is 1.8 years (95% CI: 1.4 - 2.6 years) and for those that did not receive a blood transfusion is 3.3 years (95% CI: 2.3 - 3.6 years). None of the patients died as a direct result of blood loss and therefore the most likely explanation of this finding is a reduction in the immune capability following a transfusion.

Table 11. Statistics for univariate analysis of clinicopathological variables (n = number and DF = degrees of freedom)

Variable		n	Percentage Surviving			χ^2 Statistic	DF	P value
			1 yr	3 yrs	5 yrs			
Sex	Male	58	95	53	21	0.60	1	0.437
	Female	47	83	47	19			
Age	<60	50	84	42	18	0.70	1	0.401
	60+	55	95	58	22			
Dukes' Stage	A	4	100	75	25	9.82	4	0.044
	B	22	91	64	23			
	C1	24	79	29	13			
	C2	5	80	60	40			
	D	50	94	52	20			
Synchronous Metastases	Yes	48	94	54	21	0.04	1	0.842
	No	57	86	47	19			
Primary Operation Curative	Yes	100	89	50	20	0.05	1	0.826
	No	5	100	60	20			
Adjuvant Therapy (Primary)	Yes	27	81	44	15	1.95	1	0.162
	No	78	92	53	22			
Blood Transfusion (Liver)	Yes	24	83	29	17	3.60	1	0.058
	No	81	91	57	21			
Liver Tumour Diameter	<5 cm	53	96	57	35	6.64	1	0.010
	>5 cm	52	82	43	20			
Number of Nodules	1	48	85	52	23	5.01	3	0.171
	2	23	91	70	22			
	3	18	94	39	17			
	>3	16	94	31	13			
Adjuvant Therapy (Liver)	Yes	29	79	31	14	5.58	1	0.018
	No	76	93	57	22			
Additional Therapy (Liver)	Yes	51	87	41	10	7.02	1	0.008
	No	54	93	59	30			

Figure 15a & 15b. Kaplan Meier survival curves for gender and age (+ censored values)



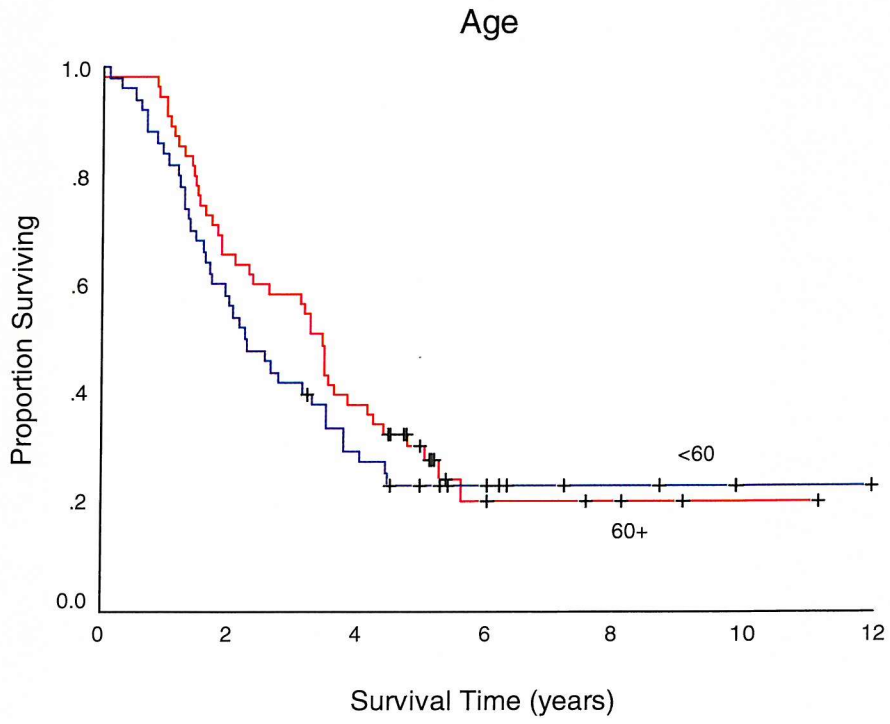
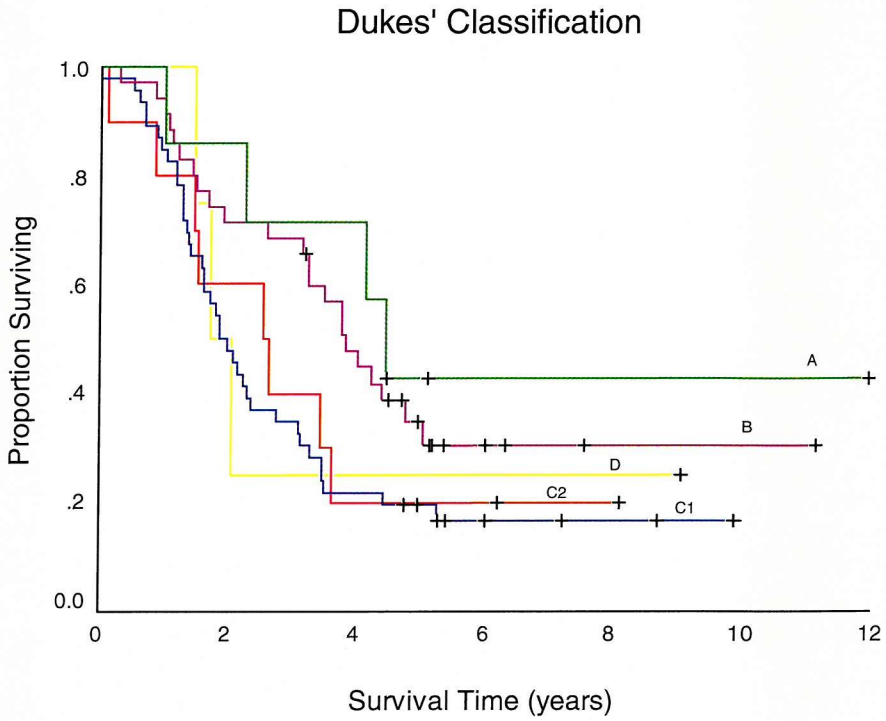


Figure 15c & 15d. Kaplan Meier survival curves for Dukes' classification and tumour diameter (+ censored values)



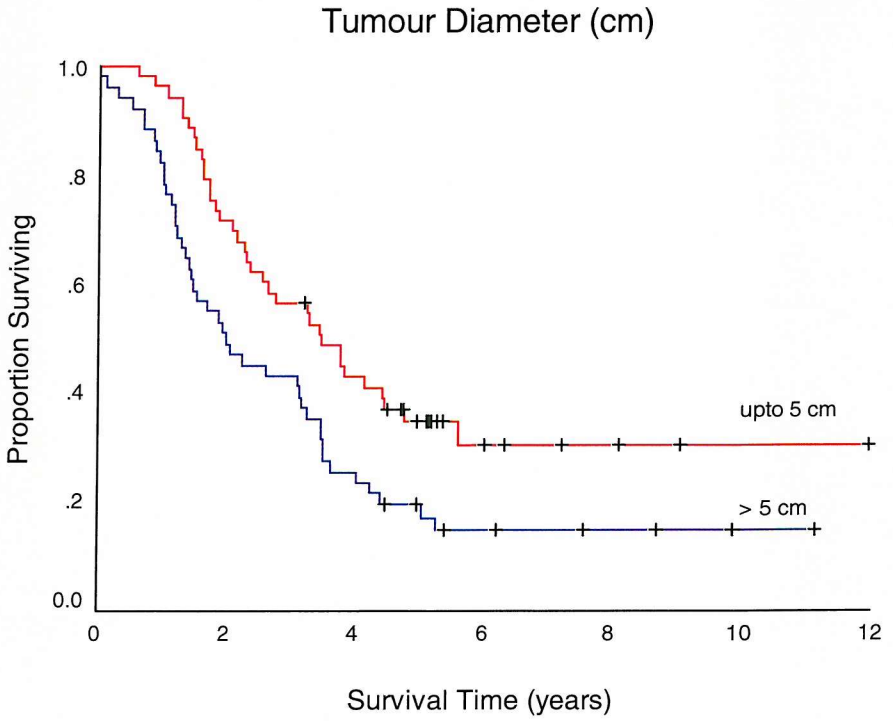
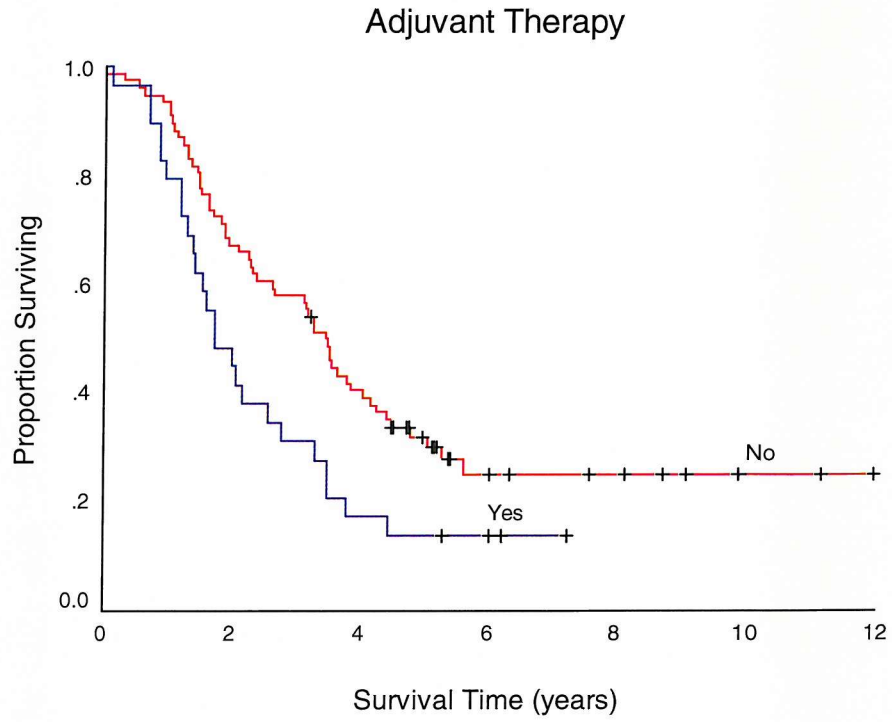


Figure 15e & 15f. Kaplan Meier survival curves for adjuvant therapy and additional therapy (+ censored values)



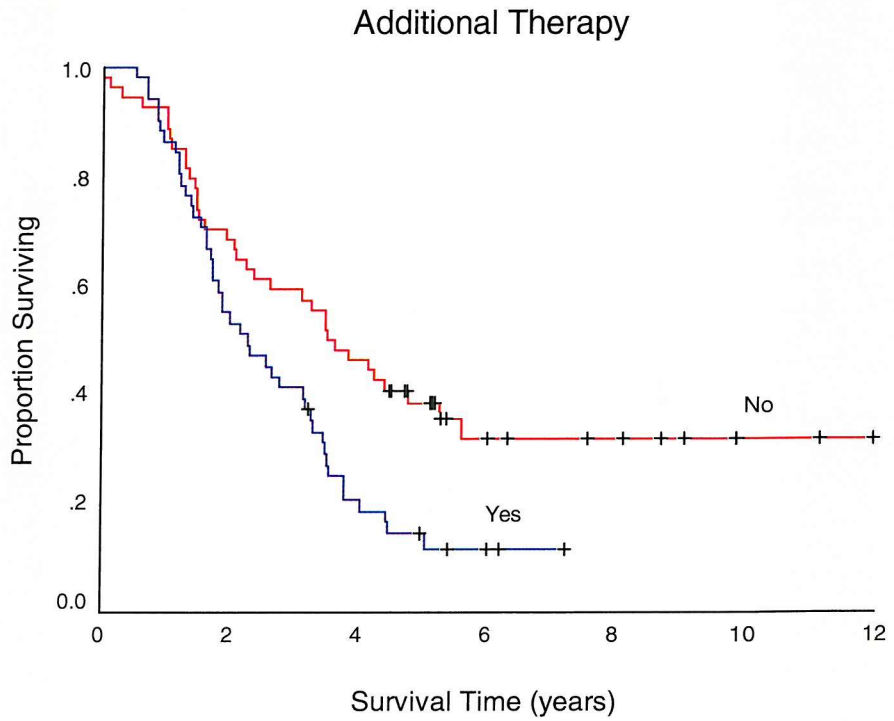
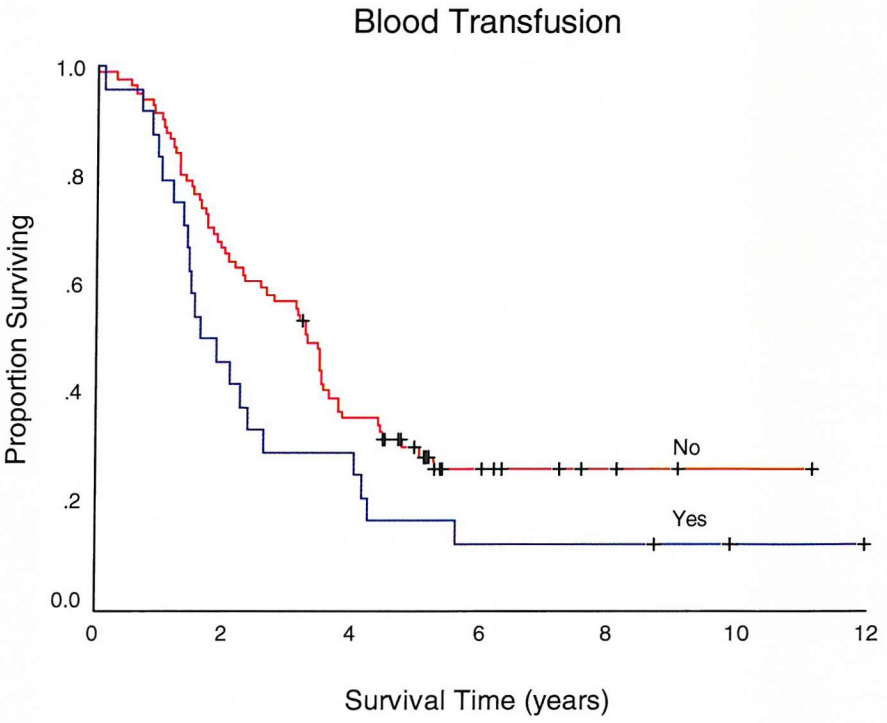


Figure 15g. Kaplan Meier survival curve for blood transfusion (+ censored values)



Enzymes

The log-rank test was also used to assess the three matrix metalloproteinases and two tissue inhibitors of matrix metalloproteinases individually. Each enzyme was assessed to see if there was a difference in survival between the levels of expression. The four different areas of the slide were considered for each enzyme. There was no survival difference between the levels of expression for the matrix metalloproteinases, however, there were significant differences for the expression of tissue inhibitors of matrix metalloproteinases in the tumour (Table 12). Tissue inhibitor of matrix metalloproteinase –1 showed significant differences in survival between the levels of expression for both adjacent tumour (χ^2=10.27, df=3, P=0.016) and distant tumour (χ^2=8.98, df=3, P=0.030). Similarly tissue inhibitor of matrix metalloproteinase –2 also showed significant differences for both adjacent tumour (χ^2=12.48, df=3, P=0.029) and distant tumour (χ^2=14.40, df=3, P=0.013).

Table12. Summary of log-rank test for MMP-1 with 1,3 and 5 year survival rates (DF = degrees of freedom)

Area	Score (No. of patients)		Percentage Surviving 1 yr	3 yrs	5 yrs	χ^2 Statistic	DF	p value
Adjacent	**0**	(n=80)	88	46	20			
Tumour	**1**	(n= 4)	100	50	25			
	2	(n= 6)	100	67	17	0.76	5	0.980
	3	(n= 7)	86	57	29			
	4	(n= 6)	100	67	17			
	5	(n= 2)	100	100	0			
Distant	**0**	(n=77)	87	45	18			
Tumour	**1**	(n= 9)	100	44	33			
	2	(n= 7)	100	71	14	2.07	5	0.839
	3	(n= 8)	88	63	38			
	4	(n= 2)	100	100	0			
	5	(n= 2)	100	100	0			
Adjacent	**0**	(n= 0)	-	-	-			
Liver	**1**	(n=15)	60	40	27			
	2	(n=54)	96	61	22	4.58	3	0.205
	3	(n=31)	90	39	16			
	4	(n= 5)	100	40	0			
	5	(n= 0)	-	-	-			
Distant	**0**	(n= 1)	100	100	100			
Liver	**1**	(n=18)	67	44	22			
	2	(n=65)	95	55	18	4.60	3	0.203
	3	(n=21)	90	38	19			
	4	(n= 0)	-	-	-			
	5	(n= 0)	-	-	-			

Table12 (cont). Summary of log-rank test for MMP-7 with 1,3 and 5 year survival rates (DF = degrees of freedom)

Area	Score (No. of patients)		Percentage Surviving 1 yr	3 yrs	5 yrs	χ^2 Statistic	DF	p value
Adjacent	0	(n=63)	87	49	21			
Tumour	1	(n=17)	88	53	29			
	2	(n= 7)	86	71	0	4.94	5	0.423
	3	(n=11)	100	45	18			
	4	(n= 6)	100	50	17			
	5	(n= 1)	100	0	0			
Distant	0	(n=51)	88	49	20			
Tumour	1	(n=21)	90	52	33			
	2	(n= 8)	88	75	0	5.38	5	0.371
	3	(n=16)	88	44	19			
	4	(n= 8)	100	50	13			
	5	(n= 1)	100	0	0			
Adjacent	0	(n= 0)	-	-	-			
Liver	1	(n= 0)	-	-	-			
	2	(n=104)	89	50	20	1.32	1	0.250
	3	(n= 1)	100	100	0			
	4	(n= 0)	-	-	-			
	5	(n= 0)	-	-	-			
Distant	0	(n= 0)	-	-	-			
Liver	1	(n= 0)	-	-	-			
	2	(n=104)	89	50	20	1.32	1	0.250
	3	(n= 1)	100	100	0			
	4	(n= 0)	-	-	-			
	5	(n= 0)	-	-	-			

Table12 (cont). Summary of log-rank test for MMP-9 with 1,3 and 5 year survival rates (DF = degrees of freedom)

Area	Score (No. of patients)		Percentage Surviving 1 yr	3 yrs	5 yrs	χ^2 Statistic	DF	p value
Adjacent Tumour	0	(n=67)	93	52	21	3.65	4	0.456
	1	(n=19)	79	32	16			
	2	(n=10)	80	60	20			
	3	(n= 7)	100	57	29			
	4	(n= 2)	100	100	0			
	5	(n= 0)	-	-	-			
Distant Tumour	0	(n=73)	90	49	22	1.79	4	0.774
	1	(n=19)	84	42	11			
	2	(n= 9)	89	67	11			
	3	(n= 3)	100	67	67			
	4	(n= 1)	100	100	0			
	5	(n= 0)	-	-	-			
Adjacent Liver	0	(n=28)	93	61	21	0.81	4	0.937
	1	(n=28)	93	54	21			
	2	(n=15)	93	47	13			
	3	(n=23)	83	39	13			
	4	(n=11)	82	45	36			
	5	(n= 0)	-	-	-			
Distant Liver	0	(n=60)	88	53	23	0.72	4	0.949
	1	(n=24)	92	42	29			
	2	(n=10)	90	30	0			
	3	(n=10)	90	70	0			
	4	(n= 1)	100	100	0			
	5	(n= 0)	-	-	-			

Table12 (cont). Summary of log-rank test for TIMP-1 with 1,3 and 5 year survival rates (DF = degrees of freedom)

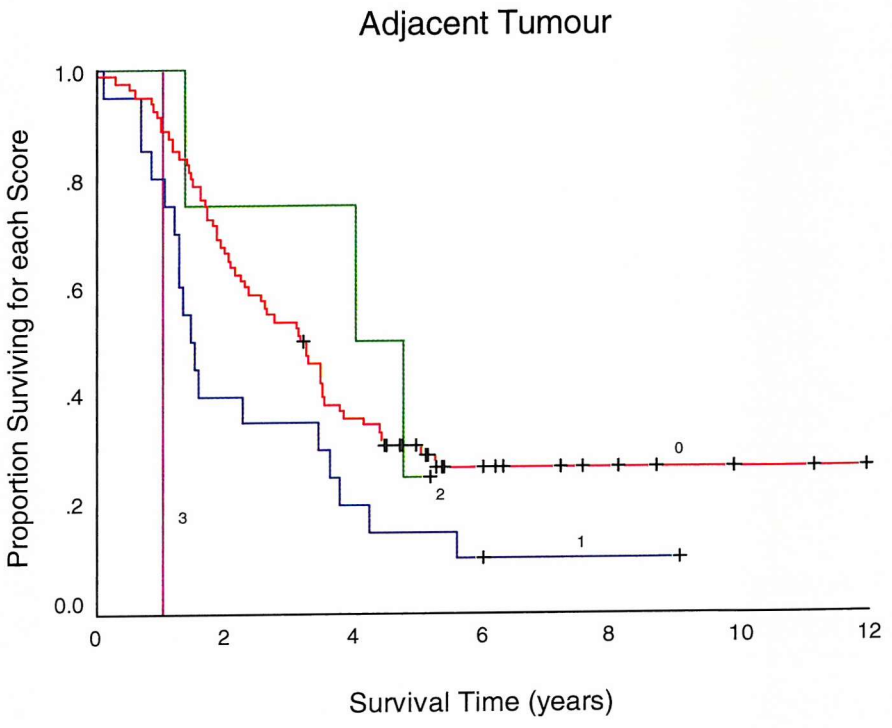
Area	Score (No. of patients)		Percentage Surviving 1 yr	3 yrs	5 yrs	χ^2 Statistic	DF	p value
Adjacent	**0**	(n=80)	91	54	31			
Tumour	**1**	(n=20)	80	35	15			
	2	(n= 4)	100	75	25			
	3	(n= 1)	100	0	0	10.27	3	0.016
	4	(n= 0)	-	-	-			
	5	(n= 0)	-	-	-			
Distant	**0**	(n=79)	92	51	27			
Tumour	**1**	(n=17)	82	47	23			
	2	(n= 8)	88	63	38			
	3	(n= 1)	0	0	0	8.98	3	0.030
	4	(n= 0)	-	-	-			
	5	(n= 0)	-	-	-			
Adjacent	**0**	(n=30)	93	60	30			
Liver	**1**	(n=24)	96	54	4			
	2	(n=23)	87	39	17			
	3	(n=13)	92	62	23	4.70	4	0.320
	4	(n=15)	73	33	27			
	5	(n= 0)	-	-	-			
Distant	**0**	(n=55)	93	55	24			
Liver	**1**	(n=21)	86	48	19			
	2	(n=21)	86	43	19			
	3	(n= 5)	100	40	0	3.77	4	0.439
	4	(n= 3)	67	67	0			
	5	(n= 0)	-	-	-			

Table12 (cont). Summary of log-rank test for TIMP-2 with 1,3 and 5 year survival rates (DF = degrees of freedom)

Area	Score (No. of patients)		Percentage Surviving 1 yr	3 yrs	5 yrs	χ^2 Statistic	DF	p value
Adjacent	**0**	(n= 3)	100	67	33			
Tumour	**1**	(n=16)	88	56	44			
	2	(n=24)	88	54	21			
	3	(n=33)	94	42	21	12.48	5	0.029
	4	(n=26)	88	58	32			
	5	(n= 3)	67	0	0			
Distant	**0**	(n= 7)	100	86	57			
Tumour	**1**	(n=16)	88	56	8			
	2	(n=22)	91	45	14			
	3	(n=39)	87	38	22	14.40	5	0.013
	4	(n=19)	95	68	34			
	5	(n= 2)	50	0	0			
Adjacent	**0**	(n=82)	89	52	22			
Liver	**1**	(n=17)	100	47	18			
	2	(n= 3)	67	33	0			
	3	(n= 2)	50	50	0	2.70	4	0.609
	4	(n= 1)	100	0	0			
	5	(n= 0)	-	-	-			
Distant	**0**	(n=101)	89	51	22			
Liver	**1**	(n= 3)	100	67	33			
	2	(n= 0)	-	-	-			
	3	(n= 0)	-	-	-	1.76	2	0.414
	4	(n= 1)	100	0	0			
	5	(n= 0)	-	-	-			

However, as the Kaplan-Meier survival curves show, there is no obvious clinical significance as the curves for TIMP-1 (Figure 16a & 16b) and TIMP-2 (Figure 16c & 16d) have a random order with no survival advantage to either low or high expression. When the results for TIMP-1 adjacent tumour are re-analysed comparing no staining (score 0) against staining (scores 1-5) there is a difference that suggests a survival advantage for patients who do not express TIMP-1 (Figure 17). The associated log-rank test for the differences in survivor functions is significant at the 10 per cent level but not quite at the 5 per cent significance level (χ^2=3.54, df=1, P=0.060). The median survival is 3.2 years (95% CI: 2.3 – 4.1 years) for those patients who do not express TIMP-1 in adjacent tumour and 1.54 years (95% CI: 1.16 – 1.92 years) for those who do express TIMP-1 in adjacent tumour. Although not reaching conventional significance levels the confidence intervals for median survival would suggest that TIMP-1 expression in the adjacent tumour probably does have an important role in determining survival.

Figure 16a & 16b. Kaplan-Meier survival curves for TIMP-1 (+ censored values)



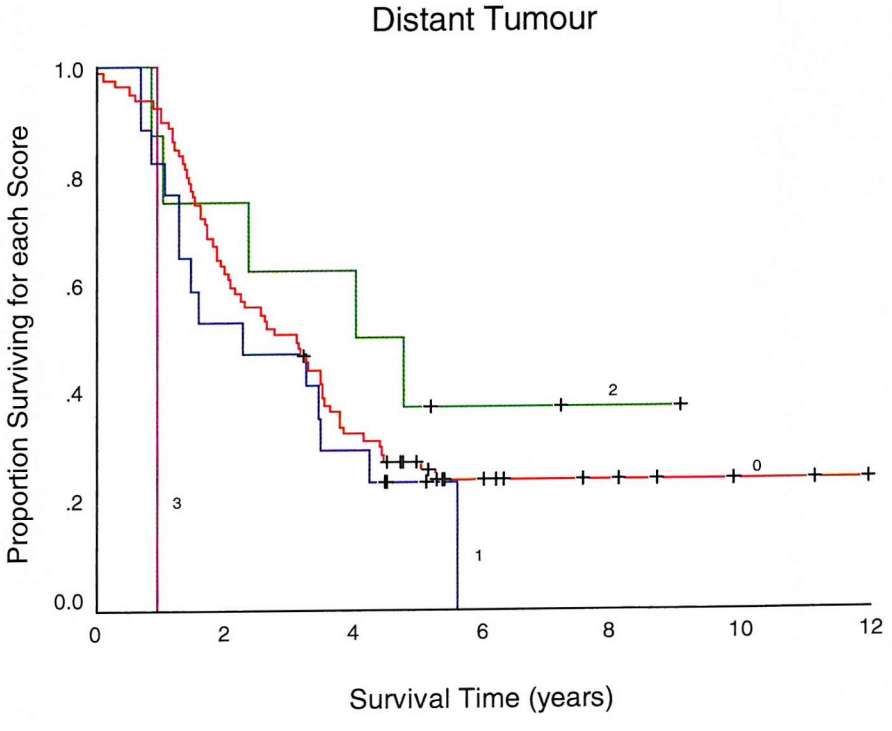
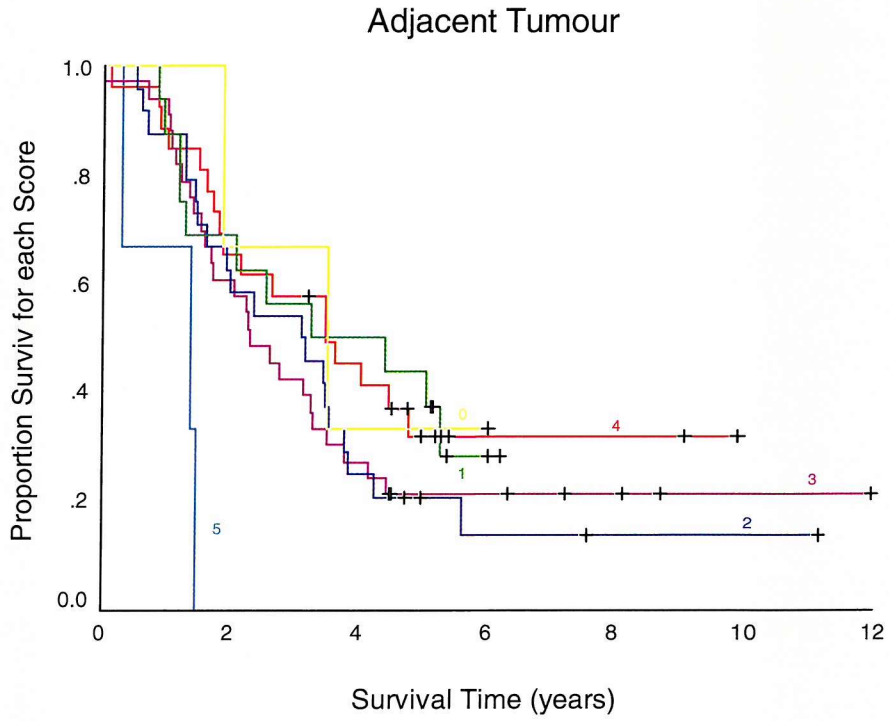


Figure 16c & 16d. Kaplan-Meier survival curves for TIMP-2 (+ censored values)



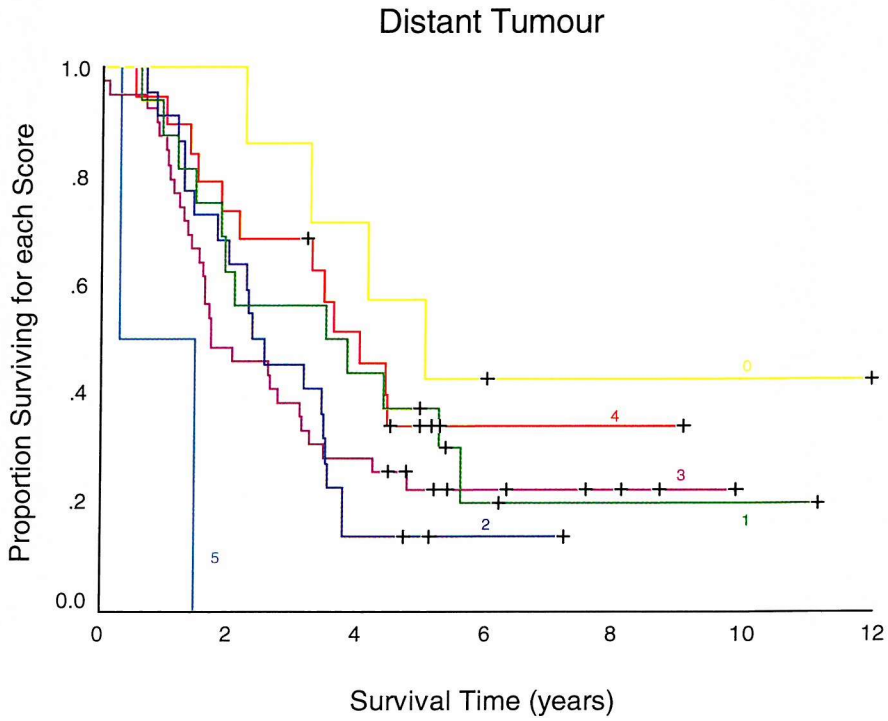
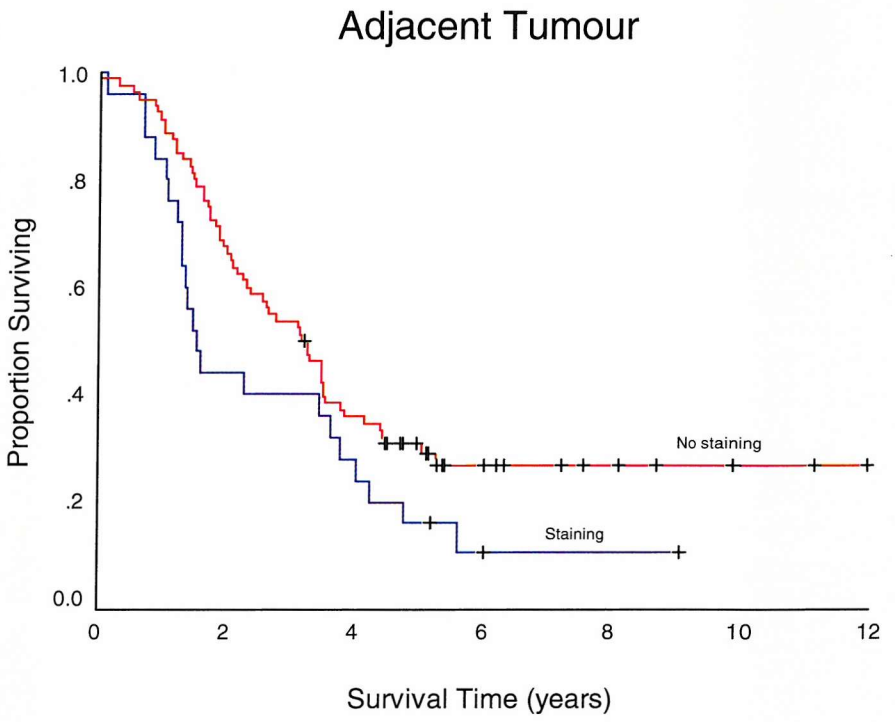


Figure 17. Kaplan-Meier survival curves for TIMP-1 comparing staining versus no staining (+ censored values)



Expression of single enzymes on their own gave no clinically significant survival advantage to any one group. However, there was a suggestion that TIMP-1 expression in the adjacent tumour may correlate with reduced survival. It is probably unrealistic to believe that an enzyme in isolation would provide all the answers and it is more likely that combinations of enzymes are important. To investigate this further the scores for TIMP-1 in adjacent tumour were analysed with those for MMP-1, -7 and –9 both in tumour and liver. When combining the score for TIMP-1 in adjacent tumour and the score for MMP-1 in adjacent liver a clinically significant pattern of survival emerges (Table13). The lower combined scores have a significant survival advantage over the higher combined scores (χ^2=10.80, df=4, P=0.029) and this is appreciated more readily in the Kaplan-Meier survival curves (Figure 18).

The survival advantage seen in the lower combined scores only remains clinically relevant if the influence of each enzyme changes linearly across the range of combined scores. This is readily seen to be the case in Table 14, which shows that as the combined score increases so does the proportion and value of the TIMP-1 input. This suggests therefore that high expression of TIMP-1 in the adjacent tumour combined with high expression of MMP-1 in the adjacent liver is associated with a worse outcome.

Table 13. Summary of log-rank test for combined scores of TIMP-1 in adjacent tumour and MMP-1 in adjacent liver

Combined Score	n	Percentage surviving			χ^2 Statistic	DF	p-value
		1 yr	3 yrs	5 yrs			
1	12	67	50	33			
2	43	93	58	32			
3	34	91	44	29	10.80	4	0.029
4	13	92	54	7			
5	3	100	33	0			

Figure 18. Kaplan-Meier survival curves for the combined scores of TIMP-1 in adjacent tumour and MMP-1 in adjacent liver (+ censored values)

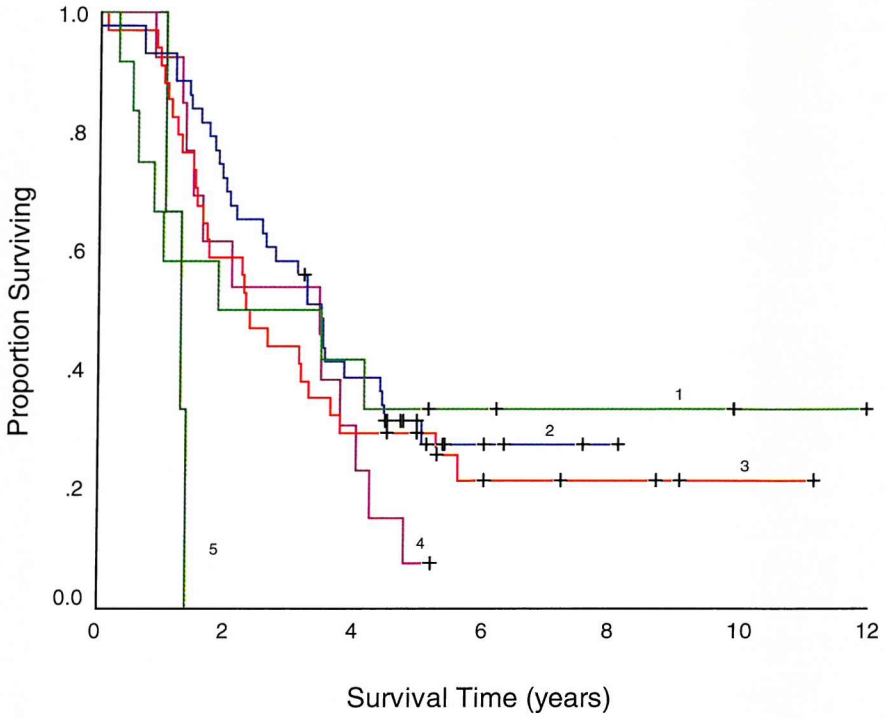


Table 14. TIMP-1 contribution to the combined scores (n = number of patients)

Combined Score	Number of Patients	Contribution of TIMP-1		% of Combined Scores that Include Contribution from TIMP-1
		n	Score	
1	12	0	-	0
2	43	3	1	7
3	34	10	1	29
4	13	6	1	69
		3	2	
5	3	1	1	100
		1	2	
		1	3	

Multivariate Analysis

A Cox Proportional Hazards model was fitted for TIMP-1 (adjacent tumour) alone, TIMP-1 (adjacent tumour) comparing expression versus no expression, and then lastly the combined TIMP-1 (adjacent tumour) and MMP-7 (adjacent liver). Only these models have been produced as the log-rank test showed that these three data sets were the only clinically significant ones. Although TIMP-2 was statistically significant this could not be converted into clinical significance and so is not considered here. A univariate Cox Proportional Hazards model was fitted for each of these two sets of variables in order to assess their impact on survival. This model is described as the unadjusted model. Models adjusting for age and sex, and age, sex, Dukes' stage, tumour diameter, adjuvant therapy and additional therapy were then fitted. This allowed examination of the impact of the enzymes in the presence of the other prognostic factors identified by the log-rank test.

Cox Proportional Hazards Model for TIMP-1

The results for the Cox Proportional Hazards model for TIMP-1 adjacent tumour is shown (Table 13). The unadjusted analysis assessing the effect of TIMP-1 at the adjacent tumour achieves significance at the 10% level (P=0.082). When the impact of TIMP-1 at the adjacent tumour is assessed in the presence of age and sex, the effect on survival becomes non-significant at the 10% level with the P value increasing (P=0.180). The estimates of the hazard ratios and their 95% confidence intervals for the unadjusted analysis and the analysis adjusting for age and sex are very similar. When the impact of TIMP-1 at the adjacent tumour is assessed in the presence of age, sex, Dukes' stage, tumour diameter, adjuvant therapy and additional therapy the effect of TIMP-1 on survival becomes significant at the 0.1% level (P=0.001). The hazard ratios estimated from this model are greater than the hazard ratios for the other two models. Again, the relationship between the levels of TIMP-1 and survival does not appear to be linear. The reference category is those patients not expressing TIMP-1 (score 0). Those patients with a score of 1 have a relative hazard that is 3.12 times that for those patients with a score of 0. Patients with a score of 2 have the lowest risk with a hazard that is 9% more than that for patients with a score of 0 and the patient

with a score of 3 has the highest risk with a relative hazard of 12.39. As we would expect the five-year survival rates decrease as the hazard increases, with those patients with a score of 0 having the highest survival rate and the lowest hazard ratio. It should be noted that the significant result here should be treated with caution, as there are only 4 subjects with a score of 2 and only a single patient with a score of 3. The corresponding Kaplan-Meier curves seen previously show the same pattern of survival for the four categories of enzyme levels and the associated log-rank test achieves significance at the 5% level (log-rank P value is 0.016).

Table13. Cox Proportional Hazards Model for TIMP-1 (adjacent tumour)

Score	N	5 year survival (95% CI)	Unadjusted	Adjusted for age and sex	Adjusted for age, sex, Dukes' stage, tumour diameter, adjuvant & additional therapy
			Relative Hazard (95% CI)	Relative Hazard (95% CI)	Relative Hazard (95% CI)
0	80	30.8% (21.0- 41.1%)	1.00	1.00	1.00
1	20	15.0% (3.7- 33.5%)	1.80 (1.06, 3.06)	1.75 (1.00, 3.05)	3.12 (1.65, 5.90)
2	4	25.0% (0.8- 58.2%)	0.84 (0.26, 2.67)	0.85 (0.27, 2.72)	1.09 (0.31, 3.79)
3	1	0	8.52 (1.11, 65.5)	8.03 (1.03, 62.7)	12.39 (1.35, 113)
P value for each model			0.082	0.180	0.001

Cox Proportional Hazards Model for TIMP-1 comparing expression versus no expression in adjacent tumour

The results of the Cox Proportional Hazards model comparing expression versus no expression of TIMP-1 in adjacent tumour are shown (Table 14). The unadjusted analysis assessing the effect of expression of TIMP-1 at the adjacent tumour achieves significance at the 10% level (P=0.072). When the impact of TIMP-1 at the adjacent tumour is assessed in the presence of age and sex, the effect on survival becomes non-significant at the 10% level with the P value increasing (P=0.215). The estimates of the hazard ratios and their 95% confidence intervals for the unadjusted analysis and the analysis adjusting for age and sex are very similar. When the impact of expression of TIMP-1 at the adjacent tumour is assessed in the presence of age, sex, Dukes' stage, tumour diameter, adjuvant therapy and additional therapy the effect of TIMP-1 on survival becomes significant at the 0.1% level (P=0.001). The hazard ratios estimated from this model are greater than the hazard ratios for the other two models. The reference category is those patients not expressing TIMP-1 (score 0). Those patients who express TIMP-1 in the adjacent tumour have a relative hazard that is 2.36 times that for those patients who do not express TIMP-1 in the adjacent tumour. The five-year survival rate for patients who do not express TIMP-1 (30.8%) is almost twice that of patients who do express TIMP-1 (16.0%). The corresponding Kaplan-Meier curves seen previously show the same pattern of survival when comparing the presence or absence of staining and the associated log-rank test almost achieves significance at the 5% level (log-rank P value is 0.060).

Table 14. Cox Proportional Hazards model for TIMP-1 comparing expression versus no expression in adjacent tumour

Stain	N	5 year survival (95% CI)	Unadjusted	Adjusted for age and sex	Adjusted for age, sex, Dukes' stage, tumour diameter, adjuvant & additional therapy
			Relative Hazard (95% CI)	Relative Hazard (95% CI)	Relative Hazard (95% CI)
No	80	30.8% (- %)	1.00	1.00	1.00
Yes	25	16.0% (-%)	1.60 (0.98 – 2.62)	1.60 (0.94 – 2.61)	2.36 (1.33 – 4.18)
P value for each model			0.072	0.215	0.001

Cox Proportional Hazards Model for TIMP-1 and MMP-1

The scores for TIMP-1 adjacent tumour and MMP-1 adjacent liver were combined as in the univariate analysis. The results of the Cox Proportional Hazards model are shown (Table 15). The unadjusted analysis assessing the effect of combining TIMP-1 at the adjacent tumour and MMP-1 at the adjacent liver does not achieve significance at the 10% level (P=0.160). When the impact of this combined score is assessed in the presence of age and sex, the effect on survival decreases further with the P value increasing (P=0.276). The estimates of the hazard ratios and their 95% confidence intervals for the unadjusted analysis and the analysis adjusting for age and sex are very similar. When the impact of the combined score is assessed in the presence of age, sex, Dukes' stage, tumour diameter, adjuvant therapy and additional therapy the effect of TIMP-1 at the adjacent tumour and MMP-1 at the adjacent liver on survival becomes significant at the 5% level (P=0.021). The hazard ratios estimated from this model are greater than the hazard ratios for the other two models. The relationship between the combined scores and survival appear to be linear. The reference category is those patients with a score of 2. Those patients with a score of 1 have a relative hazard that is 68% less than those patients with a score of 2. Patients with scores 3-5 have increasing relative hazards compared to the reference category (43%, 65% and 5.12 times respectively). As we would expect the five-year survival rates decrease as the hazard increases, with those patients with a score of 1 having the highest survival rate and the lowest hazard ratio. It should be noted that the significant result here should be treated with caution as there are only 3 subjects with a score of 5 and the confidence intervals are wide for each result. The corresponding Kaplan-Meier curves seen previously show the same pattern of survival for the five categories of enzyme levels and the associated log-rank test achieves significance at the 5% level (log-rank P value is 0.029).

The log-rank associated survivor functions for the interaction between levels of TIMP-1 at the adjacent tumour and MMP-1 at the adjacent liver was found to have a significant association with survival (P=0.029). However, this interaction does not achieve significance for the unadjusted Cox Proportional Hazards model (P=0.160). This is likely to be because there is insufficient data to demonstrate a significant relationship using the Cox Proportional Hazards model. The fact that there is a

difference between the P values for the log-rank and Cox proportional hazards model means that the association between this interaction and survival is tenuous and no firm conclusions can be drawn.

The confidence intervals around the relative hazards for all enzymes (Table 14) are very wide, suggesting that there are insufficient numbers of patients in each category to detect significant differences in survival.

Table 15. Cox Proportional Hazards model for TIMP-1 (adjacent tumour) and MMP-1 (adjacent liver)

Score	n	5 year survival (95% CI)	Unadjusted	Adjusted for age and sex	Adjusted for age, sex, Dukes' stage, tumour diameter, adjuvant & additional therapy
			Relative Hazard (95% CI)	Relative Hazard (95% CI)	Relative Hazard (95% CI)
1	12	33.3% (6.7- 60.0%)	0.89 (0.51, 2.42)	0.91 (0.50, 2.40)	0.32 (0.20, 4.06)
2	43	31.6% (17.5- 45.6%)	1.00	1.00	1.00
3	34	29.4% (14.1- 44.7%)	1.26 (0.74, 2.13)	1.23 (0.72, 2.09)	1.43 (0.82, 2.51)
4	13	7.7% (0-22.2%)	1.53 (0.78, 3.00)	1.51 (0.77, 2.96)	1.65 (0.79, 3.48)
5	3	0	6.17 (1.76, 21.5)	5.60 (1.57, 20.1)	5.12 (1.41, 18.6)
P value for each model			0.160	0.276	0.021

Conclusion

These results show no clear marker of prognosis among the 5 enzymes studied. However, there is a suggestion that TIMP-1 levels in the adjacent tumour may be an important marker of prognosis particularly when analysed in combination with MMP-1 levels in the adjacent liver. Further studies with larger patient numbers are required to help to evaluate this further.

Chapter Five

Discussion

Matrix metalloproteinases have long been associated with cancer, and there is no doubt that they are major functional contributors to the metastatic process. The nature of their contribution originally was assumed to be primarily facilitation of the breakdown of physical barriers between a primary tumour and distant sites for metastases. Original models for the role of the matrix metalloproteinases in cancer were based around the notion that these enzymes had increased levels associated with a decrease in the levels of their corresponding inhibitors. However, the simplistic expectation that malignant tumours would have increased matrix metalloproteinase expression accompanied by decreased tissue inhibitors of matrix metalloproteinase levels is often not met. In several cases, malignant tumours have been shown to have increased rather than decreased tissue inhibitors of matrix metalloproteinase levels (Grignon et al, 1996; McCarthy et al, 1999). The tissue localisation of both specific matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in and around a tumour can be complex, with variable expression both within the tumour and the adjacent stromal cells. This complexity is further increased by the ability of matrix metalloproteinases to activate one another.

Tumour cell invasion and metastasis are now regarded as multi-step phenomena, involving proteolytic degradation of basement membranes and extracellular matrix, altered cell adhesion and physical movement of tumour cells. Angiogenesis is essential both for tumour growth and for successful tumour invasion and metastasis. Growth and development of blood vessels within tumours requires the same factors that are crucial to tumour cell invasion and the matrix metalloproteinases play a central role in all of these processes. Individual matrix metalloproteinases may have different, possibly contradictory, roles in angiogenesis. Proteolysis of the extracellular matrix is a prerequisite for angiogenesis, and activated matrix metalloproteinases (specifically MMP-2) are present in endothelial cells of blood vessels at sites of angiogenesis. However, several matrix metalloproteinases (MMP-2, MMP-3, MMP-7 and MMP-9) have been shown to be capable of proteolytic cleavage of plasminogen to form angiostatin, an endogenous angiogenesis inhibitor, which specifically inhibits proliferation of endothelial cells (Patterson & Sang, 1997; O'Reilly et al, 1999). The matrix metalloproteinases are responsible for the degradation of the constituents of basement membranes and the extracellular matrix. Through interactions with an array of cell adhesion molecules, matrix metalloproteinases are implicated in altered

adhesion between the tumour cell and its environment, and recently have been shown to play a role in the movement of cells through the extracellular matrix (Curran & Murray, 2000). In addition to their function in the breakdown of the extracellular matrix, matrix metalloproteinases also have growth regulatory effects on both primary and secondary tumours. *In vitro* studies have demonstrated degradation of insulin-like growth factor receptor proteins by matrix metalloproteinases. This may contribute to the observed growth regulatory functions of the matrix metalloproteinases (Fowlkes et al, 1995). There is also experimental evidence that matrix metalloproteinases are involved in the early stages of tumour growth and development. Following the administration of the synthetic inhibitor batimastat, a 48 per cent reduction in the number of adenomas was observed in Min mice (Goss et al, 1998).

With the role for matrix metalloproteinases and their inhibitors in cancer accepted but far from understood it was the intention of our work to investigate the expression patterns of MMP-1, MMP-7, MMP-9, TIMP-1 and TIMP-2 in colorectal liver metastases. The clinical relevance of these expression patterns was then investigated by comparing them with patient outcome. The staining patterns observed were diverse with clear positive staining in some patients and negative staining in others. This diversity in staining between patients gave encouragement that a clinically relevant difference might be identified.

Correlation between levels of expression for different enzymes was only identified in four instances. Three out of these four involved parallel overexpression of MMP-9 and TIMP-1, although in the survival analysis this did not lead to a clinically significant predictor of outcome. Parallel overexpression of MMP-9 and TIMP-1 mRNA has been reported previously in primary colorectal cancer and liver metastases, suggesting the possible co-regulation of these two important genes *in vivo* (Zeng et al, 1993). It is possible that the expression of TIMP-1 is in response to MMP-9, however this conclusion is probably too simplistic. It is far more likely that TIMP-1 is acting in a stimulatory role rather than inhibitory, a notion which is supported by the fact that TIMP-1 is known to have growth promoting properties (Gasson et al, 1985; Bertaux et al, 1991). TIMP-1 is also known to account for a significant portion of the growth factor activity of serum (Hayakawa et al, 1992), and is capable of stimulating a wide range of human and bovine cell lines, including those

derived from tumours (breast cancer, leukaemia and Burkitt's lymphoma). Further studies have also shown that TIMP-1 stimulates the secretion of collagenase from human skin fibroblasts (Clark et al, 1994). Therefore, in addition to its role as a metalloproteinase inhibitor, TIMP-1 probably also functions as a growth factor in the pathogenesis of a variety of diseases. The precise mechanism and control of TIMP's multiple function is currently not known.

The staining patterns for MMP-9 and TIMP-1 were predominantly in inflammatory cells that morphologically had the appearances of macrophages and neutrophils. The most abundant staining was seen at the tumour/liver interface. It has long been recognised that MMP-9 originates from both neutrophils (Gallegos et al, 1995) and macrophages (Zeng & Guillem, 1995). It is reported that tumour-associated macrophages may be involved in the development of liver metastases (Heuff et al, 1993). As many tumours produce factors such as macrophage colony-stimulating factor (Walter et al, 1991) and as tumour-associated macrophages may bear receptors for macrophage colony-stimulating factor (Bottazzi et al, 1990), tumour cells may stimulate the migration and growth of tumour-associated macrophages to the tumour edge. This may lead to increased local MMP-9 production thus facilitating tumour invasion. This notion is supported by the identification of agents such as lipopolysaccharide, which can stimulate macrophages to produce several matrix metalloproteinases including MMP-9 (Xie et al, 1994), and also by work which demonstrates that metastatic colorectal cancer cells can stimulate human monocytes to produce MMP-9 (Swallow et al, 1996). Further support for an important role of macrophage derived matrix metalloproteinases in invasion comes from studies using macrophage metalloproteinase deficient mice. Macrophages in these mice have a markedly diminished capacity to degrade extracellular matrix components and are unable to penetrate reconstituted basement membrane *in vitro* or *in vivo* (Shipley et al, 1996).

The survival analysis performed showed that some of the clinicopathological variables were highly clinically significant. These included three variables associated with the hepatic surgery: tumour diameter, adjuvant therapy and additional therapy. In addition blood transfusion either during or after hepatic surgery also appeared highly relevant although not reaching the conventional 5% level. All of these results are

predictable from the published literature, but provide confirmation that the series of patients studied was typical. Adjuvant therapy, additional therapy and blood transfusion are not variables that can assist in pre-operative decision making for obvious reasons. Tumour diameter, however, is a measurable pre-operative variable and the findings suggest those patients with tumour greater than 5cm have a worse outcome. This finding supports previous work, but as discussed in the introduction not every study identifies tumour diameter as a prognostic factor. Therefore the use of tumour diameter as a predictor of outcome cannot be relied upon.

The role of matrix metalloproteinases in colorectal cancer is well documented and further recent evidence suggests that they may provide important prognostic information (Inuzuka et al, 2000; Bodey et al, 2000). The role of matrix metalloproteinases in colorectal cancer liver metastases has not been widely published and this work provides original descriptions for the expression of some of the matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases. As yet no data has been published which examines the prognostic role of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases following hepatic resection for colorectal cancer liver metastases. The survival analysis performed in our work shows no clear role for MMP-1, MMP-7 or MMP-9 alone in predicting outcome following hepatic surgery. There was, however, a suggestion that the tissue inhibitors of matrix metalloproteinases might be important predictors as the log-rank test associated with survivor functions for both enzymes showed statistical significance. The resulting Kaplan Meier survival curves were not linear and therefore it was impossible to extrapolate this significance into a clinical one. When the results for TIMP-1 were reanalysed comparing expression against no expression in the adjacent tumour, it was found that there was a survival advantage for those patients not expressing TIMP-1 that was significant at the 10% level (p=0.060). Further analysis particularly concentrating at the tumour/liver interface showed that the expression of TIMP-1 in adjacent tumour and MMP-1 in adjacent liver correlated with a clinically significant survival advantage for the low expressers (p=0.029).

The Cox Proportional Hazards model for TIMP-1 maintains significance at the 10% level in the univariate unadjusted model (p=0.082) although as before this is not linear as shown by the variable relative hazards. The model adjusting for the known

prognostic indicators becomes highly significant (p=0.001). The Cox Proportional Hazards model for TIMP-1 comparing expression with no expression also maintains significance at the 10% level (p=0.072). Those patients expressing TIMP-1 in the adjacent tumour have a relative hazard that is 60% greater than those patients not expressing TIMP-1 in the adjacent tumour. The Cox Proportional Hazards model for the combined TIMP-1 and MMP-1 score does not maintain significance (p=0.160) in the univariate unadjusted model. However, when controlling for the other prognostic variables the model becomes significant (P=0.021). There is a close connection between the log-rank test and the Cox Proportional Hazards model when testing the null hypothesis of no difference in survival between two groups and they would be expected to give similar results. The results for TIMP-1 alone maintain this approximation but the combined score results are very different. In addition, the widths of the confidence intervals around estimated relative hazards are wide. The variability of the results and the wide confidence intervals strongly suggest that the number of patients in the study was insufficient to confidently detect differences in survival functions.

The concept that the tissue inhibitors of matrix metalloproteinases may have a role in cancer progression and, indeed, be markers of poor prognosis is not new. High levels of tissue inhibitors of matrix metalloproteinases have been identified in a number of tumours compared to normal tissue levels, including gastric cancer (Murray et al, 1998), renal carcinoma (Kugler, 1999) and cervical carcinoma (Davidson et al, 1999). In breast carcinoma tissue inhibitors of matrix metalloproteinases were originally shown to be associated with lymph node metastases (Ree et al, 1997). More recently, further research has identified TIMP-1 in breast carcinoma using the enzyme-linked immunosorbent assay and high concentrations were associated with poor outcome (McCarthy et al, 1999). Outcome was measured as disease free interval and overall survival. Using the same technique, high levels of TIMP-1 have also indicated poor prognosis in lung cancer patients (Yllsirnio et al, 2001). The simple theory that tissue inhibitors of matrix metalloproteinases are associated with better outcome because of their inhibitory role is clearly not the whole picture. These multi-functional proteins play a complicated role in cancer progression and metastasis, and currently our understanding of this role is poor. This lack of understanding is clearly highlighted in work looking at the synthetic matrix

metalloproteinase inhibitor batimastat, which was designed to treat cancer. Treatment with batimastat was found to cause human breast carcinoma cells to metastasise to the liver of nude mice (Kruger et al, 2001).

Immunohistochemistry is a straightforward research tool in which it is relatively easy to obtain immunoreactivity, but it can be extremely difficult to interpret what the staining represents. Even when the immunoreactivity is believed to be representative of the target protein rather than either artefact or cross-reactivity, it can be difficult to present the results in a meaningful way and understand what the staining patterns represent. In an attempt to validate the results most immunohistochemical studies use two independent observers. In this work we have used image analysis, which is an accepted method although still open to questions regarding bias.

One of the drawbacks of this study is the inability to distinguish between the pro and active forms of the enzymes. The matrix metalloproteinases, except for stromelysin 3 (MMP-11), are secreted as inactive zymogens (proMMP's), and extracellular activation mechanisms are required for their function. ProMMP's can be activated by various factors including organomercurials, acid exposure and serine proteinases (Nagase & Okada, 1997). Several studies have demonstrated that matrix metalloproteinases have the ability to activate one another (Cao et al, 1995; Fridman et al, 1995). Stromelysin (MMP-3) has been shown to activate proMMP-9, and proMMP-3 can be activated by plasmin (Inuzuka et al, 2000) and cathepsin B. MT-MMP's, which are found on the cell membrane of tumour cells, have been shown to activate MMP-2 (Sato et al, 1994). The complex of proMMP-2 and TIMP-2 binds to activated MT-MMP and this binding ultimately results in activation of MMP-2 (Himelstein et al, 1994; Cao et al, 1995). The active MMP-2 species may in turn activate proMMP-9 (Fridman et al, 1995). Furthermore work examining the expression of MMP-9 in colorectal liver metastases has demonstrated the presence of proMMP-9 in both liver metastases and normal liver parenchymal tissue. However, the active form of MMP-9 was only detected in the liver metastases (Zeng & Guillem, 1998). This suggests that activation is a pivotal and therefore identification of the active form may be of fundamental importance.

One of the issues relating to archival tissue is enzyme degradation. Certainly as far as the matrix metalloproteinases are concerned there is no information available regarding this matter. In our work some of the most abundant staining was observed in the older specimens with enough variety throughout to suggest that degradation of these enzymes with time is not a problem in wax embedded formalin fixed tissues.

Tumour localisation studies can only give information regarding one point in time, and yet we are attempting to understand a very dynamic biological process. Therefore, there are difficulties in interpreting these results. For example, is over-expression of a particular enzyme or inhibitor an indication of a functional role for it in the malignant process or is it a sign of the host response. The localisation and interplay between matrix metalloproteinases and their inhibitors *in vivo* is complex and as yet poorly understood. It seems unlikely that tumour behaviour can be predicted from the presence or absence of matrix metalloproteinases at a single biopsy. However, as production and activation of matrix metalloproteinases requires a cascade of reactions, by determining which additional factors are required for matrix metalloproteinase expression it may be possible to develop a panel of probes whose combined presence or absence will predict tumour behaviour. One promising approach to address this question involves the use of transgenic or knockout mice to investigate the effects of altered host levels of specific matrix metalloproteinases or tissue inhibitors of matrix metalloproteinases (Khoka et al, 1995).

If matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases are proven to have a prognostic role in patients with colorectal cancer metastases then it is clear that the enzyme levels need to be measured before surgery. Biopsy of metastases prior to hepatectomy is not advisable because of the risk of tumour seeding, and even if it were acceptable it is doubtful that a single biopsy would accurately identify enzyme levels given the heterogeneity of their expression. An alternative may be to measure plasma levels. Previous work has shown that high plasma levels of MMP-9 and TIMP complex were associated with decreased survival in colorectal cancer patients (Zucker et al, 1999). Elevated serum concentrations of TIMP-1 have also been shown to correlate with poor survival in patients with lung cancer (Ylisirnio et al, 2001).

Identification of prognostic matrix metalloproteinases in colorectal liver metastases could lead to targeted drug therapy. Synthetic, potent, low molecular weight matrix metalloproteinase inhibitors have been developed, and over the past five years have begun clinical testing in patients with cancer, rheumatoid arthritis, osteoarthritis and acute macular degeneration. There have been a number of disappointments with the halting of trials of Ro 32-3555 in patients with rheumatoid arthritis and of BAY 12-9566 in patients with cancer. There have, however, been some successes with perhaps the clearest indication of efficacy being seen in results of a phase III trial of marimastat in patients with advanced gastric cancer (Brown, 2000).

Research into matrix metalloproteinase inhibitors suitable for use as an anticancer therapy continues, with many novel substances being proposed. An antibacterial agent called hypothemycin, which inhibits Ras-inducible genes, including MMP-1, MMP-3 and MMP-9, has been described. This transcriptional regulation represents a new approach to inhibition of the matrix metalloproteinases (Tanaka et al, 1999). Antisense oligonucleotides to MMP-7 have been tested on human colon cancer cell lines. The antisense oligonucleotide inhibited both the secretion of MMP-7 by cultured cells and their *in vitro* invasion through the basement membrane (Miyazaki et al, 1999). An MMP-9 ribozyme has also been evaluated in an experimental model that involves inhibition of the synthesis of matrix metalloproteinases (Hua & Muschel, 1996). A further possibility involves a gene delivery system, which is activated by matrix metalloproteinases expressed preferentially by tumour cells (Peng et al, 1997). More recently a specific treatment for colorectal liver metastases has been described using adenoviral transfer of TIMP-2 into the liver tissue (Brand et al, 2000). In this work, a nude mouse model of colorectal liver metastases is transduced to overexpress TIMP-2 in the liver prior to, or following, tumour challenge by metastatic cells *in vivo*. Transduction of approximately 50 per cent of hepatocytes resulted in 95 per cent reduction in metastases after tumour challenge compared with controls. Furthermore, TIMP-2 gene transfer into liver with pre-existing metastatic spread resulted in a 77 per cent reduction in tumour cell growth.

Future research is likely to concentrate on the development of specific MMP targets in different diseases. Tissue inhibitors of matrix metalloproteinases have been shown to be associated with advanced disease or poor survival. Indeed this work suggests that

high levels of expression of TIMP-1 in the adjacent tumour may be associated with poor survival. The role of tissue inhibitors of matrix metalloproteinases in cancer progression is poorly understood but synthetic inhibitors of these naturally occurring inhibitors may provide successful targeted therapy.

Considerable information is now available about the role of matrix metalloproteinases and their inhibitors in tumour progression and metastasis, but the challenge that remains, is the application of this knowledge with clinical relevance. Individual matrix metalloproteinases have been shown to be of prognostic significance in several types of tumours. Precise information about which matrix metalloproteinases are critical to tumour invasion and metastasis may enable the development of drugs aimed at specific matrix metalloproteinases or their inhibitors. If, as some recent studies suggest, matrix metalloproteinases are involved in the early stages of tumour development, it may be possible to use inhibitors as tumour prevention agents. Advancing our understanding of this important group of enzymes will enable us to fully exploit their potential clinical applications.

This work has provided new information regarding matrix metalloproteinases and their role in cancer. The expression of MMP-1, MMP-7, MMP-9, TIMP-1 and TIMP-2 in colorectal cancer liver metastases has not been described before. This description alone may provide a useful comparison for further research into the role of matrix metalloproteinases and colorectal cancer metastases. Probably due to patient numbers, this work has not been able to establish clinically significant roles for matrix metalloproteinases or tissue inhibitors of matrix metalloproteinases as prognostic markers in patients with colorectal cancer liver metastases. However, these results do suggest that future research should concentrate on examining the expression of the tissue inhibitors of matrix metalloproteinases and their role in liver metastases from colorectal cancer. Also, any future work should probably also look at assessing plasma levels of these enzymes to allow easier application to the clinical setting.

Our understanding of the role of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in cancer remains limited although continuing research will hopefully advance this knowledge. The aim of cancer research is to gain a full understanding of the tumour biology so that it may be applied clinically to the benefit

of the patients. The role of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases has clearly been identified although the understanding of this role is far from clear. There is a significant gap in our knowledge of the tumour biology for colorectal liver metastases. Further work needs to concentrate on examining the variable expression patterns of the matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases. In particular the tumour/liver interface seems to be the most logical site to focus on, and this is supported by this work in which the positive findings were all related to enzyme levels expressed at the interface.

Immunohistochemistry remains a very useful tool for this work as it is both straightforward and reproducible. As a primary research tool it provides the ability to examine large numbers of patients and enzymes, thereby producing a good overall picture of enzyme expression and more importantly the variability of enzyme expression. The main drawback remains the interpretation of the staining particularly in relation to quantification. Therefore, this method may lead to a more focused exploration of the expression of these enzymes by more powerful research methods.

The most important end point is the application of our understanding in the clinical setting. We will not be able to examine colorectal liver metastases on patients before their surgery as pre-operative biopsies are considered to be oncologically dangerous. We must therefore develop methods that allow us to identify the prognostic value of these enzymes before the tumour is removed. It is possible that the plasma levels of the enzymes may allow correlation with outcome. Further work should therefore also concentrate on examining both the pre-operative plasma levels as well as the post-operative liver specimen

Our original hypothesis was that the expression of matrix metalloproteinases -1, -7 and -9 and tissue inhibitors of matrix metalloproteinases -1 and -2 would have prognostic significance in patients with colorectal liver metastases. This work has shown that for tissue inhibitors of matrix metalloproteinase-1 this may well be true but for the other four enzymes we have found no evidence to support this hypothesis. This study has been limited by the number of patients available and further work will certainly require greater numbers to obtain clinically significant results.

Appendices

Appendix I

Patients Details for Primary Disease

Key

Procedure performed

1 Right hemicolectomy
2 Extended right hemicolectomy
3 Transverse colectomy
4 Left hemicolectomy
5 Sigmoid colectomy
6 Hartmann's
7 Anterior resection
8 AP Resection
9 Subtotal colectomy

Adjuvant therapy

0 None
1 Preoperative radiotherapy & postoperative chemotherapy
2 Postoperative radiotherapy
3 Postoperative radiotherapy & postoperative chemotherapy
4 Preoperative & postoperative chemotherapy
5 Postoperative chemotherapy

Patient No.	Date of Primary Operation	Age at Primary Operation (years)	Procedure Performed	Dukes' Classification	Curative for Primary Disease	Synchronous Metastases	Adjuvant Therapy	Time to Diagnosis of Metastases (months)
1	01/01/91	54	4	B	yes	no	0	29.5
2	03/09/91	79	7	A	yes	no	0	24.3
3	01/04/92	50	8	C_1	yes	no	5	10.2
4	01/05/92	49	7	D	yes	yes	0	0.0
5	12/11/91	53	5	D	yes	yes	0	0.0
6	03/12/92	51	7	D	yes	yes	5	0.0
7	16/02/93	60	1	D	yes	yes	0	10.2
8	01/10/91	53	5	C_1	yes	no	0	26.7
9	01/06/90	57	9	B	yes	no	0	38.1
10	01/05/90	67	7	B	yes	no	2	36.5
11	01/09/92	55	1	C_1	yes	no	0	12.2
12	16/06/92	52	5	D	yes	yes	0	0.0
13	16/01/92	58	5	C_2	yes	no	5	6.1
14	03/10/91	47	7	C_1	yes	no	3	8.8
15	06/02/92	46	4	D	yes	yes	0	0.0
16	13/07/89	83	7	B	yes	no	0	31.5
17	06/02/92	56	7	D	yes	yes	0	0.0
18	29/08/90	61	8	C_2	yes	no	5	17.5
19	01/02/92	51	8	D	yes	yes	0	0.0
20	01/11/90	45	5	C_1	yes	no	0	11.1
21	01/08/90	60	5	D	no	yes	0	0.0

Patient No.	Date of Primary Operation	Age at Primary Operation (years)	Procedure Performed	Dukes' Classification	Curative for Primary Disease	Synchronous Metastases	Adjuvant Therapy	Time to Diagnosis of Metastases (months)
22	11/01/91	41	7	D	yes	yes	0	0.0
23	11/10/89	76	5	D	yes	yes	0	0.0
24	11/10/90	56	7	D	yes	yes	0	0.0
25	25/04/88	67	7	B	yes	no	0	36.3
26	01/11/90	61	4	B	yes	no	5	1.0
27	09/01/90	34	7	B	yes	no	0	15.9
28	01/03/90	49	7	C_1	yes	no	0	20.9
29	23/05/90	65	4	D	no	yes	0	0.0
30	01/02/90	57	7	D	yes	yes	0	0.0
31	14/07/89	50	1	C_1	yes	no	0	5.3
32	01/05/86	57	6	C_1	yes	no	0	45.3
33	01/09/83	55	4	B	yes	no	0	74.1
34	29/11/89	66	4	C_1	yes	no	0	4.1
35	01/12/88	43	7	C_1	yes	no	0	0.0
36	13/09/85	61	7	B	yes	no	0	46.1
37	02/08/88	63	7	D	yes	yes	0	0.0
38	25/04/87	60	4	D	yes	yes	0	0.0
39	03/06/87	55	7	D	yes	yes	0	0.0
40	09/03/88	69	1	D	yes	yes	0	0.0
41	01/09/86	59	7	D	yes	yes	0	1.1
42	09/07/86	47	7	D	yes	yes	0	27.0

Patient No.	Date of Primary Operation	Age at Primary Operation (years)	Procedure Performed	Dukes' Classification	Curative for Primary Disease	Synchronous Metastases	Adjuvant Therapy	Time to Diagnosis of Metastases (months)
43	25/07/88	49	4	D	no	yes	0	0.0
44	06/02/88	70	3	D	yes	yes	0	0.0
45	28/01/87	48	4	D	yes	yes	5	14.3
46	01/03/94	55	1	B	yes	no	0	1.0
47	16/10/89	65	4	C_2	yes	no	0	18.7
48	24/07/92	70	7	D	yes	yes	0	22.7
49	04/06/94	57	5	D	yes	yes	0	0.0
50	12/10/93	49	3	D	yes	yes	5	0.0
51	29/12/92	50	2	D	yes	yes	5	19.2
52	01/11/91	55	7	C_2	yes	no	5	33.5
53	17/04/91	54	7	D	yes	yes	0	0.0
54	24/03/89	66	7	C_1	yes	no	5	49.0
55	01/08/92	62	5	B	yes	no	0	23.3
56	30/12/92	69	4	C_1	yes	no	0	22.1
57	24/02/94	49	7	C_1	yes	no	5	9.3
58	10/11/94	67	7	D	yes	yes	0	0.0
59	23/11/94	70	8	C_1	no	no	2	-0.3
60	07/05/93	61	7	D	yes	yes	0	18.1
61	01/03/94	59	5	B	yes	no	0	10.3
62	01/09/91	61	5	C_1	yes	no	0	42.9
63	24/06/94	44	5	D	yes	yes	5	0.0

Patient No.	Date of Primary Operation	Age at Primary Operation (years)	Procedure Performed	Dukes' Classification	Curative for Primary Disease	Synchronous Metastases	Adjuvant Therapy	Time to Diagnosis of Metastases (months)
64	26/02/93	73	7	B	yes	no	0	25.4
65	12/03/95	65	1	C_1	yes	no	0	1.9
66	09/06/93	69	7	B	yes	no	0	21.0
67	23/08/94	61	5	A	yes	no	0	6.8
68	01/06/94	69	1	C_1	yes	no	0	12.5
69	13/10/94	56	7	D	yes	yes	5	1.6
70	19/10/93	66	5	C_1	yes	no	5	16.6
71	22/07/95	51	7	D	yes	yes	5	0.0
72	09/03/95	69	1	B	yes	no	0	4.6
73	01/06/92	60	7	B	yes	no	0	39.7
74	19/09/95	81	7	D	yes	yes	0	0.0
75	29/09/92	70	4	C_1	yes	no	0	35.0
76	04/12/93	67	7	C_2	yes	no	4	20.9
77	01/12/85	38	1	C_1	yes	no	0	119.1
78	15/02/93	49	8	C_1	yes	no	3	9.6
79	24/03/94	72	7	B	yes	no	0	15.5
80	24/07/95	54	7	D	yes	yes	5	0.0
81	07/02/94	66	6	D	yes	no	3	4.5
82	19/08/95	47	1	D	yes	yes	5	0.0
83	29/09/95	51	7	D	yes	yes	0	0.0
84	05/06/95	65	2	B	yes	no	0	6.3

Patient No.	Date of Primary Operation	Age at Primary Operation (years)	Procedure Performed	Dukes' Classification	Curative for Primary Disease	Synchronous Metastases	Adjuvant Therapy	Time to Diagnosis of Metastases (months)
85	12/05/94	59	4	B	yes	no	0	17.1
86	26/08/95	60	5	D	yes	yes	0	0.0
87	16/11/95	68	5	D	yes	yes	0	0.0
88	30/05/95	72	7	B	yes	no	0	4.7
89	23/01/96	77	7	D	yes	yes	0	0.0
90	04/03/96	49	9	D	yes	yes	0	0.0
91	17/04/96	47	7	D	yes	yes	0	0.0
92	16/12/94	28	8	A	yes	no	0	17.0
93	11/06/96	57	7	D	no	yes	0	-0.4
94	04/12/95	61	6	D	yes	yes	0	0.0
95	20/06/96	52	4	D	yes	yes	0	0.0
96	26/10/94	67	5	D	yes	yes	0	0.0
97	23/05/94	50	4	B	yes	no	0	23.9
98	23/06/92	63	7	C_1	yes	no	5	46.5
99	07/11/95	67	5	D	yes	yes	5	0.0
100	28/12/95	68	1	C_1	yes	no	0	4.9
101	11/08/96	50	1	B	yes	no	0	-1.3
102	01/01/92	48	8	D	yes	no	0	48.7
103	03/01/96	47	7	D	yes	yes	5	0.0
104	17/04/96	57	7	C_1	yes	no	1	2.9
105	02/12/91	65	4	A	yes	no	0	58.1

Appendix II

Patients Details for Secondary Disease

Key

Procedure performed

1 Extended right hemihepatectomy
2 Right hemihepatectomy
3 Left hemihepatectomy
4 Left lateral segmentectomy
5 Large wedge
6 Segmentectomy

Adjuvant therapy

0 None
1 Preoperative chemotherapy
2 Preoperative & postoperative chemotherapy
3 Postoperative chemotherapy
4 Radiotherapy
5 Preoperative chemotherapy & radiotherapy

Additional therapy (for progression or recurrence)

0 None
1 Systemic chemotherapy
2 Radiotherapy
3 Regional chemotherapy

Cause of death

0 Dead without disease
1 Dead with and because of disease
2 Dead - other cause but with disease
3 Dead - with disease but ? Cause
4 Dead ? Disease ? Cause

Patient No.	Date of Liver Operation	Age at Liver Surgery	Procedure Performed	Curative for Liver Metastases	Adjuvant Therapy	Reoperation for Recurrence	Additional Therapy	Survival (years)	Cause of Death
1	18/10/93	57	2	yes	0	no	1	1.25	1
2	29/12/93	81	2	yes	0	no	0	1.02	0
3	17/05/93	52	1	yes	2	no	1	7.25	alive
4	08/03/93	50	2	yes	0	no	0	3.54	1
5	18/01/93	54	2	yes	0	no	1	1.70	1
6	08/02/93	52	2	yes	3	no	1	1.73	1
7	24/01/94	61	4	yes	0	no	1	5.08	1
8	07/02/94	56	2	yes	0	no	1	1.31	1
9	04/10/93	60	2	yes	0	yes	1	3.19	1
10	12/07/93	71	2	yes	0	no	2	3.28	0
11	06/12/93	66	2	yes	0	no	0	0.61	1
12	05/07/93	53	2	yes	0	no	0	1.95	1
13	21/09/92	59	3	yes	1	no	0	0.12	4
14	10/08/92	48	2	yes	2	no	1	0.72	1
15	05/05/92	47	6	yes	3	no	3	1.38	1
16	14/09/92	86	2	yes	0	no	0	7.58	alive
17	08/06/92	56	2	yes	0	no	1	3.52	1
18	01/06/92	62	2	yes	0	no	0	8.14	alive
19	03/08/92	52	2	yes	0	no	1	2.27	1
20	03/02/92	46	2	no	3	no	1	0.96	1
21	14/01/91	60	2	yes	0	no	0	9.08	alive

Patient No.	Date of Liver Operation	Age at Liver Surgery	Procedure Performed	Curative for Liver Metastases	Adjuvant Therapy	Reoperation for Recurrence	Additional Therapy	Survival (months)	Cause of Death
22	11/01/91	41	6	yes	0	yes	0	9.90	alive
23	11/03/91	77	6	yes	0	no	3	1.89	1
24	07/10/91	56	2	yes	0	no	0	8.73	alive
25	13/05/91	70	2	yes	0	no	0	2.61	1
26	22/02/91	63	3	no	4	no	1	0.85	1
27	26/06/91	36	2	yes	4	no	1	3.82	1
28	25/11/91	51	3	yes	0	no	1	3.16	1
29	26/11/90	66	2	yes	0	no	0	4.17	1
30	28/05/90	57	6	yes	0	no	1	3.79	1
31	05/02/90	50	2	yes	3	no	1	1.21	1
32	12/03/90	61	2	yes	0	no	0	5.27	1
33	12/02/90	61	3	yes	0	no	0	11.15	alive
34	17/09/90	67	2	yes	0	no	0	2.38	1
35	03/05/90	45	2	no	0	no	0	2.25	1
36	02/10/89	66	2	yes	0	no	0	4.26	1
37	06/02/89	63	3	yes	0	no	0	2.11	1
38	17/04/89	60	2	yes	0	no	0	1.44	4
39	12/04/89	58	5	yes	0	no	0	11.98	alive
40	16/05/88	69	2	yes	0	no	0	1.49	1
41	31/10/88	61	6	yes	0	no	0	5.64	1
42	22/08/88	49	2	yes	0	no	0	1.36	1

Patient No.	Date of Liver Operation	Age at Liver Surgery	Procedure Performed	Curative for Liver Metastases	Adjuvant Therapy	Reoperation for Recurrence	Additional Therapy	Survival (months)	Cause of Death
43	03/10/88	49	6	yes	0	no	1	2.67	1
44	24/08/88	70	6	yes	0	no	1	1.63	1
45	15/10/90	52	2	yes	0	no	1	4.05	1
46	18/05/94	56	2	yes	0	no	0	6.02	alive
47	17/07/91	67	2	no	3	no	1	1.54	1
48	13/06/94	72	6	yes	3	no	1	6.04	alive
49	04/07/94	57	3	yes	0	no	1	1.63	1
50	17/08/94	50	1	yes	1	no	1	0.70	1
51	12/09/94	52	6	yes	1	no	0	1.62	4
52	19/09/94	58	2	yes	1	no	1	6.21	alive
53	03/10/94	58	2	yes	0	no	0	6.34	alive
54	31/10/94	72	2	yes	1	no	2	1.42	1
55	23/01/95	65	2	yes	0	no	0	1.02	1
56	25/01/95	72	2	yes	0	no	1	1.87	1
57	01/02/95	50	2	yes	1	no	1	2.17	1
58	08/02/95	68	6	yes	0	no	0	5.17	alive
59	13/02/95	70	2	yes	0	no	0	3.50	3
60	20/02/95	63	2	yes	0	no	0	3.87	1
61	23/02/95	60	6	yes	0	no	0	4.79	1
62	26/04/95	64	6	yes	0	no	1	1.84	3
63	01/05/95	45	2	yes	1	no	0	1.30	1

Patient No.	Date of Liver Operation	Age at Liver Surgery	Procedure Performed	Curative for Liver Metastases	Adjuvant Therapy	Reoperation for Recurrence	Additional Therapy	Survival (months)	Cause of Death
64	24/05/95	75	6	No	0	no	0	1.52	3
65	12/06/95	65	2	no	0	no	1	0.89	1
66	09/07/95	71	2	yes	0	no	0	5.38	alive
67	19/07/95	62	2	yes	0	no	0	5.14	alive
68	15/08/95	70	2	yes	0	no	0	3.13	1
69	30/08/95	56	2	yes	1	no	0	5.29	alive
70	04/09/95	68	4	yes	1	no	2	1.20	1
71	25/09/95	51	2	yes	2	yes	1	2.57	1
72	29/09/95	69	3	yes	0	no	0	1.09	1
73	02/10/95	65	6	yes	0	no	0	4.72	alive
74	19/09/95	81	4	yes	0	no	0	1.29	1
75	04/10/95	73	3	yes	0	no	0	0.01	1
76	11/10/95	68	2	yes	0	yes	0	3.64	1
77	01/11/95	48	3	yes	0	no	1	5.43	alive
78	13/11/95	52	2	yes	1	no	1	2.78	1
79	15/11/95	74	3	yes	0	no	1	1.15	1
80	27/11/95	54	2	yes	1	no	1	3.30	1
81	11/12/95	68	2	yes	5	no	1	1.72	1
82	15/01/96	47	4	yes	2	no	1	0.88	1
83	17/01/96	52	4	yes	0	no	1	0.53	1
84	22/01/96	66	2	yes	0	no	1	4.97	alive

Patient No.	Date of Liver Operation	Age at Liver Surgery	Procedure Performed	Curative for Liver Metastases	Adjuvant Therapy	Reoperation for Recurrence	Additional Therapy	Survival (months)	Cause of Death
85	05/02/96	61	2	yes	0	no	0	5.21	alive
86	11/03/96	60	2	yes	0	no	1	3.56	1
87	15/04/96	68	5	yes	0	yes	0	4.76	alive
88	22/04/96	63	2	yes	0	no	0	4.53	alive
89	08/05/96	77	2	yes	0	no	0	4.41	1
90	29/05/96	49	6	yes	0	no	1	3.22	alive
91	03/06/96	47	1	yes	0	no	0	1.04	1
92	10/06/96	29	2	yes	0	no	2	4.48	1
93	11/06/96	58	3	no	0	no	0	1.48	1
94	12/06/96	61	2	yes	0	no	2	3.45	1
95	20/06/96	52	3	yes	0	no	1	4.96	alive
96	21/06/96	68	2	yes	0	no	0	3.28	1
97	24/06/96	55	2	yes	0	no	0	0.31	1
98	01/07/96	67	2	yes	1	no	0	3.50	1
99	01/08/96	67	2	yes	1	yes	1	3.50	1
100	05/08/96	69	2	yes	0	no	1	2.33	1
101	09/09/96	51	2	yes	0	no	0	4.53	alive
102	16/09/96	53	3	yes	3	no	0	2.06	1
103	23/09/96	47	2	yes	1	yes	1	4.47	1
104	09/10/96	58	3	yes	4	no	1	2.02	3
105	16/10/96	70	2	yes	0	no	0	4.49	alive

Appendix III

Reagents

Endogenous peroxidase inhibitor

Hydrogen peroxide (30%) 0.2 ml
Methanol 11.8ml

0.01M citrate buffer pH 6.0

Citric acid crystals 3.15g
Distilled water 1500ml
Adjust pH to 6.0 with 1M sodium hydroxide (approximately 37.5ml)

TRIS buffered saline pH 7.6

Sodium chloride 80g
TRIS hydroxymethyl methylamine (TRIS) 6.05g
Distilled water 10L
Adjust pH to 7.6 with 1M hydrochloric acid (approximately 38ml)

Liquid DAB chromagen

Deionised water 4.5ml
10X substrate buffer 0.5 ml
Chromagen (mix well) 4 drops, and mix
Hydrogen peroxide 2 drops, and mix

Harris' haematoxylin

Haematoxylin 1g
Alcohol 10ml
Aluminium potassium sulphate 20g
Distilled water 200ml
Mercuric oxide 0.5g
Glacial acetic acid 8ml

1% acid alcohol

70% alcohol 990ml
10M hydrochloric acid 10ml

Appendix IV

Visual and Image Analysis Scores

Visual and image analysis scores for MMP-1

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
1	1	1	3	3	12338	371	0.316
2	0	0	3	2	19226	632	0.312
3	0	0	3	2	19820	580	0.318
4	0	0	3	2	17042	482	0.295
5	4	3	2	2	9736	337	0.315
6	0	0	4	3	26131	823	0.326
7	0	0	1	1	2898	147	0.287
8	0	0	2	2	11018	315	0.314
9	4	3	3	2	14016	474	0.312
10	0	0	2	2	9997	309	0.320
11	3	3	3	3	12595	512	0.291
12	0	0	3	3	15301	547	0.273
13	0	0	3	2	17225	448	0.306
14	0	0	2	2	6431	260	0.276
15	0	0	3	3	19840	570	0.317
16	0	0	2	2	7554	285	0.287
17	0	0	1	1	2561	105	0.295
18	0	0	1	1	3726	196	0.292
19	0	0	2	1	8896	353	0.287
20	0	0	1	1	3951	56	0.296
21	2	2	1	1	2438	66	0.307
22	0	0	2	2	6840	342	0.312
23	2	2	4	3	28480	686	0.298
24	0	1	2	2	6757	283	0.325
25	0	1	3	3	16070	540	0.308
26	0	0	3	3	14388	513	0.271
27	0	0	3	3	16320	531	0.296
28	0	0	3	3	13888	611	0.320
29	3	3	2	2	6681	274	0.277
30	0	0	2	2	6719	281	0.325
31	0	0	2	2	9566	291	0.316
32	3	3	1	1	3991	182	0.290
33	0	0	2	2	10759	313	0.290
34	0	0	2	2	8010	384	0.270
35	0	0	3	2	13559	473	0.284

Visual and image analysis scores for MMP-1

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
36	0	0	1	1	1093	54	0.325
37	0	0	3	3	14372	389	0.275
38	0	0	2	2	9028	320	0.283
39	0	0	2	1	8368	260	0.287
40	0	0	3	2	15589	526	0.310
41	0	0	2	2	9667	333	0.289
42	0	0	2	2	8395	295	0.274
43	5	5	3	2	14126	493	0.308
44	0	0	2	2	6835	269	0.324
45	0	0	4	3	27965	624	0.323
46	0	0	2	2	8439	339	0.292
47	0	1	2	2	8929	283	0.297
48	0	0	4	3	24190	752	0.312
49	0	0	2	2	10780	273	0.287
50	0	0	1	1	2325	124	0.316
51	0	0	1	1	2136	108	0.293
52	0	0	1	1	1875	179	0.322
53	0	0	2	2	9701	321	0.305
54	2	1	2	1	9004	370	0.319
55	4	1	1	1	4189	103	0.297
56	4	3	2	2	9129	257	0.271
57	0	0	2	2	6813	296	0.278
58	3	3	3	3	14666	487	0.302
59	2	2	3	3	17631	586	0.311
60	0	0	2	2	10370	359	0.288
61	0	0	3	2	15103	539	0.274
62	0	0	1	0	3117	155	0.317
63	2	2	1	1	1586	80	0.322
64	0	0	2	2	10624	356	0.278
65	1	1	2	2	10676	303	0.300
66	0	0	2	2	8695	342	0.323
67	0	0	3	3	16745	575	0.294
68	0	0	3	3	15709	606	0.282
69	0	0	3	3	14519	410	0.283
70	0	0	2	2	6512	272	0.314

Visual and image analysis scores for MMP-1

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
71	2	2	2	2	8073	354	0.323
72	0	0	2	2	7023	372	0.274
73	0	0	3	3	17681	497	0.280
74	0	0	2	2	9518	377	0.306
75	0	0	3	2	18265	568	0.304
76	0	0	2	2	10492	284	0.306
77	0	0	2	2	10168	378	0.278
78	0	0	2	2	9578	283	0.301
79	0	0	2	2	9181	387	0.272
80	0	0	2	2	7934	313	0.299
81	0	0	2	2	8232	303	0.314
82	0	0	3	2	11998	397	0.280
83	0	0	1	1	3562	57	0.294
84	0	0	3	2	15992	514	0.326
85	1	1	2	2	10301	301	0.292
86	0	0	2	2	9847	342	0.273
87	0	0	2	2	9219	305	0.274
88	0	0	2	2	8426	387	0.307
89	0	0	2	2	9396	293	0.310
90	0	0	2	2	8533	323	0.289
91	0	0	2	2	7461	345	0.308
92	3	3	2	2	8046	296	0.315
93	3	2	3	2	12607	597	0.303
94	3	2	3	2	18859	415	0.302
95	4	4	2	2	9888	343	0.304
96	0	0	2	2	7095	325	0.308
97	0	0	1	1	1614	58	0.314
98	0	0	2	1	7137	382	0.282
99	4	4	4	3	24438	703	0.300
100	1	1	3	3	17973	579	0.295
101	5	5	3	2	12245	393	0.283
102	0	0	2	2	8741	317	0.323
103	0	0	2	2	8937	277	0.291
104	0	0	2	2	6740	330	0.302
105	0	0	2	2	8493	336	0.299

Visual and image analysis scores for MMP-7

Patient No.	Visual Score				Image Analysis Score (Adjacent Tumour)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
1	0	0	2	2	135	15	0.324
2	0	0	2	2	911	67	0.294
3	0	0	2	2	223	15	0.296
4	3	3	2	2	4806	271	0.310
5	0	0	2	2	318	25	0.264
6	1	1	2	2	1056	51	0.245
7	0	0	2	2	201	14	0.289
8	0	1	2	2	356	24	0.325
9	0	0	2	2	241	29	0.294
10	0	0	2	2	121	20	0.331
11	0	0	2	2	311	26	0.293
12	0	0	2	2	242	12	0.324
13	0	0	2	2	133	28	0.288
14	0	0	2	2	340	29	0.316
15	0	0	2	2	85	17	0.292
16	2	3	2	2	2096	112	0.322
17	0	0	2	2	234	29	0.299
18	1	1	2	2	1044	45	0.331
19	1	1	2	2	775	32	0.288
20	0	0	2	-	157	15	0.287
21	0	0	2	-	338	24	0.301
22	0	0	2	2	302	20	0.299
23	0	0	2	2	124	26	0.283
24	0	0	2	2	66	6	0.323
25	1	1	2	2	2205	130	0.243
26	0	0	2	2	292	16	0.276
27	0	0	2	2	151	29	0.284
28	3	3	2	2	4663	237	0.330
29	0	0	2	2	138	10	0.291
30	1	2	2	2	331	28	0.286
31	0	0	2	2	287	10	0.264
32	0	0	2	2	177	22	0.325
33	0	0	2	2	206	11	0.296
34	1	2	2	2	517	34	0.322
35	0	0	2	2	240	17	0.247

Visual and image analysis scores for MMP-7

Patient No.	Visual Score				Image Analysis Score (Adjacent Tumour)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
36	0	0	2	2	90	29	0.331
37	0	0	2	2	258	27	0.298
38	0	0	2	2	164	26	0.315
39	0	0	2	2	260	13	0.306
40	0	1	2	2	190	22	0.318
41	0	0	2	2	342	12	0.296
42	0	1	2	2	101	7	0.327
43	0	0	2	2	116	16	0.254
44	1	1	2	2	629	47	0.327
45	0	1	2	2	309	11	0.254
46	3	3	2	2	4101	171	0.321
47	0	0	2	2	275	19	0.278
48	0	0	2	2	78	28	0.286
49	1	1	2	2	354	47	0.327
50	0	0	2	2	334	11	0.322
51	0	0	2	2	179	28	0.325
52	0	0	2	2	99	21	0.268
53	0	0	2	2	138	17	0.248
54	0	0	2	2	340	6	0.262
55	0	1	2	2	321	18	0.248
56	0	3	2	2	288	10	0.270
57	1	1	2	2	790	56	0.273
58	0	0	2	2	257	18	0.300
59	0	0	2	2	252	24	0.318
60	1	1	2	2	751	36	0.307
61	3	4	2	2	6927	275	0.331
62	1	3	2	-	535	49	0.258
63	1	2	2	2	726	47	0.311
64	4	4	2	2	10210	493	0.310
65	2	2	2	2	1840	77	0.321
66	4	4	2	2	8842	284	0.329
67	2	2	2	2	2129	109	0.320
68	1	1	2	2	438	29	0.303
69	0	1	2	2	215	25	0.244
70	0	0	2	2	184	10	0.309

Visual and image analysis scores for MMP-7

Patient No.	Visual Score				Image Analysis Score (Adjacent Tumour)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
71	0	1	2	2	159	6	0.297
72	3	3	2	2	4514	226	0.309
73	4	4	2	2	7737	264	0.242
74	5	5	2	2	8177	497	0.311
75	4	4	2	2	12041	359	0.255
76	3	3	2	2	3835	272	0.251
77	4	4	2	2	12186	506	0.309
78	3	3	2	2	4799	169	0.259
79	0	1	2	2	229	26	0.270
80	0	0	2	2	227	10	0.327
81	3	3	2	2	3020	145	0.282
82	2	3	2	2	1426	95	0.295
83	1	3	2	2	1010	41	0.300
84	0	0	3	3	236	14	0.267
85	0	1	2	2	210	26	0.302
86	3	4	2	2	4218	155	0.309
87	0	0	2	2	144	24	0.326
88	1	2	2	2	642	44	0.322
89	2	2	2	2	1609	125	0.293
90	4	4	2	2	9240	306	0.303
91	0	0	2	2	237	23	0.259
92	0	1	2	2	350	25	0.280
93	3	3	2	2	5477	188	0.243
94	0	0	2	2	146	24	0.253
95	0	1	2	2	120	15	0.323
96	0	0	2	2	119	15	0.320
97	0	0	2	2	345	22	0.257
98	2	2	2	2	1828	127	0.288
99	0	0	2	2	286	23	0.276
100	0	0	2	2	306	25	0.279
101	1	1	2	2	989	53	0.277
102	3	3	2	2	6271	186	0.317
103	2	3	2	2	1504	99	0.300
104	1	3	2	2	970	34	0.289
105	0	0	2	2	191	10	0.326

Visual and image analysis scores for MMP-9

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
1	2	2	1	1	224	10	0.308
2	1	1	4	1	7508	411	0.303
3	1	0	0	1	45	7	0.255
4	0	0	1	1	361	11	0.310
5	3	3	4	1	6752	379	0.324
6	0	0	1	1	192	18	0.339
7	0	0	0	0	69	9	0.271
8	0	0	1	1	287	17	0.319
9	4	2	4	0	7039	407	0.288
10	0	0	3	2	2867	241	0.273
11	2	0	2	1	1255	77	0.321
12	0	0	4	0	6483	275	0.340
13	0	0	0	0	90	5	0.301
14	0	0	0	0	49	5	0.316
15	0	2	2	1	2248	142	0.334
16	0	2	1	0	302	19	0.290
17	0	0	1	0	340	10	0.313
18	0	0	1	0	411	23	0.303
19	0	0	4	1	7788	343	0.330
20	1	0	1	0	393	14	0.305
21	1	1	1	0	529	18	0.289
22	1	0	2	0	1390	82	0.326
23	3	1	2	0	2579	165	0.315
24	1	1	1	0	319	18	0.278
25	0	0	4	1	8234	449	0.323
26	0	0	0	0	57	4	0.291
27	0	1	1	3	531	14	0.313
28	1	0	3	0	2917	177	0.320
29	3	1	2	1	1951	116	0.292
30	0	1	1	0	400	18	0.325
31	0	0	1	1	146	10	0.287
32	2	2	2	0	1108	89	0.302
33	0	0	1	0	338	23	0.330
34	1	1	4	1	8074	345	0.292
35	1	0	0	0	55	4	0.303

Visual and image analysis scores for MMP-9

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
36	0	0	3	0	3476	194	0.302
37	0	0	3	0	3354	181	0.288
38	0	0	3	3	3934	165	0.285
39	0	0	1	0	180	15	0.260
40	1	2	1	1	265	10	0.279
41	0	0	4	0	7457	306	0.271
42	1	0	1	0	237	17	0.323
43	4	4	2	0	1661	66	0.318
44	0	0	4	1	7083	301	0.271
45	0	1	2	0	1673	105	0.269
46	1	0	3	0	3236	173	0.276
47	0	0	0	0	43	10	0.321
48	0	0	2	2	2520	145	0.301
49	0	0	0	0	68	10	0.261
50	2	1	4	1	6973	300	0.328
51	0	0	3	0	3734	217	0.267
52	0	0	0	0	84	4	0.290
53	0	0	1	0	548	18	0.268
54	2	1	0	0	56	6	0.259
55	1	1	0	0	43	5	0.306
56	3	1	2	0	1930	70	0.297
57	0	0	3	3	2993	217	0.262
58	3	0	0	0	74	9	0.313
59	2	0	2	1	1297	70	0.335
60	0	1	3	0	3466	237	0.262
61	0	0	4	0	7742	368	0.293
62	0	0	0	0	42	5	0.326
63	2	2	1	0	179	15	0.330
64	0	0	0	0	77	7	0.309
65	0	0	3	1	4400	182	0.278
66	0	0	3	1	3609	214	0.318
67	0	0	1	0	452	21	0.334
68	0	0	3	1	4347	184	0.255
69	0	0	0	0	93	3	0.340
70	0	0	0	0	37	6	0.279

Visual and image analysis scores for MMP-9

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
71	1	1	1	0	487	17	0.300
72	0	0	1	3	620	10	0.278
73	0	0	1	0	329	19	0.283
74	0	0	1	1	600	23	0.308
75	0	0	0	0	79	4	0.307
76	0	0	1	2	223	21	0.315
77	0	0	0	0	66	6	0.320
78	0	0	3	3	2938	189	0.319
79	0	0	3	0	4263	188	0.333
80	0	0	3	3	3994	218	0.303
81	0	0	3	0	3560	172	0.304
82	1	0	3	2	4361	193	0.273
83	1	0	3	0	3022	161	0.323
84	0	0	1	2	278	20	0.259
85	2	3	3	1	3642	198	0.291
86	0	0	0	4	94	5	0.302
87	0	0	2	3	1000	71	0.312
88	0	2	0	2	90	9	0.304
89	0	0	0	1	88	9	0.303
90	0	0	3	3	3585	180	0.332
91	3	3	3	0	2866	152	0.327
92	1	0	1	0	210	16	0.257
93	1	0	0	0	35	3	0.296
94	1	1	3	3	4551	245	0.308
95	2	1	0	0	57	3	0.259
96	0	0	0	0	56	10	0.300
97	0	0	0	0	40	9	0.260
98	0	0	0	0	95	9	0.288
99	3	1	0	0	92	10	0.316
100	0	0	1	2	593	19	0.282
101	2	1	3	3	3417	180	0.324
102	0	0	2	2	1132	77	0.252
103	0	0	0	0	79	6	0.338
104	0	0	2	2	1282	61	0.318
105	0	2	2	2	2516	127	0.275

Visual and image analysis scores for TIMP-1

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
1	1	1	0	0	60	2	0.264
2	1	0	4	1	7646	346	0.255
3	0	0	1	1	324	16	0.292
4	0	0	3	2	3107	139	0.271
5	1	1	4	1	6656	283	0.300
6	0	0	3	2	3627	167	0.264
7	0	0	2	0	1131	132	0.324
8	0	0	2	2	1450	102	0.318
9	1	1	3	1	4748	139	0.325
10	0	0	4	2	6725	333	0.321
11	0	0	3	2	2501	154	0.313
12	0	0	4	1	6290	287	0.257
13	0	0	0	0	54	2	0.265
14	1	0	1	0	326	15	0.286
15	0	2	2	0	910	93	0.254
16	2	2	2	0	1700	111	0.301
17	0	0	1	0	454	19	0.317
18	0	0	2	0	1886	121	0.269
19	1	2	4	2	6122	274	0.327
20	0	1	0	0	62	9	0.303
21	0	0	0	0	49	3	0.333
22	0	0	1	0	196	10	0.271
23	0	0	3	1	3681	161	0.273
24	1	0	3	1	3737	153	0.327
25	0	0	3	2	4299	149	0.276
26	0	0	0	0	71	6	0.308
27	0	3	2	0	917	103	0.306
28	2	0	4	0	6581	323	0.267
29	0	0	4	2	8185	278	0.278
30	0	0	1	0	465	17	0.293
31	1	1	2	2	1733	121	0.327
32	1	1	4	2	6946	368	0.289
33	0	0	0	0	73	6	0.278
34	1	0	4	4	8061	268	0.264
35	0	0	1	0	366	9	0.306

Visual and image analysis scores for TIMP-1

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
36	0	0	2	1	1530	92	0.301
37	0	0	2	0	2247	118	0.310
38	0	0	0	2	61	8	0.304
39	0	0	0	0	47	4	0.331
40	0	2	0	0	57	3	0.287
41	0	0	4	1	7666	365	0.308
42	0	1	1	0	76	13	0.329
43	0	0	1	0	114	14	0.325
44	1	0	4	1	7424	363	0.327
45	1	1	1	0	426	12	0.318
46	1	0	3	0	5463	181	0.267
47	0	0	0	0	78	3	0.315
48	0	0	1	3	419	10	0.284
49	0	0	0	0	53	3	0.305
50	1	1	4	2	6562	361	0.262
51	1	1	3	2	2550	128	0.296
52	0	0	0	0	77	8	0.304
53	0	0	0	0	43	7	0.273
54	0	0	1	0	148	13	0.301
55	0	0	1	0	88	10	0.263
56	0	0	2	0	1188	114	0.256
57	0	0	2	1	1117	118	0.315
58	0	0	1	0	137	9	0.321
59	0	0	2	1	1094	99	0.319
60	0	0	2	2	1779	111	0.264
61	0	0	4	3	7004	271	0.310
62	0	0	0	0	54	5	0.309
63	0	1	1	1	184	16	0.278
64	0	0	0	0	40	9	0.293
65	2	2	1	0	450	11	0.332
66	0	0	1	2	119	13	0.335
67	0	0	2	1	1411	108	0.297
68	0	0	2	0	1075	112	0.314
69	0	0	1	0	445	10	0.301
70	0	0	0	0	48	9	0.334

Visual and image analysis scores for TIMP-1

Patient No.	Visual Score				Image Analysis Score (Adjacent Liver)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
71	0	1	1	0	480	9	0.258
72	0	0	0	0	58	9	0.323
73	0	0	0	0	36	8	0.251
74	0	0	0	0	58	7	0.296
75	1	1	0	0	49	3	0.322
76	0	0	0	0	48	9	0.269
77	1	1	0	0	59	3	0.264
78	0	0	1	2	197	9	0.299
79	0	0	2	1	975	118	0.267
80	1	0	4	4	6688	334	0.273
81	0	0	2	1	1105	104	0.283
82	1	2	3	2	3328	132	0.251
83	0	0	4	1	7149	275	0.258
84	0	0	1	0	221	18	0.287
85	2	2	2	1	1317	76	0.330
86	0	0	2	3	1709	120	0.333
87	0	0	3	2	3055	187	0.335
88	0	1	2	2	1267	109	0.332
89	0	0	0	0	76	3	0.267
90	0	0	2	4	2052	106	0.265
91	3	2	2	0	1041	95	0.277
92	0	0	1	1	487	17	0.314
93	0	0	0	0	63	7	0.326
94	1	1	3	3	4014	168	0.254
95	0	0	0	0	68	4	0.250
96	0	0	0	0	61	4	0.295
97	0	0	0	0	37	8	0.337
98	0	0	1	1	380	15	0.279
99	0	0	0	0	64	7	0.267
100	0	0	0	1	54	5	0.296
101	0	0	3	2	5641	152	0.288
102	0	0	2	0	1740	111	0.256
103	0	0	0	0	40	4	0.273
104	0	0	1	3	442	10	0.289
105	0	1	1	2	340	12	0.257

Visual and image analysis scores for TIMP-2

Patient No.	Visual Score				Image Analysis Score (Adjacent Tumour)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
1	5	5	4	4	24953	454	0.339
2	3	3	0	0	6039	367	0.313
3	4	3	0	0	23176	671	0.266
4	4	3	0	0	20100	630	0.331
5	2	1	0	0	3623	216	0.280
6	1	1	0	0	487	55	0.247
7	3	0	1	1	4587	151	0.152
8	2	2	0	0	4272	182	0.292
9	2	3	0	0	3826	214	0.273
10	1	2	0	0	409	60	0.311
11	2	1	0	0	3769	128	0.320
12	1	1	0	0	763	52	0.300
13	3	0	0	0	6207	349	0.274
14	2	2	0	0	2605	149	0.277
15	2	2	0	0	2559	161	0.311
16	4	4	3	1	12123	526	0.335
17	3	0	1	0	5574	266	0.266
18	4	3	1	0	21113	677	0.253
19	4	4	0	0	21292	501	0.319
20	1	3	0	0	339	24	0.320
21	0	1	0	0	41	3	0.318
22	3	3	1	0	7506	371	0.308
23	3	2	0	0	7515	299	0.292
24	3	3	1	0	8140	354	0.287
25	3	3	0	0	6314	321	0.333
26	3	3	0	0	7120	332	0.335
27	1	1	0	0	1220	66	0.315
28	5	4	0	0	20426	683	0.329
29	3	3	0	0	5216	286	0.294
30	3	2	0	0	5576	317	0.325
31	3	2	0	0	6588	330	0.280
32	3	3	0	0	5927	370	0.262
33	2	3	0	0	2310	168	0.245
34	4	3	3	0	13353	500	0.290
35	3	3	1	0	5743	270	0.287

Visual and image analysis scores for TIMP-2

Patient No.	Visual Score				Image Analysis Score (Adjacent Tumour)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
36	2	1	0	0	2360	182	0.294
37	3	3	0	0	7011	322	0.253
38	3	3	0	0	5146	329	0.280
39	0	1	0	0	165	11	0.313
40	3	2	0	0	7915	276	0.314
41	2	1	0	0	3651	184	0.337
42	3	3	0	0	5696	311	0.266
43	2	2	0	0	3137	122	0.270
44	3	3	0	0	6639	359	0.252
45	1	2	0	0	775	37	0.239
46	1	0	0	0	853	59	0.261
47	0	0	0	0	81	6	0.288
48	2	3	0	0	3118	178	0.277
49	1	0	0	0	514	35	0.336
50	2	2	0	0	2842	181	0.275
51	3	3	0	0	5647	354	0.242
52	1	1	0	0	743	38	0.271
53	3	3	0	0	5297	297	0.260
54	3	3	0	0	8581	337	0.281
55	4	4	2	0	19263	625	0.295
56	4	4	1	0	12807	565	0.247
57	3	3	0	0	7767	273	0.307
58	3	3	0	0	8180	304	0.330
59	3	4	1	0	5872	274	0.297
60	4	4	1	0	12599	647	0.240
61	4	3	1	0	21926	668	0.335
62	1	4	0	0	973	34	0.297
63	2	2	0	0	2783	139	0.287
64	2	1	0	0	2552	214	0.253
65	4	3	0	0	13494	625	0.325
66	4	2	1	0	13185	657	0.325
67	2	3	0	0	3702	120	0.245
68	4	4	0	0	14956	624	0.252
69	4	3	2	0	16506	571	0.316
70	1	1	0	0	542	47	0.307

Visual and image analysis scores for TIMP-2

Patient No.	Visual Score				Image Analysis Score (Adjacent Tumour)		
	Tumour		Liver		Count Area (μm^2)	Count Number	Field Area (mm^2)
	Adjacent	Distant	Adjacent	Distant			
71	1	2	0	0	468	70	0.244
72	2	3	0	0	2307	187	0.325
73	4	4	0	0	13811	565	0.294
74	1	1	0	0	447	23	0.339
75	2	2	0	0	3323	167	0.249
76	1	2	0	0	671	77	0.336
77	3	3	1	1	8578	338	0.337
78	2	2	2	0	2944	142	0.285
79	3	3	0	0	5479	362	0.316
80	4	4	0	0	21065	622	0.319
81	4	3	1	0	19695	635	0.262
82	4	2	0	0	11808	559	0.256
83	2	4	0	0	3628	138	0.320
84	2	1	0	0	4139	124	0.289
85	4	3	0	0	15309	455	0.290
86	2	2	0	0	3084	179	0.250
87	4	3	1	0	11380	487	0.341
88	3	4	0	0	5548	261	0.338
89	1	1	0	0	782	75	0.271
90	4	4	0	0	15021	648	0.327
91	3	3	0	0	7749	292	0.298
92	4	4	1	0	19109	510	0.333
93	2	1	0	0	3404	142	0.314
94	2	2	0	0	4315	200	0.312
95	4	4	0	0	20964	556	0.265
96	1	0	0	0	1439	53	0.245
97	5	5	0	0	19663	492	0.342
98	4	3	0	0	20096	548	0.290
99	4	4	0	0	11208	547	0.292
100	3	2	0	0	4761	284	0.306
101	4	4	0	0	19122	502	0.283
102	3	3	1	0	7591	328	0.253
103	3	4	0	0	8378	351	0.265
104	2	2	0	0	2370	113	0.315
105	3	3	1	0	7260	270	0.326

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