UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

SURGERY

Doctor of Medicine

THE EXPRESSION OF MATRIX METALLOPROTEINASES (MMPs) AND THEIR INHIBITORS (TIMPS) IN METASTATIC COLORECTAL CANCER (CRC).

By Stephen Richard Kelly

Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are enzymes involved in extracellular matrix breakdown and remodelling and have been implicated in tumour invasion and metastatic spread. This thesis investigates their role in patients with colorectal cancer (CRC) liver metastases.

The expression pattern of MMPs (Collagenases MMPs 1,8,13; Gelatinases MMPs 2,9; Stromelysins MMPs 3,7) and TIMPs (TIMPs 1 and 2) was examined in fresh tissue and paraffin sections from 30 patients undergoing hepatic resection for colorectal carcinoma metastases and 3 patients with benign liver lesions, using semiquantitative RT-PCR and immunohistochemistry. It was also examined, using immunohistochemistry, in the paraffin sections of the primary tumours giving rise to these liver metastases.

MMP 1, MMP 9, MMP 3 and MMP 7 mRNAs were highly expressed in colorectal liver metastases. Immunohistochemistry confirmed these findings, MMPs 1 and 9 being expressed by tumour stromal cells, whereas MMPs 3 and 7 were expressed by adenocarcinoma and stromal cells. TIMP 1 and TIMP 2 mRNA was expressed by both colorectal carcinoma metastases and distal liver. The expression of TIMP 1 and 2 mRNA in the adjacent liver was markedly lower than in both the metastasis and distal liver. In the benign lesions TIMP 1 and 2 mRNA expression appeared equal throughout. All the primary tumours expressed MMP 1,8,2,9,3,7 and TIMPs 1 and 2. Tumour stromal cells expressed MMP 1 (fibroblasts and macrophages), MMP 8 (fibroblasts), MMP 2 (fibroblasts), MMP 9 (macrophages). MMPs 3 and 7 were expressed by both adenocarcinoma cells and tumour stromal macrophages. TIMP 1 was expressed by macrophages in the tumour stroma and TIMP 2 by fibroblasts.

High expression of MMPs 1,9,3 and 7 in the CRC metastasis and down regulation of TIMP 1 and 2 in the immediately adjacent liver tissue may facilitate local growth of hepatic metastatic disease. Further studies are required to elucidate the mechanisms involved in this complex relationship between metastasis, liver, MMP and TIMP.

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PREFACE AND ACKNOWLEDGEMENTS

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DEFINITIONS AND ABBREVIATIONS USED

MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinase
CRC	colorectal carcinoma
CEA	carcinoembryonic antigen
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
DMSO	dimethylsulphoxide
IHC	immunohistochemistry
IVVM	intravital videomicroscopy

Chapter 1: COLORECTAL CANCER

1.1 Introduction

Colorectal cancer (CRC) is the second most common cause of death from malignancy in the Western world. There are 700,000 new cases of CRC each year world wide and 400,000 deaths attributable to it. In the U.K. CRC remains a major health problem with 28,904 new cases diagnosed in 1994 and 15,740 deaths, (ONS, 2000) making it the third commonest cancer, and second commonest cause of death due to cancer in the UK. The overall relative five year survival is currently only 50% (ONS, 2000).

1.2 Aetiology and Pathogenesis

Although hereditary factors are important in about 5% of cases of CRC, the majority of cases appear to be related to environmental influences. Dietary factors such as red meat and animal fat may be important promoters (Kritchevsky D 1993), whereas vegetables are protective (Weisburger JH 1991). It is postulated that a high dietary animal fat intake is associated with an increase in faecal bile acids and neutral steroids, which are degraded by certain anaerobic bacteria to produce carcinogens. (Nagao M 1993)
It is generally accepted that most, if not all adenocarcinoms of the large bowel arise from pre-existing adenomas (Muto T 1975). The adenoma-carcinoma sequence is a multi-step process involving sequential mutations or deletions of a number of genes including APC, K-ras, DCC and p53. (Vogelstein B 1988; Fearon ER 1990). It is now known that there are at least two completely different pathogenetic pathways for colorectal cancer (Lengauer C 1997). Both are thought to begin with inactivation or loss of the adenomatous polyposis coli (APC) gene, followed by the sequential loss of other tumour-suppressor genes and activation of cellular oncogenes.

The first pathway, which is thought to be the pathway in most sporadic cancers (80-85% of colorectal neoplasms) is the basis of familial adenomatous polyposis (FAP). In FAP there is a germline mutation in the APC gene, which is located on chromosome 5 and plays a central role in regulating proliferation and cellular adhesion by regulating the rate of degradation of β-catenin (Bodmer WF 1987). As every cell in FAP patients already has one mutation at an APC allele a very large number of adenomatous polyps will occur once an inactivating event occurs in the other wild type APC allele. This occurs at an earlier age (20-30 years) than in the normal population due to the presence of the preexisting mutations; the number of polyps and age of developing them depending on the exact site of the mutation in the APC gene (Wu JS 1998).

The second pathway for tumour development in the large bowel requires inactivation of the DNA mismatch repair (MMR) system. Loss of DNA MMR leads to a hypermutable state in which simple repetitive DNA sequences (microsatellites) are unstable during DNA replication (Ionov Y 1993). If these microsatellite sequences are located in the coding regions of genes involved in the regulation of cell growth, programmed cell death, and DNA MMR genes themselves, it leads to the accumulation of a large number of mutations granting the cell autonomy from normal growth controls (Boland CR 2000). The hereditary example of this pathway is hereditary nonpolyposis colorectal cancer (HNPCC) which makes up 5% of all colorectal cancers. This occurs when there is an inactivating germline mutation in one of the DNA MMR genes (Aaltonen LA 1998). Around 15% of all colorectal cancers have microsatellite instability (MSI), due to a defective DNA MMR system but only 20-25% of these are a result of HNPCC (Aaltonen LA 1998).

About 70% of adenomas are in the last 60cm of the colon and rectum (Granqvist S 1981). 60-70% of adenomas are tubular, 10% are villous and the rest are tubulovillous (Muto T 1975). Around 15% of patients have greater than 2 adenomas, the likelihood of

multiple polyps increasing with age and peaking at about 60 years of age. Four factors predispose to the development of carcinoma: size, histological type, presence of epithelial dysplasia and number of polyps. Large villous adenomas are more likely to develop malignancy than the commoner small tubular adenomas (relative risk 3.6% vs 0.5%) (Atkins WS 1992).

In the National Polyp Study, 1,418 patients were followed up for an average of 5.9 years after removal of all identifiable polyps at colonoscopy. There were only 5 cancers, all malignant Dukes A polyps, and no deaths from CRC. They calculated a 76 to 90% decrease in the incidence of colorectal cancer. (Winaner SJ 1993.)

1.3 Screening for Colorectal cancer

Thus in colorectal cancer there is a window of opportunity when effective intervention is possible and this has lead to support for screening programmes for CRC (Toribara NW 1995). However the question remains who should you screen and with what method.

Patients at high risk of developing CRC include those with familial adenomatous polyposis (FAP) syndrome, those with hereditary non-polyposis colorectal cancer (HNPCC) syndrome, and those with a predisposing disease (e.g long standing ulcerative colitis) (Standards Committee of American Society of Colon and Rectal Surgeons 1999).

1.3a Familial adenomatous polyposis (FAP) syndrome

FAP accounts for less than 1% of all colorectal cancers. It results from mutations in the adenomatous polyposis coli (APC) gene on chromosome 5 and is inherited as an autosomal dominant condition (Bodmer WF 1987). Its estimated occurrence is 1/8300 to 1/14025 live births; one third of cases arise as spontaneous mutations; and it is characterised by hundreds of adenomas in the colon and numerous extracolonic

manifestations. FAP family members should undergo regular sigmoidoscopy starting in adolescence (Standards Committee of American Society of Colon and Rectal Surgeons 1999). Previously total colectomy with ileorectal anastomosis was performed when the patient was in their mid 20s. However recent widespread acceptence and proven safety of the ileoanal pouch procedure has led to total colectomy, mucosectomy and handsewn ileoanal pouch formation being performed for pateints with FAP in their late teens/early twenties (Daniels IR 1999). Upper GI endoscopic screening is also advised as there is a high incidence of upper GI tumours (Box JC 1995). Both gastric and duodenal polyps and carcinomas occur, usually in the patients fifth and sixth decades, the treatment of which is not as straight forward or successful as the colonic polyps (Wallace MH 1998).

Data supports the use of molecular techniques (genetic testing) to establish the presymptomatic diagnosis of FAP (Powell SM 1993) and National Polyposis Registries have been established to coordinate this and collect data. Genetic testing has also been used as a guide to the surgical management of FAP (Vasen HF 1996, Wu JS 1998). Several investigators have tried to establish whether there is a correlation between a specific APC gene mutation and clinical phenotype, (Nugent KP 1994; Gayther SA 1994; Caspari R 1994) but other studies do not support this (Paul P 1993).

The site of the APC mutation, however does appear to have direct influence on the expression of congenital hypertrophy of the retinal pigment epithelium (CHRPE) an extra colonic manifestation of FAP (Olschwang S 1993; Wallis YL 1994), and the attenuated form of APC (AAPC) also appears to result from mutations within a specific region of the APC gene (Spirio L 1993).

1.3b Hereditary non-polyposis colorectal cancer (HNPCC) syndrome

HNPCC accounts for 5% of all colorectal cancers. It is characterised by early age of onset, right sided colorectal cancer, multiple primary sites (synchronous and

metachronous) in a non-polyposis pattern, and the propensity to develop carcinoma of the endometrium, ovary, stomach, small bowel, ureter and renal pelvis. The condition is caused by a dominantly inherited alteration in the DNA mismatch repair (MMR) genes (Marra G 1995). Defective repair may result in the accumulation of more mutations, leading to instability in the length of microsatellite sequences in the DNA from HNPCC tumours (microsatellite instability, MSI) (Moore J 1999). So far six MMR genes have been identified: hMLH1, hMSH2, hMSH6, hPMSI, hPMS2 and hMSH3. The syndrome is diagnosed using the Amsterdam criteria: at least 3 family members with CRC, at least 2 generations affected (one a first degree relative of the other two), and at least one individual less than 50 years of age at the time of diagnosis (Vasen HF 1991). Colonoscopic screening should begin at least 5 years before the earliest onset of CRC in the family (Thorson AG 1999).

Individuals from kindreds with known mutations in MMR genes may be directly tested for these mutations because they are inherited in the germline. In HNPCC kindreds in which the mutation is not known the first thing that should be established is whether tumours are RER +. In RER – cases it is extremely unlikely that a mutation in a MMR gene will be found (Wijnen JT 1998).

1.3c Sporadic cancers

Sporadic cancers account for about 95% of all cases of colorectal cancer. The vast majority of cancers in this group occur after 50 years of age and for this reason asymptomatic people over 50 have been labelled as an average risk group and may benefit from screening (Standards Committee of American Society of Colon and Rectal Surgeons 1999). In the United Kingdom two methods are now available for screening these individuals in the population: faecal occult blood testing (FOBT) and flexible sigmoidoscopy.

FOBT is aimed at the detection of early asymptomatic cancers. Sensitivity is limited because FOBT fails to detect 20-50% of cancers and upto 80% of polyps. Specificity is also low because the ingestion of red meat, some raw vegetables and fruit may yield false positive results. Sensitivity is increased by rehydrating the slides but at the expense of specificity. The main advantage of FOBT is its low cost, although the subsequent radiological or endoscopic investigation of those who test positive increases costs.

The results of three randomised control trials using FOBT have been published. In the Minnesota study (Mandel JS 1993) there was a 33% reduction in colorectal cancer related mortality in patients randomised to annual screening. However as rehydration slides were used false positive results were common and as a result almost 40% of patients underwent colonoscopy during the study. The reduction in mortality observed may simply be a reflection of the high proportion of patients undergoing colonoscopy.

In the Nottingham study (Hardcastle JD 1996) patients underwent biennial screening and the compliance rate was 59%. Slides were not rehydrated, reducing the number of false positive results. Only 4% required radiological or colonoscopic investigation. Overall there was an increase in the proportion of Dukes' stage A tumours detected and a 15% reduction in CRC related mortality. 28% of all cancers occuring in the screened group were interval cancers emphasising the low sensitivity of the test. Similar results were obtained in the Danish study (Kronberg O 1987).

Flexible sigmoidoscopy is a more expensive investigation but has the advantages of being highly sensitive and lesions can be removed endoscopically, making it both diagnostic and therapeutic. The progression from polyp to carcinoma is probably a slow and orderly one, taking 10-15 years to complete. A single flexible sigmoidoscopy in people in their late fifties may therefore offer protection from distal cancers until they are almost 70 years old and this approach is being investigated in a large MRC funded multi-

centre trial (Atkin WS 1993). This trial has reported its results from the recruitment and screening phase (Atkin WS 2002). The study contacted 354,262 people aged between 55-64 years in 14 UK centres to ask if they would attend for flexible sigmoidoscopy screening. 55% (194,726) said yes and this group was randomly allocated to screening or no screening (control group). 71% (40,674 of 57,254) of the people invited to sigmoidoscopy screening attended. 5% of these (2,131 people) were classified as high risk on the basis of the sigmoidoscopy result and were referred for colonoscopy. Distal adenomas were detected in 4931 (12.1%) and distal cancer in 131(0.3%). Proximal adenomas were found in 386 (18.8%) of those having colonoscopy and proximal cancer in nine cases (0.4%). 62% of cancers were Dukes' stage A or locally excised. There was one perforation following flexible sigmoidoscopy and four following colonoscopy. It will be some time before the trial provides data on the incidence of and mortality from colorectal cancer, but these results indicate that the test is acceptable, safe and provides clinically useful information.

1.4 Treatment of Colorectal cancer

The treatment of colorectal cancer is dependent on the stage of the disease at presentation. Once colorectal cancer has become invasive there are five methods of tumour spread:

1) direct invasion of adjacent structures
2) lymphatic permeation and dissemination
3) venous embolisation
4) transperitoneal seeding
5) intraluminal implantation

A number of staging classifications have been designed that enumerate the characteristics of the primary tumour and the presence or absence of regional and distant

metastases in order to allow appropriate therapy to be chosen and predict the probability of survival. Despite many attempts to modify it, the main staging system remains the Dukes classification (Dukes C 1932) consisting of Dukes A (confined to bowel wall), Dukes B (growth through the bowel wall into the surrounding fat but lymph nodes clear), Dukes C (lymph nodes involved). The TNM classification is also in widespread use giving information about the tumour (T 1-4), the nodal involvement (N 1-3) and any metastases (M 0-1).

1.4a Surgery/Radiotherapy

The treatment of CRC is primarily surgical resection, the goal of which is a wide resection of the involved segment of the colon or rectum together with removal of regional lymphatic drainage. This can only be deemed curative for the small number of patients (6-10%) with Dukes' A carcinoma. The outlook for patients with CRC remains poor largely because of the advanced stage of the disease at presentation. Hopefully screening programmes (if adopted) will improve this but advanced CRC will remain a problem in surgical practice as the compliance rate in the Minnesota study was 75% and in the Nottingham study only 59%.

Efforts are continuing to be made to try and improve the survival from colorectal cancer. Previously, despite apparently curative resection about 25% of patients with rectal cancer developed pelvic recurrence usually within 24 months of surgery. The likelihood of pelvic recurrence increases with stage, histological grade and presence of microscopic tumour involvement at the resection margins. A number of series have shown that pelvic recurrence rates can be kept below 10% if total mesorectal excision (TME) is performed (MacFarlane JK 1993) and this has become standard practice amongst specialist colorectal surgeons. There is now some evidence that adjuvant radiotherapy given either pre or post operatively reduces local recurrence rates (Swedish Rectal Cancer Trial 1997). It was felt that some of this improvement with radiotherapy was simply due to compensation for poor

surgical technique and a surgical teaching initiative to train surgeons in TME has recently been shown, in a population-based study, to have a major effect on cancer outcomes, decreasing the proportion of APEs and reducing LR rates by more than 50% with early evidence of a decline in rectal cancer mortality (Lehander Martling A 2000).

However the Dutch Colorectal Cancer Group (Kapiteijn E 2001) has recently published a randomised controlled trial in which 1861 patients with resectable rectal cancer were randomised to receiving preoperative radiotherapy (25 Gy in 5 fractions) followed by total mesorectal excision (924 patients) or total mesorectal excision alone (937 patients). At 2 years follow up there was no difference in survival (82% and 81.8% respectively $p=0.84$). The local recurrence rate at 2 years was significantly less in the radiotherapy plus surgery group at 2.4% compared to surgery alone group with a recurrence rate of 8.2% ($p<0.001$). This backs up the feeling that preoperative radiotherapy halves the local recurrence rate following surgery for rectal cancer but that surgery should still be carried out precisely using the techniques advocated by Heald.

Furthermore, despite the feeling among many surgeons that short course RT is harmless, the total radiation dose is very similar to long course RT. Indeed one prospective audit reported a high anastomotic leak rate (after anterior resection) and a high perineal wound infection rate (after abdominoperineal resection) (Lele S 2000). There is as yet no long term data on the incidence of small bowel damage and pelvic or femoral fractures following short course RT. To try and clearly define the role of radiotherapy in rectal cancer the MRC has launched a study (CR07) comparing preoperative radiotherapy for all patients, with selective post operative radiotherapy for those with evidence of circumferential resection margin involvement at the time of surgery.

An exciting recent development is the use of 3mm slice MRI with phased-array coil to delineate the projected circumference of the TME resection margin in relation to

the cancer which may facilitate preoperative identification of patients at risk of recurrence who would benefit from preoperative RT (Beets-Tan RGH 2001).

A further development is the increasing popularity of laparoscopic surgery to perform colorectal operations, especially in North America. Initially these were for benign conditions but enthusiasts quickly turned the technique to colorectal cancer. However a number of oncological concerns very soon became apparent including the staging and extent of the resection performed and the incidence of port site recurrences (Young-Fadok 1999). As a result of these concerns upto seven major trials are ongoing to try and delineate the role of laparoscopic surgery in colorectal cancer.

The American NIH-supported Laparoscopic Versus Open Colectomy for Cancer Trial requires 1,200 patients to be randomly assigned to laparoscopic or open arms (Nelson H 1994). Randomisation is stratified based on tumour site, primary surgeon, and American Society of Anesthesiology (ASA) operative risk classification. Eligible patients are those with confirmed adenocarcinoma of the right, left or sigmoid colon (transverse colon and rectal lesions having been left out due to technical difficulties). All surgeons involved have performed more than 20 laparoscopic procedures and have agreed to perform resections to standardized guidelines, random audits of videotaped laparoscopic procedures are performed to ensure these guidelines are not being broken. The primary aim is to determine if overall survival and disease-free survival are equivalent, regardless of the approach used. The secondary aim is to assess the safety of laparoscopic colectomy in terms of early and late morbidity and perioperative mortality. The tertiary aim is to perform a formal cost-benefit analysis, using Q-TwiST (quality-adjusted time without symptoms of disease and toxicity of treatment) methodology. Preliminary results are encouraging and after 4 years of the trial 62 surgeons from 45 centres in North America have acrued 530 patients. The two arms are well matched and available data suggest that there is no significant difference in the length of resected bowel, proximal and distal

margins, and mesenteric clearance (measured by length of vascular pedicle and number of lymph nodes). There is also an independent Data Monitoring Committee with the power to close the trial if there is a statistically significant difference in recurrences in either arm and this has not happened in the 4 years of the trial.

The United Kingdom MRC Classic trial differs from the NIH trial in that it allows entry of patients with rectal cancer and stage IV (Dukes' D) disease and in pathological examination of specimens including radial margins for colon as well as rectal specimens. The only randomised trial to report so far is a single centre trial from Barcelona (Lacy AM 2002). Despite the question of the generalisation of their results to a wider patient and surgical population, they showed that for non-metastatic colon cancer, in their hands laparoscopy-assisted colectomy is more effective for treatment of colon cancer in terms of morbidity, hospital stay, tumour recurrence and cancer-related survival compared with open colectomy. They postulated the beneficial effect on tumour recurrence and cancer-related survival may stem from the lesser surgical and immunological insult caused by laparoscopic surgery but cautioned against widespread adoption of laparoscopy-assisted colectomy as the standard surgical approach until the multicentre randomised trials have reported their results.

1.4b Chemotherapy

Further attempts to improve the survival of patients with CRC has seen the introduction of adjuvant chemotherapy, the rationale for which is attempting to eradicate the remaining microscopic tumour foci when the tumour burden is at its lowest. 5-fluorouracil is the mainstay of treatment. It acts during the S phase of the cell cycle via a cytotoxic metabolite (fluorodeoxyuridine monophosphate – FdUMP) that binds to thymidylate synthase, thereby impairing pyrimidine synthesis. In 1988 a meta analysis of more than 6000 patients included in randomised clinical trials suggested that a regimen

containing 5-FU administered over an extended period of time reduced the mortality rate by about 17%, equivalent to an increase in 5 year survival of 3% (Buyse M 1988).

In 1990 the American Intergroup Study reported a reduction in overall mortality of one third in patients with Dukes C tumours with the administration after surgery of a one year course of 5-FU and levamisole, although levamisole alone was of no benefit. (Moertel CG 1990) These results were maintained in the final report at a median follow up of 6.5 years (Moertel CG 1995), with a reduction in tumour recurrence of 41%. On the basis of the 1990 report the National Cancer Institute in the USA advocated that all patients with Dukes C colon cancer should be treated with 5-FU and levamisole for 1 year.

An alternative approach has been to combine 5- FU with folinic acid. It has been shown that folinic acid can stabilize the ternary complex of 5-FdUMP and thymidylate synthase, thereby increasing the degree of thymidine depletion and cytotoxicity of 5-FU. Studies of 5-FU combined with high dose folinic acid for 6 months after operation have demonstrated a significant improvement in survival in patients with Dukes C colonic cancer. (Francini G 1994; IMPACT 1995) Provisional results from ongoing trials indicate that there is no increased benefit from 12 months of therapy compared with the benefit obtained by 6 months of treatment (O'Connell MJ 1996), that there is no significant therapeuic difference between the addition of levamisole or of folinic acid to 5-FU (Woolmark N 1999), and that the addition of levamisole to 5-FU and folinic acid provides no advantage (Haller DG 1996). Overall, 5-FU containing adjuvant chemotherapy appears to generate a survival benefit in patients with Dukes C colonic carcinoma.

Despite this evidence the extent of benefit remains uncertain, particularly in certain subgroups. The UKCCCR (UK Coordinating Committee for Cancer Research) launched the QUASAR (Quick and Simple and Reliable) study in 1994 to randomise a large heterogenous group of CRC patients. Patients considered to be at low risk (uncertain indication) were randomised to no treatment or chemotherapy; patients at high risk

(certain indication) received 5-FU (370mg/m^2) and randomised to either high dose (175mg) or low dose (25mg) folinic acid +/- levamisole (50mg). Between 1994 and 1997 4927 patients were enrolled. The primary endpoint was mortality from any cause, analysed by intention to treat. Survival was similar with high-dose and low dose folinic acid (70.1% vs 71.0% at 3 years; p=0.43), as were 3 year recurrence rates (36% vs 34.9% at 3 years; p=0.16). This study showed that the use of levamisole and the use of higher over lower doses of folinic acid did not confer any additional survival or recurrence benefit over low dose (Quasar Collaborative Group, 2000).

The National Surgical Adjuvant Breast and Bowel Project (NSABP) R-02 trial randomised patients with Dukes' B and C carcinoma of the rectum to either receive post operative adjuvant chemotherapy alone (n=348) or chemotherapy with radiotherapy (n=346). All female patients (n=287) received 5 FU and leucovorin. 200 male patients received 5 FU and leucovorin while 207 male patients received 5 FU, semustine and vincristine (MOF). Average follow up was for 93 months. Postoperative radiotherapy reduced the incidence of local recurrence from 13% to 8% at 5 year follow up (p=0.02) but showed no improvement in disease free survival (p=0.90) or overall survival (p=0.89). Male patients who received 5 FU and leucovorin had significantly better 5 year disease free survival (55%) compared to the group receiving MOF chemotherapy (47%; p=0.009). No significant difference was reported in overall 5 year survival (65% vs 62%, respectively; p=0.17) (Woolmark N 2000).

An alternative approach for adjuvant chemotherapy is regional chemotherapy using the portal vein as a route of administration. The liver is the commonest site of distant spread of colorectal tumours and as 60% of 5-FU is extracted by the liver on first pass only small amounts of drug escape into the systemic circulation, making toxicity low. A meta-analysis of ten published trials of portal vein infusion (Piedbois P 1995) indicated a 20% reduction in risk of death from colorectal cancer with a significantly greater benefit

in patients with Dukes C carcinoma compared with Dukes B. However a randomised controlled multicentre trial investigating the efficacy of PVI (portal-vein infusion) has shown no benefit over surgery alone. (Rougier P 1998). The UKCCCR Adjuvant X-ray and 5-FU Infusion Study (AXIS) trial recruited nearly 4000 patients and again has shown no overall benefit with PVI for rectal and colon cancer together (AXIS Collaborators 2003). However separating colonic tumours and combining them in a meta-analysis incorporating previous trials of PVI gives an absolute survival benefit for patients with colonic cancer of 5.8%.

1.5 Postoperative surveillance and recurrence

The goals of postoperative surveillance are the detection of recurrent tumour at a stage when it is still curable and the detection and prevention of metachronous carcinoma. Almost all recurrent tumours are seen within 5 years of resection for colorectal carcinoma: 85% recur in the first 30 months with the rest detected in the next 30 months. (Kelly CJ 1992). Therefore follow-up evaluation must be most intensive in the first several years following resection when the patient is at highest risk. Patients at greatest risk of recurrence are those with advanced disease stage and higher grades of neoplasia. Recurrent colorectal cancer may present as local regional tumour, regional or distant tumour or distant tumour only. The most common site of recurrent disease is distant, with liver or lung metastases or both.

The methods of surveillence include colonoscopy for recurrent or metachronous polyps and cancers, tumour markers (CEA), and US scan or CT scan looking for liver metastases. At present there is no universal surveillence policy and studies have failed to show any survival advantage.

Kjeldsen and colleagues randomised 600 patients to either six monthly follow up or to follow up visits at five and ten years only (Kjeldsen B 1997). Investigations included

chest xray and colonoscopy, no routine liver imaging was performed. Recurrence rates were similar in both groups (26%) but the recurrences were detected on average nine months earlier in the intensive group. However no difference existed in overall survival (68% vs 70%) or cancer related survival (Kjeldsen B 1997).

Schoemaker evaluated intensive follow up with yearly chest xray, colonoscopy and liver CT scan in addition to standard followup based on clinical examination, FOB, liver function tests and CEA measurement (Schoemaker D 1998). At five years fewer patients in the intensive group died but the result was not significant.

1.6 Management of Advanced disease

1.6a Liver metastases

Even though colorectal cancer metastases primarily to the regional lymph nodes historically up to 25% of patients had synchronous hepatic metastases (Bengmark S 1969) at the time of detection of the primary lesion. Metachronous liver metastases can develop following a curative colorectal resection and the cumulative incidence of such metastases is 6%, 17% and 20% after first, third and five years following resection of the primary tumour. However it is unlikely that a patient will develop liver metastases after 5 years (Launois B 1994). Untreated, the prognosis in this group is poor, with median survival less than 12 months, and it is estimated that 40-67% of patients with colorectal cancer die due to liver secondaries (Parker SL 1997). A large prospective study of prognostic factors in patients with unresected hepatic metastases from colorectal cancer identified six independent determinants of survival: percentage liver volume replaced by tumour (LVRT), grade of malignance of primary tumour, presence of extrahepatic disease, mesenteric lymph node involvement, serum carcino-embryonic antigen, and age (Stangl R 1994). Prolonged follow up of untreated patients with liver metastases, including solitary lesions confirms survival beyond 5 years is rare. These grim statistics have led to a degree

of nihilism among clinicians about the treatment options for patients with hepatic metastases (Logan S 1982).

These results contrast with those from most series of hepatic resection for colorectal liver metastases in which five year survival rates of 25-44% are reported. Some authors report 10 and even 20 year survivors (Scheele J 1990, Adson MA 1984). These results have led to an increased acceptance of surgery for hepatic metastases in the last 20 years. They are due to refinements of surgical technique and advances in metabolic, haemodynamic and respiratory support allowing hepatic surgery to be performed safely with minimal blood loss; and the realisation that there are a proportion of patients in whom metastatic spread from colorectal cancer is confined to the liver. Two factors appear to be clearly associated with poorer outcome following liver resection: involved resection margins and the presence of extrahepatic disease (including hilar and coeliac axis lymph nodes) at the time of liver resection. Some studies have shown that a resection margin of > 1cm compared to <1cm is an advantage (Nordlinger B 1996, Scheele J 1995, Shirabe K 1997), although others have reported that a smaller margin does not affect prognosis so long as it is clear of tumour (Rees M 1997, Ohlsson B 1998). None of the other factors related to the patients, their primary tumour or the metastases themselves have been conclusively shown to adversely effect long-term survival following liver resection for metastatic colorectal carcinoma (Memon MA 2001).

Unfortunately only 10-20% of patients with colorectal liver metastases have potentially resectable liver metastases (Scheele J 1990). Alternative treatment strategies for patients with unresectable liver metastases from colorectal cancer include regional chemotherapy (via a hepatic artery catheter), cryosurgery, radio-frequency tissue ablation, percutaneous ethanol injection, radiation therapy, arterial embolisation and chemo-embolisation (Cascinu S 1998). Randomized trials comparing hepatic arterial chemotherapy with conventional systemic chemotherapy have shown that response rates

achieved with intra-arterial therapy are significantly greater than those achieved by intravenous therapy but there has been no consistent survival advantage. The MRC has just completed a multicentre randomised trial of intrahepatic arterial versus conventional intravenous fluorouracil and folinic acid for colorectal cancer liver metastases (Kerr DJ 2003). In both arms 5-FU modulated by folinic acid is given as a 48 hour infusion. Theoretically, as only a proportion of the 5-FU administered intra-arterially will be absorbed in the liver the remaining 5-FU will enter the lungs and eventually the systemic circulation, thereby achieving both a regional and systemic effect. They found there was no advantage for the intra-arterial regimen in the 290 patients studied: median overall survival was 14.7 months compared to 14.8 in the intravenous group. However 50 (37%) of the allocated patients did not start intra-arterial treatment and in another 39 (29%) the catheter blocked early.

1.6b Disseminated disease

5-FU has a short half-life and because only 3% of the cells are in S-phase at any one time bolus administration is relatively ineffective. The efficacy of 5-FU can be enhanced by prolonging the duration of binding to thymidylate synthase either by administration of 5-FU as an infusion or by biochemical modulation of 5-FU by folinic acid.

The de Gramont regimen combines bolus and infusional 5-FU with a two hour infusion of high dose folinic acid and is widely used in advanced disease in the UK. An alternative approach is to use protracted venous infusion for several weeks or months. The 5-FU is administered via an indwelling central venous line using a portable pump enabling patients to be treated continuously at home. Response rates of 30-35% have been achieved using this regimen.

A number of new agents are also available. These include a new thymidylate synthase inhibitor, raltitrexed, which is given as an intravenous bolus once every three weeks. Preliminary studies suggest that response rates are similar to that of conventional 5-FU/folinic acid schedules. In the UK the de Gramont regimen, protracted venous infusion and raltitrexed are currently being assessed in a randomised clinical trial (MRC CR06). Raltitrexed is also currently being compared with 5-FU/folinic acid as adjuvant therapy in colon cancer in a pan-European study (PETACC). Oxaloplatin and irinotecan, a topoisomerase I inhibitor are currently being evaluated. The addition of oxaloplatin to the de Gramont regimen (Folfox 1-6) increases the response rate in advanced disease (Bleiberg H 1998), and neoaduvant treatment with folfox regimens increases the operability of colorectal cancer liver metastases. Irinotecan, as second line therapy, has recently been shown to prolong median survival by 2.5 months and improve quality of life (QoL) compared with best supportive care or infusional 5-FU (Rougier P 1998; Cunningham D 1998). Douillard et al investigated the addition of irinotecan to 5 FU and folinic acid as treatment in patients with metastatic colorectal cancer. Patients (n=387) previously untreated with chemotherapy (other than adjuvant) were randomised to receive 5 FU and calcium folinate with or without irinotecan given by once weekly or fortnightly infusion. Patients in the irinotecan group showed significantly higher response rates (49 vs 31%, $p<0.001$ for evaluable patients, 35 vs 22%, $p<0.005$ by intention to treat), longer time to progression (median 6.7 vs 4.4 months, $p<0.001$) and longer overall survival (median 17.4 vs 14.1 months, $p=0.031$) (Douillard JY 2000). Irinotecan combinations should therefore be considered as first line treatment in metastatic colorectal cancer.
The irinotecan study group randomised 683 patients to three treatment regimens. 231 were assigned to receive irinotecan, fluorouracil and leucovorin; 226 to receive fluorouracil and leucovorin; 226 to receive irinotecan alone. Compared to fluorouracil and leucovorin, combination therapy with irinotecan, fluorouracil and leucovorin resulted in a significantly

longer progression free survival (7 vs 4.3 months, p=0.004), a higher response rate (39% vs 21%, p<0.001) and longer overall survival (14.8 vs 12.6 months, p=0.04). The results of irinotecan alone were similar to fluorouracil and leucovorin (Saltz LB 2000). It was concluded that treatment with irinotecan, fluorouracil and leucovorin was superior to fluorouracil and leucovorin.

However preliminary results from two current trials, N9741 and C89803 have shown an unexpectedly high mortality rate associated with the use of identical combinations of irinotecn, fluorouracil and leucovorin, resulting in suspension of enrolment (Sargent DJ 2001).

Oral fluoropyrimidines (capecitabine) have been used in the advanced disease setting and they are as effective as 5-FU but are safer and the oral route makes them more convenient for most patients. The use of capecitabine, oxaliplatin and irinotecan in the adjuvant setting is being investigated in clinical trials (Tebbutt NC 2002).

Chapter 2: TUMOUR INVASION AND METASTASIS

2.1 The extracellular matrix

Recently a greater understanding of tumour invasion and metastasis (Hart I 1992) has lead to the investigation of novel approaches in research and treatment of cancer which include modification of the tumour/host interaction. The key characteristic of a malignant tumour cell is its ability to invade and destroy surrounding tissue and metastasise to distant loci. The first barrier the malignant tumour cell meets is the extracellular matrix (ECM). The ECM is divided into two components: the basement membrane and the interstitium.

2.1a The Basement membrane

Basement membranes are thin, continuous extracellular structures that separate organ cells, epithelia and endothelia from the interstitium. They are laid down during organogenesis by adjacent cells which remain attached to them via specific cellular receptors (Flug M 1990). They act as an interface between histologically different tissues and are involved in numerous processes such as tissue repair, ultrafiltration (e.g. glomerular basement membrane of the kidney), cell attachment and physical support. They also act as a barrier for non-blood derived cells and are essential for maintaining the polarity of epithelial cells resting on them. The basement membrane is composed of three layers:

1) lamina lucida : adjacent to epithelial cells
2) lamina densa or basal lamina
3) lamina fibroreticularis: attaches the lamina densa to the surrounding connective tissue

Type IV collagen accounts for up to 60% of the total protein content of basement membranes and lends the structure some flexibility. This is due to non-collagenous

interruptions in the triple helical structure. However this leaves type IV collagen open to proteolytic attack. Table 1 shows the main constituents of the basement membrane.

Table 1: Main constituents of the basement membrane.

Component	Size of molecule (M_r x 10^{-3})	Molecular structure	Features
Type IV collagen	550-600	$[\alpha 1(IV)]_2\alpha 2(IV)$	Basement membrane specific, major structural component, assembles into network
Laminin	1000	A,B1,B2	Basement membrane specific, facilitates cell binding to matrix
Heparan sulphate	500	Core protein (M_r 350,000) 3 to 6 side chains (M_r 65,000)	Interact with type IV collagen, laminin and fibronectin. Role in filtration of macromolecules
Condroitin sulphate	200-300	Core protein (M_r 30,000). 10-20 side chains (M_r 15,000)	
Entactin	150	Single chain	Basement membrane specific

The result is a highly meshed, tough matrix which cannot be penetrated, with respect to tumour invasion, without mechanical or chemical degradation.

2.1b The interstitium

Interstitial connective tissue is a complex structure of cells located in a meshwork of collagen fibres and hyaluronic acid, glycoprotein and proteoglycan ground substance (Tryggvason K 1987). It has major mechanical and supportive functions and is present in different forms such as bone, tendons, ligaments, fasciae and stroma. Depending on its form it may contain fibroblasts, osteoblasts, chondrocytes and macrophages. Table 2 gives the main constituents of the interstitium and their distribution.

Collagen fibres make up to 90% of the organic constituent of interstitial matrices with collagen types I,II,and III predominating. Unlike type IV collagen found exclusively in basement membrane these do not have non-collagenous interruptions in their structure and therefore are rigid rods more resistant to proteolysis.

Fibronectin is a major component and has a variety of functions including mediation of cell adhesion and binding to cell surface receptors. Elastin provides the interstitium with elasticity when formed into fibres and these are relatively resistant to proteolytic attack.

Table 2: Main constituents of the interstitium.

Component	Structure	Tissue distribution
Type 1 collagen	$[\alpha 1(I)]_2\alpha 2(I)$, triple helix, forms fibrils	Bone, cornea, tendon, dermis, dentin, ligament, heart valves, intestinal, large vessel and uterine walls
Type II collagen	$[\alpha 1(II)]_3$, triple helix, forms fibrils	Hyaline cartilage, vitreous body, nucleus pulposus
Type III collagen	$[\alpha 1(III)]_3$, triple helix, forms fibrils	Dermis, large vessel and uterine walls, gingiva, heart valves
Hyaluronic acid	Glucoronic acid and N-acetyl glucoamine disaccharide chain	Vitreous body, cartilage, umbilical cord
Fibronectin	Two subunit chains (M_r 210,000 and 250,000)	Dermis, tendon, vessel walls, bone, plasma
Elastin	One polypeptide (M_r 72,000) cross links to form elastic fibre	Large arteries, ligaments, dermis, lung

2.2 Tumour invasion and metastasis

Until the late 1970s it was uncertain whether tumour invasion was a passive or active process (Liotta LA 1992). It was thought that as a byproduct of the high internal pressure of a tumour coupled with a decreased tendency of tumour cells to adhere to one another, tumour cells were pushed into surrounding tissues. However this did not explain how benign tumours such as leiomyomas of the uterus grow to a large size and high internal pressure without invading surrounding tissue (recognised by an intact basement membrane). In addition basement membranes do not possess pores large enough to allow tumour cells through passively.

It is now recognised that tumour invasion and metastasis is a highly active process requiring complex steps. The behavior of the malignant tumour cell is described by its ability to cross tissue boundaries, interact with cells of various tissue compartments and metastasise to distant loci. The change from quiescent tumour to invasive tumour is accompanied by the acquisition of angiogenic properties (Arbiser JL 1997), mediated by growth factors. This allows rapid tumour growth and increases the likelihood of metastases. New capillaries in tumours are leaky and possess pores large enough for tumour cells to intravasate.

A three step mechanism has been proposed to describe how interactions between tumour cells and the extracellular matrix result in malignant invasion (Liotta LA 1991):

1) tumour cell attachment to basement membrane or stromal proteins

2) secretion of enzymes that locally degrade these proteins

3) locomotion of tumour cells through degraded areas

The first step is binding of the tumour cell to the basement membrane. This is mediated by tumour cell surface receptors of the integrin and non-integrin varieties (Hart I 1992) which recognise proteins such as laminin, type IV collagen and fibronectin, as found in the basement membrane. Evidence for this is provided by a study in which up-regulation of integrin in non-metastatic cells increased their malignant capacity (Hart I 1992). Malignant tumour cells have a reduced ability to adhere to one another. This promotes detachment from the primary tumour and reattachment to the basement membrane. Animal models have demonstrated that loss of E-cadherin (one of a group of cell adhesion molecules) expression was associated with increased metastatic behaviour (Hart I 1992).

Two to eight hours after attachment to the basement membrane a local zone of degradation is produced at the point of tumour cell contact (Liotta LA 1991).Tumour cells either secrete degradative enzymes themselves or induce host cells to do so.

Locomotion is the third step of tumour cell invasion in which the tumour cell traverses the interstitium allowing it to reach blood vessels or lymphatic channels and begin the metastatic cascade (Liotta LA 1991). An early step in locomotion is pseudopodial protrusion of the leading edge of the migrating cell. Motility is stimulated by various factors such as autocrine motility factor (produced by tumour cells), migration stimulating factor and scatter factor, produced by fibroblasts. Integrin expression is polarised at the migrating front of the tumour cell to allow adherence to ECM components and release at the rear as the cell moves forward (Hart I 1992).

Once tumour cells have intravasated they are carried in the venous or lymphatic circulation where they lodge in the next capillary bed or lymph node. It is possible to predict where 60% of metastases will arise from circulatory anatomy alone (Liotta LA 1992). Metastasising tumour cells from the colon often arise in the liver as it receives direct venous drainage from the large intestine.

Tumour cells may lodge in capillary beds or adhere to the endothelium. This may be mediated by organ specific cell adhesion molecules (Hart I 1992). Within eight to twenty four hours of adherence, tumour cells begin to invade the vessel wall and the process of invasion is repeated. If the host tissue is appropriate in terms of growth factor and blood supply the tumour cell may proliferate into a new colony (Liotta LA 1992).

Metastasis is known to be an inefficient process, fewer than 0.01% of tumour cells that escape from the primary tumour will survive to form metastatic colonies (Weiss L 1990). Various steps in the metastatic process have been thought to contribute significantly to this inefficiency. Firstly properties of the cells in the primary tumour can determine the likelihood of metastases developing, including the tendency and ability of cells to escape from the primary tumour and intravasate into the circulation. Secondly the circulation has previously been believed to be a particularly hostile environment for cancer cells, the vast majority of which have been thought to be destroyed by haemodynamic

forces. Thirdly the ability to extravasate has been considered a major rate limiting step in metastasis, with few cells that arrive in a new capillary bed thought to be able to escape from the circulation into the surrounding tissue. Fourthly cells that successfully extravasate need to be able to grow in the new site, including a requirement for induction of new blood vessels in order for the tumour to grow beyond a small size. At all steps, cells of the immune system are thought to contribute to the metastatic inefficiency (Hart I 1992).

Clinically it is important to understand the sources of metastatic inefficiency and the relative importance of each step to the overall process, so that this natural inefficiency may be exploited and enhanced therapeutically. Several successful experimental approaches have been developed to study particular aspects of metastasis, including a variety of in vivo and in vitro assays. In vivo approaches have included the development of "spontaneous" and "experimental" metastasis assays in syngeneic or immune deficient animals. In these assays tumour cell lines are injected into tissue to form a primary tumour, or directly into the blood or lymphatic circulations, with subsequent formation of metastases. The end point of the assay is to count numbers of metastases in various organs. These in vivo assays allow the effects of various genetic and molecular manipulations to be determined, and permit an association between cellular properties or specific molecules and the metastatic phenotype. In vitro assays to model steps that are believed to occur in metastasis have also been developed, such as assays to measure the ability of cells to adhere to, migrate on or invade into basement membrane molecules. These assays also permit identification of molecules that are associated with the ability to carry out these processes. Together these approaches have provided a great deal of information about molecules that are likely to contribute to metastasis (Liotta LA 1993). Two major classes of molecules have been repeatedly implicated in the metastatic process:

proteolytic enzymes and molecules involved in cellular adhesive interactions (Pupa SM 2002).

2.3 Proteolytic enzymes

Members of all four classes of naturally occurring proteinases: serine, cysteine, aspartyl and metalloproteinases (Table 3) have been associated with increased aggressiveness of tumour cells (Mignatti P 1993), and they all may function together in a cascade fashion to facilitate tumour cell movement through host barriers.

They are classified according to:

1) catalytic site

2) optimum pH

3) cation requirements

4) susceptibility to inhibitors

Among these, the members of the metalloproteinases family are capable of degrading all protein components of basement membranes, and they are reviewed in the next chapter.

Table 3: Proteases identified in malignant tissue

Protease Class	**Optimum pH**	**Examples**	**Inhibitors**	**Location**
Serine	7-9	Trypsin Plasmin Elastase Thrombin	Fluoro- Phosphate	ECM Heart Lung Kidney
Cysteine	3-8	Cathepsin B and H	n-ethyl maleimide	Most cells esp. spleen
Aspartic	2-7	Pepsin Cathepsin D	Diazo- ketones	GI system
Metallo	7-9 neutral neutral	Collagenase Gelatinase Stromelysins MT-MMP	Metal chelators TIMPs	ECM

2.3a Serine proteases

The plasminogen activators, a group of serine proteases play a role in extracellular matrix degradation. There are two major types of plasminogen activators which differ from each other with respect to catalytic, molecular and immunological properties: a tissue type (tPA) and a urokinase type (uPA) (Mignatti P 1993). The major function of plasminogen activators is to dissolve fibrin clots by converting plasminogen to the active proteinase plasmin. The catalytic activity of this system is modulated by both the Pas and by the inhibitors of both plasmin and Pas. Plasmin inhibitors include α-2-antiplasmin and α-2-macroglobulin, and PA inhibitors (PAIs) including type 1 (PAI-1) and type 2 (PAI-2). However the plasmin they produce is also able to degrade laminin (found in basement membranes) and fibronectin (found in interstitial connective tissue). Plasmin is also able to convert pro-MMP 1, 3 and 9 into their active forms. The plasminogen activators therefore play a role in tumour cell invasion and metastasis (Werb Z 1997), and uPA has been shown to be an independent prognostic marker in both breast carcinoma and colorectal carcinoma (Duffy MJ 1996).

2.3b Cysteine and aspartic proteases

Cathepsins are a group of acid lysosomal sulfhydryl proteinases which are classified either as cysteine (cathepsin B) or aspartic (cathepsin D) proteinases. They are involved in physiological processes such as turnover of cellular proteins, and studies have shown that increased expression or changes in cellular localisation are important prognostic factors and correlate with tumour progression (Yan S 1998, Tetu B 1999).

2.4 Cell adhesion molecules

Cancer cells must interact with each other as well as with host cells and structures at several points along the metastatic pathway. These interactions depend upon several classes of cell surface molecules including integrins, cadherins, selectins and the ligands

that bind to these molecules. Although originally named as adhesion molecules, it is now recognised that they do more than mediate attachment per se. There are also functional consequences of adhesive interactions due to initiation of signal transduction (Hynes RO 1982). A variety of adhesion molecules have been implicated in the metastatic process, but the mechanisms by which they contribute have been based largely on speculation and inference.

Interactions of white blood cells with endothelial cells have been extensively studied and have provided a model for interactions of circulating cancer cells with the vasculature (Tedder TF 1995). Selectins and their carbohydrate containing ligands mediate the "rolling" of leucocytes. This rolling precedes leucocyte arrest in the microvasculature and functions to slow the leucocytes, followed by formation of firm attachments to vessel walls, mediated by integrins. Integrins have been linked with the metastatic phenotype (Zetter BR 1993). Several studies have shown altered expression of specific integrins in various malignant cells. Peptides containing integrin-binding sequences have been found to intefere with metastasis, as have antibodies against integrin components. Other molecules including cadherins and CD 44 have also been shown to vary in expression in cells of differing malignancy and have been implicated functionally in metastasis.

2.5 Experimental models for studying the metastatic process

The traditionally used metastasis assays record the end-point of presence or absence of metastases, and are therefore excellent assays for determining which molecules affect this end-point. However they are less able to determine how and when these molecules contribute to the process as direct observation of the ongoing metastatic process in vivo is not possible with these techniques. Supporting evidence has been obtained from in vivo data from histological sections that capture static information from a single point

in time, but again logical inferences are required to translate information from a single point in time to the dynamic process that actually occurs.

In order to complete the picture of how metastases form, a way to directly watch the process, in vivo as it occurs over time is required. This is provided by intravital videomicroscopy (IVVM) which complements existing in vivo and in vitro assays, and permits the study of events in the metastatic process that previously have been inaccessible (Chambers AF 1995). The technique of IVVM uses a videocamera attached to a light microscope to visualise, record and quantitatively analyse the movement of blood through microcirculatory pathways of intact organs and tissues in living animals. IVVM may also be used to study interactions of leucocytes or cancer cells with vessel walls, escape of these cells from the microcirculation, and their subsequent activities in surrounding tissue.

To stabilize the image in IVVM an inverted microscope is used and cancer cells are fluorescently labelled prior to injection. By injecting cells into the venous, portal or arterial systems the microcirculation of the lung, liver or other organ systems can be targeted specifically. The lymphatic system can also be viewed by IVVM, and cancer cells in most organs such as liver and muscle can be viewed continuously for extended periods (up to six hours). High resolution views of the lung can be obtained, while briefly holding the level of inflation constant, but respiratory movement and a limited area for viewing make the extended observation of numerous cells difficult. To overcome these difficulties another respiratory organ, the chorioallantoic membrane (CAM) of chick embryo is used. As IVVM permits dynamic observations of cancer cells in intact organs of a living animal, it offers many advantages over the static views obtained by histology and ultrastructural analysis. Processes such as cell arrest within the microcirculation, cell shape changes and pseudopod formation, extravasation and migration through tissue can be followed in real time.

IVVM does have its limitations. Only a superficial layer of tissue 50-75 μm thick can be observed and while this does not cause a problem with CAM, it means that only the subcapsular layer down to the fourth layer of sinusoids can be viewed in the liver. Fortunately murine hepatic metastases from many different cell types occur at the surface of the organ and can be studied by IVVM. Secondly observations for individual animals are limited to a maximum of ~ 8 hours. This can be overcome by studying a series of animals at sequentially overlapping time periods. The major limitation, as with all these experimental models is that what is occurring in a mouse liver or chick embryo may not necessarily hold true in humans.

Results from IVVM are providing new conceptual understanding of the metastatic process, as well as the nature and timing of the contributions of molecules implicated in metastasis (e.g. proteinases and adhesion molecules) (Chambers AF 1995).

2.6 Cell arrest in the microcirculation

IVVM has shown that unlike previously thought, circulating cancer cells from solid tumours do not "roll" along and then adhere to vessel walls like leucocytes, but are initially arrested by size constraints on the inflow side of the microcirculation. They become lodged in capillaries, blocking the flow. The arrested cells do not remain in a spherical configuration but become distorted to take on the shape of the vessel. Several hours after initial arrest the cancer cells gradually move away from one side of the vessel allowing flow to resume, and stretch out along the opposite wall in preparation for the process of extravasation. IVVM has never revealed intravascular replication of cancer cells, involvement of platelets in protecting cancer cells and no evidence of cancer cells being destroyed by haemodynamic forces (Chambers AF 1995).

2.7 Cell extravasation

In both mouse liver and chick CAM it has been shown by IVVM that all cancer cells extravasate singly without observable disruption of the microvasculature. The mechanism of extravasation, and ability to do so, appeared to be the same for all cell types examined independent of metastatic ability (Chambers AF 1995). In the liver, after flattening out on the vessel wall, the cells begin their move into the hepatocyte layer by sending out cytoplasmic projections through the vessel wall and between hepatocytes. There follows a migration of the cell which eventually displaces the hepatocytes and in some cases wraps around the sinusoid.

2.8 Post-extravasation migration of cancer cells within host tissue

The IVVM findings described above point to events that occur after extravasation as being key to the formation of successful metastases. An essential feature of metastatic cells is their ability to interact with the extracellular matrix and parenchymal cells of a target organ, promoting an uncontrolled invasion. IVVM has shown for the first time that extravasated cells, whether from cell lines of high or low metastatic potential, exhibit directed migration toward preferential sites of growth before starting to divide (Chambers AF 1995).

2.9 The importance of post-extravasation growth regulation

IVVM studies have de-emphasised cancer cell arrest in the microcirculation, cell extravasation, and post-extravasation cell migration through host tissue as rate-limiting steps in metastasis formation. However experimental metastasis assays have demonstrated marked differences in metastatic ability among the cell lines studied with IVVM (Chambers AF 1995). These findings point to post-extravasation cell growth as the major rate-limiting step in metastasis.

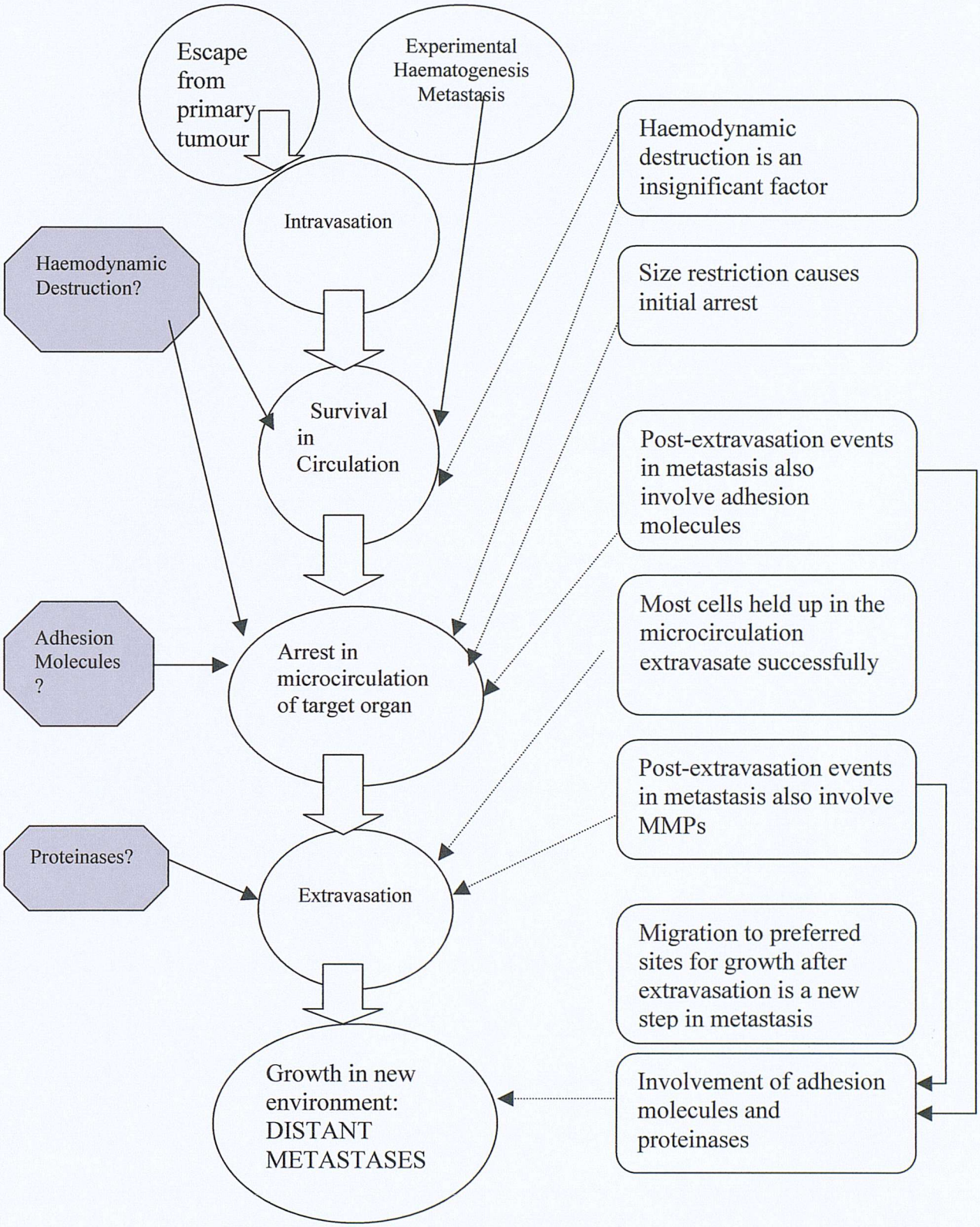
Following IVVM studies (Morris VL 1994; Koop S 1994) it appears that after cells have extravasated and migrated to appropriate sites for growth within host tissue, they must each undergo a process of activation in order for cell division to occur. Interactions with stromal cells, other cancer cells, extracellular matrix and soluble factors in the cell's immediate environment presumably determine whether a cell will grow or remain "dormant".

The new concepts emerging from IVVM studies are related to the main steps in tumour metastasis in Figure 1. The control of post-extravasation growth of individual cancer cells is a more important contributor to metastatic inefficiency than previously thought.

These concepts have led to a further examination of the role of the extracellular matrix in cancer onset and progression. Cleavage of matrix components not only removes physical barriers to cell migration but also interrupts signaling pathways between matrix and cell surface receptors effecting cell survival, proliferation and migration (Giancotti FG 1999). Proteolysis of ECM proteins triggers signaling molecules such as focal adhesion kinase (FAK) (Braga V 2000, Fashena S 2000). The breakup of the extracellular matrix also unmasks cryptic binding sites which modifies integrin-mediated anchorage, focal receptors, a function that is important in the interaction between tumour cells and laminin and which effects metastatic potential (Pellegrini R 1995).As well as revealing binding sites, proteolysis of the ECM also releases functional fragments that modulate the biological response with regard to migration of tumour cells and angiogenesis, and releases stored cytokines and growth factors (TGFβ, PDGF, b-FGF and IFN-γ) which stimulate tumour and endothelial cells (Streuli C 1999).

The role matrix metalloproteinases play in this concept of the tumour and its surrounding stroma forming a new "organ" promoting tumour growth and protecting it from immune attack will be examined in the next chapter.

Figure 1: Main steps in tumour metastasis: New concepts from IVVM



The growth stage is a more important contributor to metastatic inefficiency than previously thought as many (rather than few) cancer cells extravasate.

Chapter 3: MATRIX METALLOPROTEINASES (MMPs)

3.1 Definition and Classification of MMPs

The matrix metalloproteinases (MMPs) are a family of homologous zinc or calcium atom dependent endopeptidases whose main role is degradation and remodelling of the extracellular matrix (Parsons SL 1997). They are important in such processes as wound healing, where debridement of tissues is essential for tissue healing (Wysocki AB 1993), pregnancy (Librach CL 1991) and the normal menstrual cycle (Hampton AL 1994), and bone resorption (Delaisse J-M 1992).

However the major interest in the MMPs relates to their role in diseases where breakdown of the extracellular matrix is a key feature. Such diseases include cancer, peridontal disease (Page RC 1991) and rheumatoid arthritis (Dingle JT 1978).

The members of the MMP family have the following characteristics:

1) they are proteinases that degrade at least one component of the ECM
2) they contain a zinc ion and are inhibited by chelating agents
3) they are secreted in a latent form and require activation for proteolytic activity
4) they are inhibited by specific tissue inhibitors of metalloproteinases (TIMPs)
5) they share common amino acid similarities (Matrisian L 1990)

Initially the MMPs that had been cloned were grouped according to their substrate specificity into five subclasses: collagenases, gelatinases, stromelysins and matrilysins, membrane bound, and others (Table 4). However as the number of MMPs has grown (now greater than 21 human types) a sequential numbering system for the MMPs has been adopted, and the MMPs are now grouped according to their structure. There are eight distinct structural classes of MMPs: five are secreted and three are membrane type MMPs (MT-MMPs) (Table 5).

Table 4: Matrix metalloproteinase classification according to substrates

Class of MMP	Optimum pH	Nomenclature	Protein	Substrate
Interstitial collagenases	7-9	MMP 1 MMP 8 MMP 13	Collagenase PMN collag. Collagenase 3	Fibrillar collagens (I,II,III)
Gelatinases	Neutral	MMP 2 MMP 9	Gelatinase A Gelatinase B	Type IV and V collagens, Gelatin, fibronectin.
Stromelysins	Neutral	MMP 3 MMP 10 MMP 11 MMP 7	Stromelysin 1 Stromelysin 2 Stromelysin 3 Matrilysin (Pump)	} Laminin, fibronectin, non-fibrillar collagen. Serpin Laminin, fibronectin, non-fibrillar collagen
Membrane bound		MMP 14 MMP 15 MMP 16 MMP 17	MT-MMP 1 MT-MMP 2 MT-MMP 3 MT-MMP 4	Pro- MMP 2 Pro- MMP 2 Pro- MMP 2 ?
Others	Neutral	MMP 12	Metalloelastase	Elastin

Table 5: Matrix metalloproteinase classification according to structure

Class of MMP	Structure	Nomenclature	Common name
Secreted	Minimal domain	MMP 7 MMP 26	Matrilysin Endometase, matrilysin 2
	Simple haemopexin domain	MMP 1 MMP 3 MMP 8 MMP 10 MMP 12 MMP 13 MMP 18 MMP 19 MMP 20 MMP 22 MMP 27	Interstitial collagenase Stromelysin 1 Neutrophil collagenase Stromelysin 2 Metalloelastase Collagenase 3 Collagenase 4 (non human) Enamelysin CMMP (non human)
	Gelatin binding	MMP 2 MMP 9	Gelatinase A (72 kDa) Gelatinase B (92 kDa)
	Furin activated and secreted	MMP 11 MMP 28	Stromelysin 3 Epilsin
	Vitronectin-like insert	MMP 21	Homologue of Xenopus XMMP
Membrane type	Transmembrane	MMP 14 MMP 15 MMP 16 MMP 24	MT-MMP 1 MT-MMP 2 MT-MMP 3 MT-MMP 5
	GPI-linked	MMP 17 MMP 25	MT-MMP 4 MT-MMP 6
	Type II transmembrane	MMP 23	Cysteine array MMP

3.2 Proteolytic functions of MMPs

3.2a Collagenases

The collagenases comprise interstitial collagenase (MMP 1), neutrophil collagenase (MMP 8) and collagenase 3 (MMP 13). They specifically degrade connective tissue collagens (fibrillar collagens types I,II and III), making a single sequence specific cleavage that unwinds the helical collagen fibre and produces a denatured gelatin form that is now susceptible to cleavage by other proteases and metalloproteinases. MMP 1 shows a preference for type III collagen, while MMP 8 preferentially degrades type I collagen. The preferred form of collagen degraded by MMP 13 is currently unknown. (Duffy MJ 1998).

3.2b Gelatinases

The gelatinases (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. Degradation of type IV collagen by the gelatinases occurs within the triple helical regions. This subgroup has 2 distinct members MMP 2 (also known as gelatinase A) and MMP 9 (also known as gelatinase B). Generally these two gelatinases are thought to have similar substrate specificity. However, recently MMP2, but not MMP 9, was shown to cleave the ectodomain of fibroblast growth factor (FGF) receptor 1. MMP 2 may therefore modulate the mitogenic and angiogenic activities of FGF as well as degrading collagens. (Duffy MJ 1998).

3.2c Stromelysins

The third class of MMPs is the stromelysin class. Two members of this class, stromelysin 1 (MMP 3) and stromelysin 2 (MMP 10), are very similar in size and amino acid sequence while a third member, matrilysin (MMP 7) is a smaller truncated version of the stromelysins. These MMPs have a broad substrate specificity, degrading ECM proteoglycans, laminin, fibronectin, gelatin and the globular portion of basement

membrane collagens. The newest member of this class, stromelysin 3 (MMP 11) (Bassett P 1990) has not yet been found to degrade any matrix protein, but has serpin, a serine protease inhibitor, as its substrate. By inactivating this inhibitor stromelysin 3 may potentiate a more generalized proteolytic cascade. Another difference between MMP 11 and the other stromelysins is that it is processed intracellularly by furin (Pei D 1995). Furin is a transmembrane serine protease found in the trans-Golgi network. Thus MMP 11 can be secreted predominately in a potentially active form, a characteristic which distinguishes it from other MMPs. Most MMPs are secreted as latent proenzymes and activated extracellularly.

In view of its restricted substrate specificity and intracellular activation it has been argued that MMP 11 might represent the first member of a new MMP subgroup rather than being the fourth member of the stromelysin family. (Rio M-C 1996).

3.2d Membrane-type (MT) MMPs

The fourth class of MMPs are the membrane-type or MT- MMPs, as these proteinases possess a transmembrane domain. Four members of this class have been identified at the mRNA level, the best characterised being MT-MMP 1. This has been shown to be membrane associated rather than secreted and its principle substrate appears to be inactive MMP 2, for which it acts as a specific activator (Sato H 1994). It is also able to degrade a variety of extracellular matrix substrates (Ohuchi E 1997).

3.3 Non proteolytic functions of MMPs

As well as degrading structural components of the extracellular matrix, the MMPs help facilitate cell migration by affecting cellular signalling and functions (Streuli C 1999). This occurs because the cells have receptors for structural ECM components such as integrins. Division of ECM components by MMPs can also generate fragments with new functions, such as cleavage of laminin-5 and collagen type IV resulting in exposure of

cryptic sites that promote migration (Giannelli G 1997, Xu J 2001). Cleavage of insulin-like growth factor binding protein (IGF-BP) by MMP 3 and 9, (Manes S 1999) and of perlecan by stromelysin and collagenase (Whitelock JM 1996) releases IGF and fibroblast growth factors respectively. MMPs and the related proteinases, the ADAMS (a family of transmembrane proteinases with metalloproteinase, disintegrin (integrin-binding), cysteine-rich and epidermal growth factor (EGF)-like domains) also play an important role in the release of cell-membrane–bound precursor forms of many growth factors, including transforming growth factor-α (TGF-α) (Peschon JJ 1998). TGF-β availability is regulated by a different mechanism which involves its release from an inactive extracellular complex by MMP 2 and 9 (Yu Q 2000).

The growth factor receptors are also MMP substrates, with FGF receptor 1 being cleaved by MMP 2 (Levi E 1996), while HER2/neu (ERBB2) and HER4 (ERBB4) members of the epidermal growth factor receptor family are substrates for as yet unidentified MMPs or ADAMs. (Codony-Servat J 1999, Vecchi M 1998). The hepatocyte growth factor receptor c-MET is also a substrate for an unidentified MMP, as its shedding is blocked by the tissue inhibitor of metalloproteinase 3 (TIMP-3) (Nath D 2001). Cell adhesion molecules are also MMP substrates with the cleavage of E-cadherin by MMPs 3 and 7 (Noe V 2001) and CD44 by MT-MMP 1 (Kajita M 2001) resulting in the release of fragments of the extracellular domains and increased invasive behaviour. MT-MMP 1 (MMP 14) also cleaves the αv integrin subunit precursor leading to enhanced cancer cell migration (Deryugina EI 2001). The MMPs cleave and activate their own zymogen forms and also cleave other MMPs and proteinase inhibitors such as serpins.

3.4 Structure of MMPs

As MMP cDNA was cloned it became obvious that a gene family existed. The MMP genes are about 10 kb in length, contain 10 exons and 11 introns, and localise to

chromosome 11q22.23 where MMPs 1,3, 7, 8, 10, 12, 13, 20 and 27 are found (Massova I 1998). The gelatinases MMP 2 and MMP 9 contain 3 extra exons that encode fibronectin-like repeats, and localise to chromosomes 16 and 20 (Collier I 1991).

Analysis of the primary structure of the cloned MMPs reveals that these proteins contain several distinct domains that are conserved among the various members of the family (Figure 2) (Kahari VM 1999). The first domain is the leader sequence or N-terminal signal peptide (pre domain) that targets the molecule for secretion but is subsequently removed after it directs their synthesis to the endoplasmic reticulum and is not present in the latent enzyme. Therefore most MMPs are secreted but four contain transmembrane domains (MMP 14,15,16,24) and two are anchored to the membrane by glycosyl phosphatidylinositol (GPI) (MMP 17,25) and are expressed as cell surface enzymes (Brinckerhoff CE 2002). The pre domain is followed by a propeptide pro domain that maintains enzyme latency until it is removed or disrupted and a catalytic domain contains conserved histidine residues that are thought to be the zinc binding domain. Mutations in this in this region of rat stromelysin (transin) reveal that these residues are required for proteolytic activity, supporting the notion that they complex the critical metal ion (Sanchez-Lopez R 1988). The catalytic domain dictates cleavage-site specificity through its active site cleft, through specificity sub-site pockets that bind amino acid residues immediately adjacent to the scissile peptide bond, and through secondary substrate-binding exosites located outside the active site itself.

Except for the minimal domain MMPs (MMP 7 and 26) and MMP 23, all MMPs have a haemopexin/vitronectin-like domain that is connected to the catalytic domain by a hinge or linker region. MMP 7 and MMP 26 lack these extra domains, whereas MMP 23 has a unique cysteine/proline rich and IL 1 type II receptor like domains instead of a haemopexin domain. The role of the haemopexin domain is to influence TIMP binding, the binding of certain substrates, membrane activation, and some proteolytic activities.

Specifically for MMP 1, the haemopexin domain is required for the initial binding and orientation of the collagen fibril and local unwinding of its triple-helical structure, as both ends of MMP 1 are required for it to cleave native fibrillar collagen (Murphy G 1992, Sanchez-Lopez R 1993).

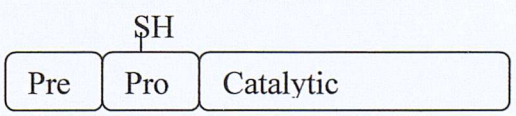
The hinge region varies in length and composition amongst the MMPs and also influences substrate specificity (Knauper V 1997).

Gelatinases A and B (MMPs 2 and 9) have three head-to-tail cysteine-rich repeats inserted in their catalytic domain. These inserts resemble the collagen-binding type II repeats of fibronectin and are required to bind and cleave collagen and elastin (Murphy G 1994, Shipley JM 1996). MMP 9 also has a unique type V collagen-like insert of unknown importance at the end of its hinge region.

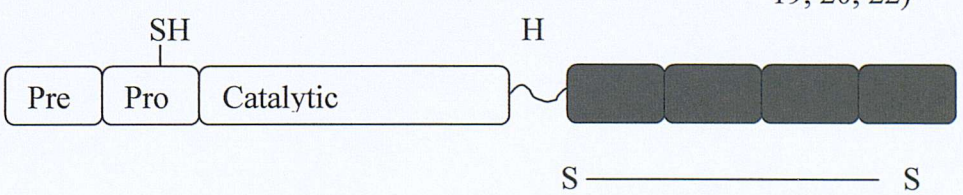
The membrane type MMPs (MT-MMPs) have a single-pass transmembrane domain and a short cytoplasmic C-terminal tail (MMPs 14,15,16 and 24) or a C-terminal hydrophobic region that acts as a glycophosphatidyl inositol (GPI) membrane-anchoring signal (MMP 17 and MMP 25) (Itoh Y 1999, Kojima S 2000). These domains play an essential role in the localisation of important proteolytic events to specific regions of the cell surface.

Figure 2: The domain structure of MMPs

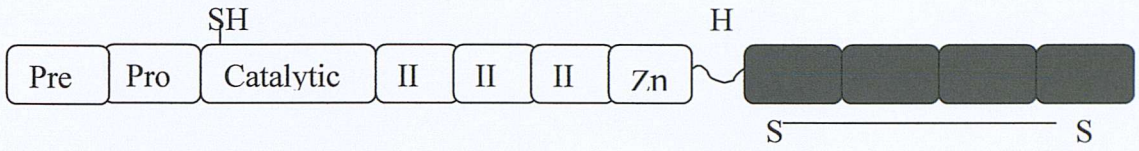
1) Minimal Domain MMPs: (MMP 7, 26)



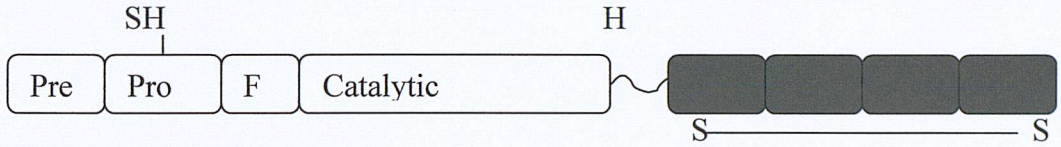
2) Simple Haemopexin Domain-Containing MMPs: (MMP 1, 8, 13, 18, 3, 10, 27,12 19, 20, 22)



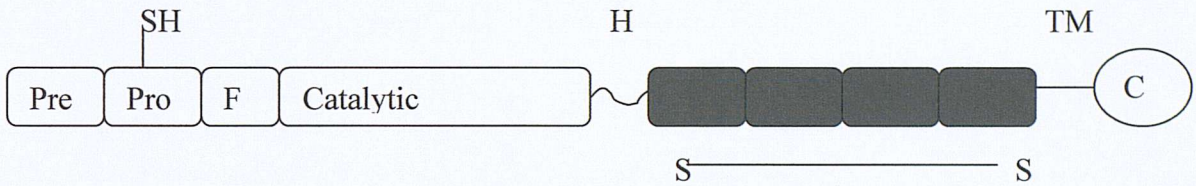
3) Gelatin-binding MMPs: (MMP 2, 9)



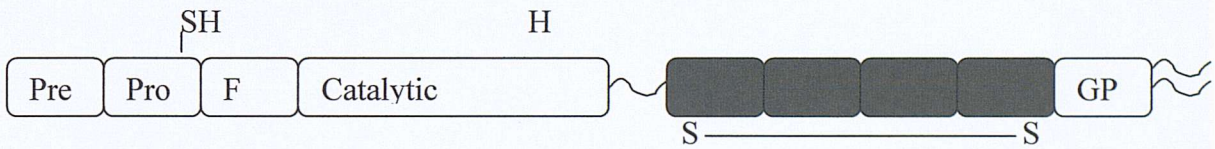
4) Furin-activated secreted MMPs: (MMP 11, 28)



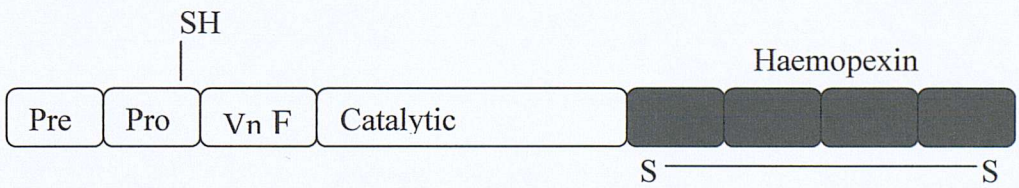
5) Transmembrane MMPs: (MMP 14, 15, 16, 24)



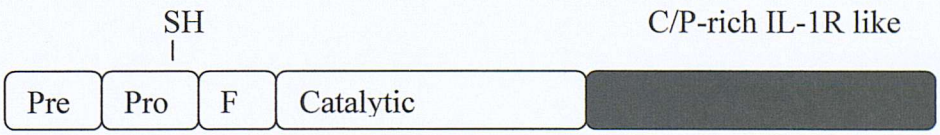
6) GPI-linked MMPs: (MMP 17, 25)



7) Vitronectin-like insert linker-less MMPs: (MMP 21)



8) Cysteine/Proline-Rich IL-1 receptor-like domain MMPs: (MMP 23)



3.5 Regulation of MMPs

Whether in physiological or pathological conditions MMPs must be present in the right cell type and pericellular location, at the right moment in suitable amounts and must be activated or inhibited as required. To achieve this the activity of MMPs is highly regulated occuring both at the level of the expression of the genes (transcriptional and post-transcriptional) and in controlling the proteolytic activity of the protein products by their activators, their inhibitors, and their cell surface localisation.

3.5a Transcriptional regulation

The differential patterns of expression of MMPs, which underlie their biological functions, are a product of tight regulation at the level of transcription. MMP 2 is the exception to this rule, being constitutively expressed and controlled through a unique mechanism of enzyme activation (Strongin AY 1995) and some post-transcriptional mRNA stabilisation (Overall CM 1991). However basal expression of MMP 2 does have some regulation as it is linked to MMP 14 and TIMP 2 expression, a fact consistent with their cooperation during MMP 2 activation and similarities in their gene promoters (Lohi J 2000).

Otherwise MMP gene expression is regulated by many stimulatory and suppressive factors that influence muliple signalling pathways. Expression of various MMPs can be up or down regulated by phorbol esters, integrin-derived signals, extracellular matrix proteins, cell stress and changes in cell shape (Kheradmand F 1998). Type 1 collagen acts as a ligand for discoidin domain-containing receptor-like tyrosine kinases that induce MMP 1 expression when they are activated by intact collagen and become inactive when they bind MMP 1 cleaved collagen (Vogel W 1997, Shrivastava A 1997). Thus MMP 1 expression can be induced by its own substrate and specifically repressed once it cleaves that substrate and is no longer needed. In addition MMP expression is regulated by several cytokines and growth factors, including interleukins,

EGF, KGF, NGF, basic FGF, VEGF, PDGF, TNF-α, TGF-β and the extracellular matrix metalloproteinase inducer (EMMPRIN). These stimuli work by inducing the expression and/or activation of c-fos and c-jun proto-oncogene products, which heterodimerize and bind activator protein-1 (AP-1) sites within several MMP gene promoters.

As well as the AP-1 complexes, other factors are involved in the regulation of MMP genes, sometimes the same signal regulating MMP genes in different ways. TGF-β suppresses the transcription of MMP 1 and 3 but induces the expression of MMP13 (Uria JA 1998). Some MMPs are only expressed by a small range of cells, normal MMP 9 production being largely limited to osteoclasts, macrophages, trophoblast cells, hippocampal neurons and migrating keratinocytes at the margins of healing wounds (Munaut C 1999). This cell specific expression seems to be controlled by regions near the 5' end of the MMP 9 gene promoter (Mohan R 1998). Cell specific induction of MMP expression can also occur, phorbol esters inducing the expression of MMP 3 rather than MMP 10 in fibroblasts but having the opposite effect in keratinocytes (Windsor LJ 1993). This shows an MMP gene's response to a given stimulus depends on both the organisation of its transcriptional promoter and its cellular context (presence or absence of other signals).

Several cis-regulatory elements influence MMP gene expression depending on their proximity to one another in the gene promoter. AP-1 sites give several MMP genes the ability to be induced by phorbol esters and act synergistically with adjacent Ets-binding sites in some genes (MMP 1), but not in others (MMP 13) (Pendas AM 1997). This difference is thought to be due to the distance between the Ets and AP-1 sites in the respective gene promoters (9 nucleotides in MMP 1 and 20 nucleotides in the MMP 13) being critical for inducability of the MMPs (Gutman A 1990). Targeted disruption of the murine Ets transcription factor results in early embryonic death and deficient MMP 9 expression, and Ets2-deficient fibroblasts do not induce MMP 3 and 13 (Yamamoto H

1998). This lack of MMP 3 and 13 expression can be restored by artificial expression of Ets 2 in these deficient fibroblasts.

Other regulatory elements within MMP gene promoters include an osteoblast-specific element in the MMP 13 gene promoter that responds to core-binding factor 1 (CBFA1) (Jimenez MJ 1999); a β-catenin regulated LEF/TCF recognition site near the MMP 7 transcription start site (Crawford HC 1999); TGF- β inhibitory elements in several MMP genes; and AP 2, Sp 1, Sp 3, NF-Kb, CCAAT/enhancer-binding protein- β, or retinoic acid response elements found in sveral MMP genes (Lohi J 2000, Ludwig MG 2000). A functioning p53-binding site has been identified in the MMP 2 gene promoter (Bian J 1997), and wild type p53 downregulates basal and inducible MMP 1 gene expression in human fibroblasts and osteogenic sarcoma cells, whereas some mutant forms do not (Sun Y 1999). Down regulation of p53 using SV40 T-antigen suppresses the expression of MMP 2,3 and 9 in human placental trophoblast-like cells (Logan SK 1996). Basal and inducible levels of MMP gene expression can also be affected by genetic variations. Common bi-allelic single-nucleotide polymorphisms (SNPs) that influence the rate of transcription have been identified within several MMP gene promoters (Ye S 2000).

A MMP 1 SNP contains one or two guanidines 1607 basepairs (bp) upstream of the transcription start site (Rutter JL 1998). Here the insertion of an extra G creates a functional Ets-binding site immediately adjacent to an AP-1 site, resulting in a 37-fold enhancement of transcription. The high-expressing 2G allele has been associated with higher levels of MMP 1 expression in vivo and is present more often in tumour cell lines and ovarian cancer patients than in the general population (Rutter JL 1998, Kanamori Y 1999). The high expressing 2G allele is retained in a large proportion of metastatic melanomas with loss of heterozygosity at this site (Noll WW 2001). Enhanced MMP 1

transcription may therefore contribute to human cancer susceptibility and progression as it does in mice (Di Colandrea TD 2000).

A MMP 2 SNP is located 1306 bp upstream of the transcription start site and contains a cytidine or thymidine allele. The less common T allele disrupts an otherwise functional Sp-1 binding site and diminishes promoter activity by about by about 50% (Price SJ 2001).

A MMP 9 SNP is located 1562 bp upstream of the transcription start site (Zhang B 1999) and contains either a cytidine or thymidine. Nuclear protein complexes bind the C allele most readily, and the less common T allele is about 1.5 fold more potent than the C allele.

A MMP 3 SNP is located 1171 bp upstream of the transcription start site and contains a run of of five or six adenosines (Ye S 2000). In this case the 6A allele binds an 89-kDa nuclear factor more readily than the 5A allele and has 50% less transcriptional activity.

An A to G transition exists 82 bp upstream from the MMP 12 transcription start site, such that the A allele has higher AP-1 binding affinity and 1.2 times higher promoter activity than the less common G allele (Jormsjo S 2000).

The MMP 3, 9 and 12 SNPs have each been associated with coronary artery disease progression despite their modest influence on gene transcription (Ye S 2000).

3.5b Post-transcriptional regulation

Post-transcriptional mechanisms can also influence MMP expression. mRNA transcripts that encode MMP 1 and 3 are stabilised by phorbol esters and EGF, whereas MMP 13 transcripts are stabilised by PDGF and glucocorticoids and destabilised by TGF-β (Delany AM 1995, Vincenti MP 2001). The turnover of MMP 1 mRNA is apparently regulated by AU-rich sequences in the 3' untranslated region, and similar sequences may also regulate the stability of other MMP transcripts (Vincenti MP 2001). A soluble and

proteolytic active form of MT-MMP 3 is generated by alternative mRNA splicing rather than membrane shedding (Matsumoto S 1997), whereas the multiple transcripts of MMP 13, 17 and 20 probably result from alternative polyadenylation.

3.5c Regulation of MMP secretion

The vast majority of MMPs are secreted once they have been translated. However there are a few instances of secretory control. MMP 8 and MMP 9 are synthesized by differentiating granulocytes in the bone marrow, stored in the specific and gelatinase granules of circulating neutrophils and released following neutrophil activation by inflammatory mediators (Hasty KA 1990). In macrophages plasmin and thrombin induce the secretion of MMP 12, but do not alter its rate of transcription (Raza SL 2000). The increased secretion is achieved by post-translational release of pre-formed MMP 12 from macrophages in response to protein kinase C activation downstream of the G protein-coupled thrombin receptor PAR-1 (protein-activated receptor-1). The PAR-1 receptor is activated after binding a ligand that is generated from its own N-terminal end by thrombin-dependent cleavage.

3.5d Activation of latent metalloproteinases:

The MMPs, in a protective arrangement similar to other proteolytic enzymes, are first synthesised as inactive proenzymes or zymogens. The latency is maintained by an unpaired cysteine sulfhydryl group near the C-terminal end of the propeptide domain. This sulfhydryl acts as a fourth ligand for the active site zinc ion, and activation requires that this cysteine to zinc switch be opened by normal proteolytic removal of the propeptide domain or by chemical disruption of the cysteine-zinc interaction (Van Wart HE 1990). Once displaced the thiol group is replaced by a water molecule that can then attack the peptide bonds of MMP targets.

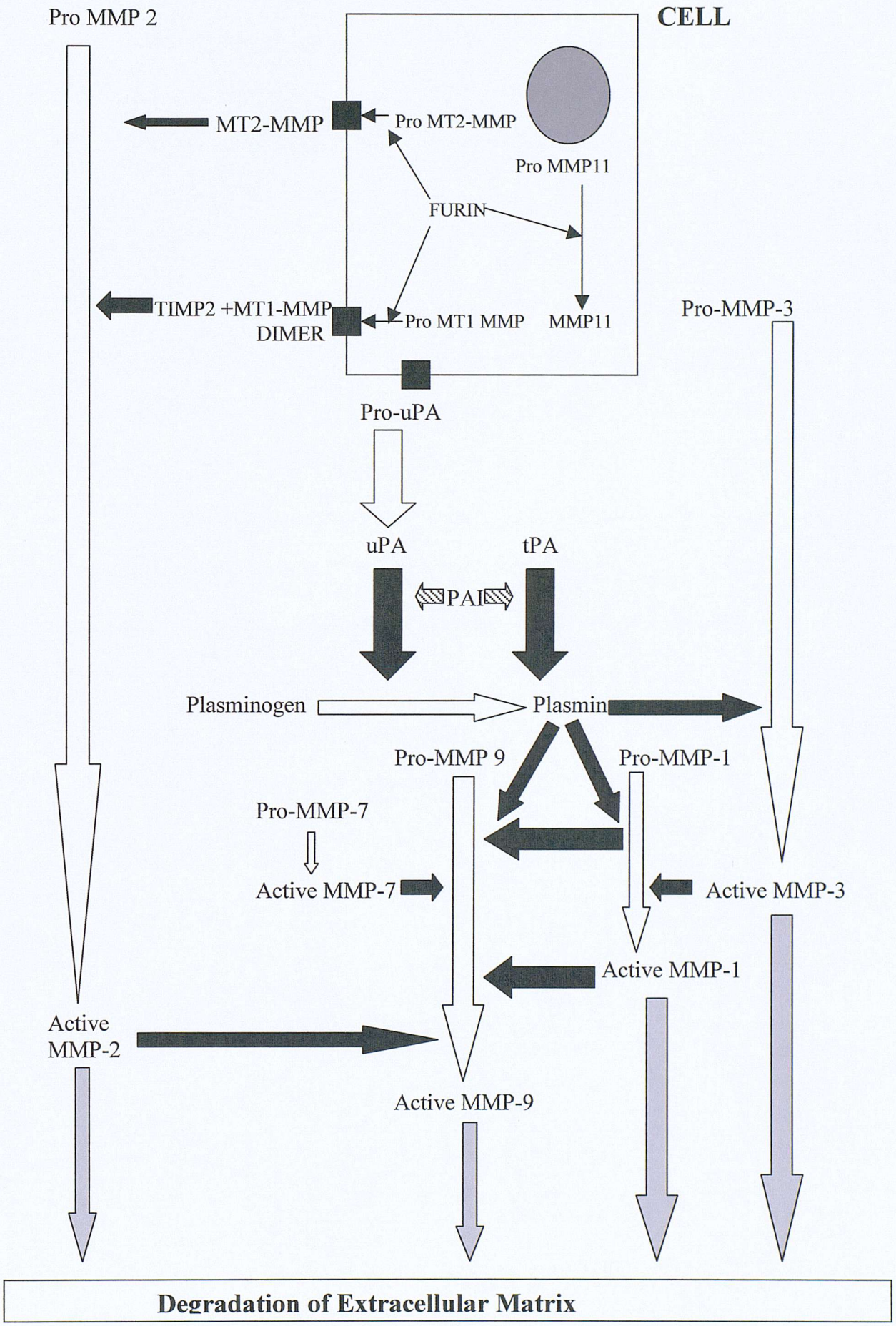
Although most MMPs are secreted as latent zymogens, MMPs 11 and 27 and the MT-MMPs contain an RXR/RR furin-like enzyme recognition motif between their

propeptide and catalytic domains. This allows them to be activated by intracellular subtilisin-type serine proteinases they reach the cell surface or are secreted (Pei D 1995). MMP 23 also has a furin-susceptible cleavage site and is a likely target of intracellular proprotein convertases, but unlike all other MMPs, it lacks the conserved cysteine that is required for enzyme latency (Gururajan R 1998). All other MMPs lack a furin-susceptible insert and are thus activated outside the cell following their secretion.

Like the coagulation and thrombolytic systems, MMP activation is regulated by a proteolytic cascade (Figure 3). Most MMPs can be activated by other already activated MMPs or by several serine proteinases that can cleave peptide bonds within MMP prodomains. The complete activation of pro-collagenase in cultured skin cells was shown to involve the interaction of the plasminogen activator urokinase and MMP 3 (He C 1989). MMP 2 is resistant to activation by serine proteases and is instead activated at the cell surface through a multistep pathway involving MT-MMPs and TIMP 2 (Strongin AY 1995). MT-MMP 1 is a very efficient MMP 2 activator (Zucker S 1998) whereas MT-MMP 2 (Miyamori H 2000) and MT-MMP 4 (English WR 2000) were not able to activate MMP 2. The mechanism involves a cell surface MT-MMP binding and being inhibited by the N-terminal domain of TIMP 2, and the C-terminal domain of the bound TIMP 2 acting as a receptor for the haemopexin domain of proMMP 2. This is followed by an uninhibited MT-MMP cleaving and activating the tethered proMMP 2. Following the initial cleavage of proMMP 2 by MT-MMP 1, a residual portion of the MMP 2 propeptide is removed by another MMP 2 molecule to yield a fully active, mature form of MMP 2 (Deryugina EI 2001). Recent work has shown that MT-MMP 2 may be able to activate pro MMP 2 by a mechanism that does not involve TIMP 2 (Morrison CJ 2001) (Figure 3).

Finally the activity of metalloproteinases on ECM substrates is dependent on the balance between the enzymes and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

Figure 3: Activation of MMPs



3.6 Tissue Inhibitors of Metalloproteinases (TIMPs)

There are four members of the TIMP family known at present, TIMP 1, TIMP 2, TIMP 3 and TIMP 4. They have been isolated, cloned and characterised (Docherty A 1985; DeClerk Y 1989; Apte SS 1994) and show structural homology. All four TIMPs have 12 conserved cysteine residues paired into 6 disulphide bonds (Murphy G 1995), and these bonds divide the TIMPs into 2 domains, each having three internal disulphide bonded loops. There is greater homology between the amino-terminal domains than the carboxyl-terminal domains. They inhibit all active MMPs by binding in a 1:1 molar ratio to form tight non-covalent complexes (Williamson RA 1994). A more complex interaction exists between TIMP 2 and MMP 2 in which the native inhibitor binds selectively to the latent proenzyme in a region distinct from its inhibitor binding site. This binding may be important in the cellular activation of MMP 2 by MT-MMP 1 (Strongin AY 1995). TIMP 1 has a similar association with MMP 9 (Williamson RA 1994; Overall CM 1994). Studies using recombinant TIMP domains and fragments of TIMPs have shown that the amino terminal domain of the TIMP retains inhibitory activity (Murphy G 1991). A specific role for the carboxyl-terminal domain has not been shown. It is believed that this may be the region which is responsible for the binding to latent forms of gelatinase (Kleiner DE 1993).

3.6a TIMP 1

TIMP 1 is a 28.5 kDa glycoprotein which forms a 1:1 complex with activated MMPs 1 and 3. It also forms a complex with latent and active forms of MMP 9 (Welgus H 1985). TIMP 1 does not inhibit MT-MMP 1 and is a poor inhibitor of MT-MMP 3. TIMP 1 can also inhibit ADAMTS-1 (Tortorella MD 1999).

3.6b TIMP 2

TIMP 2 is a 21 kDa non-glycosylated protein which has a high affinity for inactive MMP 2 (Howard E 1991). It forms a 1:1 complex with the latent and active forms of this

enzyme but it also blocks the hydrolytic activity of all activated MMPs (Stetler-Stevenson W 1989). It also inhibits MT-MMP 1 and the other MT-MMPs.

3.6c TIMP 3

TIMP 3 is the only member of the TIMP family which is found exclusively in the extracellular matrix and preferentially binds to ECM components, unlike TIMP 1 and 2 which are present in soluble forms. It is thought to be involved in tissue specific acute matrix remodelling (Uria JA 1994). TIMP 3 appears to be a more potent inhibitor of MMP 9 than other TIMPs. It is also, unlike other TIMPs, able to inhibit ADAMS 10 and 17, ADAMTS 4 and 5 (Kashiwagi M 2001). TIMP 3 is also able to bind via its C-terminal domain to heparan sulfates proteoglycans within the ECM, concentrating it to specific regions within tissues and basement membranes (Langton KP 1998). It is also a good inhibitor of tumour necrosis factor-alpha (TNF-α) converting enzyme (Mannello F 2001).

3.6d TIMP 4

TIMP 4, the most recently discovered TIMP, was found abundantly in the adult heart but only at very low levels or absent in the vast majority of other tissues (Greene J 1996). This unique expression pattern suggests that TIMP 4 may function in a tissue specific fashion in extracellular matrix haemostasis.

3.6e Non-inhibitory functions of TIMPs

Recent developments in TIMP research suggest that TIMPs are multifunctional proteins with diverse actions. TIMP 1 was first cloned as EPA (erythroid-potentiating activity), and TIMPs 1,2 and 3 have all been shown to have growth-promoting activity (Gomez DE 1997). This growth-promoting activity remains despite mutations abolishing MMP inhibitory activity, demonstrating that it is the result of a distinct function of the C-terminal domain (Chesler L 1995, Wingfield PT 1999).

TIMPs may also promote programmed cell death (Mannello F 2001). Programmed cell death (PCD) plays a crucial role in tissue homeostasis in both physiological and

pathological conditions and apoptosis is a major mechanism in this tightly controlled process. In 1994 it was shown that increased expression of TIMP 3 mRNA is found in degenerating retinitis pigmentosa retinae. This was felt to represent both restructuring of the ECM and contribute to the activation of apoptotic cell death (Jones SE 1994). More recent work has shown TIMPs bind to the cell surface and translocate to the nucleus (Ritter LM 1999), and accumulate specifically in the nucleus in a cell cycle dependent manner (Zhao WO 1998). TIMP 3 has also been shown to cause large cell cluster formation and subsequent apoptotic cell death in a colon carcinoma cell line (Bian J 1996), and that the induction of this apoptosis was due to the stabilisation of TNF-α receptors on the cell surface by TIMP 3 (Smith MR 1997) as a result of its inhibition of TNF- α converting enzyme (Amour A 1998). This prodeath function of TIMP 3 is located within the N-terminal loop and the presence of functional MMP-inhibitory activity is associated with the induction of apoptosis (Bond M 2000).

However data on TIMP 1 shows that it suppresses apoptosis in Burkitt's lymphoma cell lines (Guedez L 1998) and in human breast cell lines (Li G 1999). TIMP 2 has been shown both to promote (Lim MS 1999) and protect against apoptosis (Valente P 1998). TIMP 2 can also suppress growth factor responsiveness by interfering with the activation of tyrosine kinase-type growth factor receptors, and its ability to block mitogenic signaling is independent of its MMP inhibitory activity (Hoegy SE 2001).
At present no TIMP receptors have been identified, suggesting TIMPs may act as decoys for signaling molecules. The growth promoting and growth suppressing activities of TIMPs may not be completely MMP independent as the mutant TIMPs retain their ability to interact with MMPs via secondary non-inhibitory sites found on their C terminal domains (Howard EW 1991). The growth altering activities of TIMPs may still reflect the ability of TIMPs to indirectly modify MMP activity.

3.7 Other endogenous MMP inhibitors

There are other endogenous MMP inhibitors in the body and due to its abundance as a plasma protein, α2-macroglobulin is the major inhibitor of MMPs in tissue fluids. It has been proposed that TIMPs may act locally in the tissues, inhibiting MMPs in a reversible manner, whereas α2-macroglobulin acts systemically forming complexes with MMPs that are then irreversible cleared by receptor mediated endocytosis. Thrombospondin-2 forms a complex with MMP2 and facilitates scavenger-receptor mediated endocytosis and clearance (Yang Z 2001). Thrombospondin 1 binds to pro MMP 2 and 9, and directly inhibits their activation (Bein K 2000, Rodriguez-Manzaneque JC 2001). Thrombospondin-1 has also been reported to increase MMP 2 and 9 activation (Taraboletti G 2000).

A further class of MMP inhibitors, protein subdomains, has recently been recognised and they have structural similarity to the TIMPs. These inhibitory fragments include the C-terminal fragment of the procollagen C-terminal proteinase enhancer protein (Mott JD 2000), and the noncollagenous NC1 domain of type IV collagen (Netzer KO 1998).

RECK (reversion-inducing cysteine-rich protein with kazal motifs) is the only known membrane-bound MMP inhibitor (Oh J 2001).

3.8 Cell surface localisation

A number of mechanisms exist for localising MMPs to the cell surface and to specific cell surface subdomains, and these include the expression of membrane bound MT-MMPs; the binding of MMPs to cell surface receptors; the presence of cell surface receptors for MMP activating enzymes such as uPA, plasmin, thrombin and elastase; and the concentration of MMPs on pericellular ECM molecules. These localisation mechanisms enhance MMP activation, limit the access of MMP inhibitors, concentrate

MMPs within the vicinity of their targets, and limit the extent of proteolysis to discrete pericellular regions.

Transmembrane and GPI-linked MT-MMPs localise proteolytic activity at the cell surface and removal of the transmembrane domains of MT-MMP1,2 and 3 abolishes their ability to promote cellular invasion (Hortary K 2000). MT-MMPs can concentrate within specific cell surface domains such as invadopodia, where active ECM degradation takes place. Another mechanism of localising MMPs to the cell surface is via cell surface docking receptors. MMP2 is activated by a complex mechanism involving MT-MMP 1, integrin αvβ3 and a further, already activated MMP 2 (Deryugina EI 2001).

MMP 1 can also interact with a cell surface integrin α2β1, whereas MMP 3 and 13 do not (Stricker TP 2000). α2β1 integrin can act as both an MMP 1 inducer (Pilcher BK 1999) and an MMP 1 receptor. MMP 1 expression and localisation to the cell surface is also produced by CD147/EMMPRIN (Guo H 2000).

MMPs can also be bound to the cell surface by their own substrates. Latent proMMP 9 binds with high affinity to type IV collagen α2 chains on the surface of several cell types, ensuring its ready availability for future need (Olson MW 1998, Toth M 1999). Activated MMP 9 binds to the cell surface hyaluron receptor CD44 (Bourguignon LY 1998) and this localisation of MMP 9 to the cell surface by CD44 seems to promote tumour cell invasion and angiogenesis and may mediate the activation of latent TGF-β by MMP 9 (Yu Q 2000). MMP 7 binds to cell surface and ECM heparan sulfate moieties that enhance the stability, activation and activity of MMP 7 (Yu WH 2000).

3.9 MMP Catabolism and clearance

Further regulation of MMPs comes via proteolytic inactivation and clearance of the MMPs themselves. However little is known about the autoproteolysis of active MMPs except that some cleavages inactivate MMPs whereas others that just remove the haemopexin domain, modify the substrate specificity (Woessner JF 2000). Haemopexin

domain removal also cancels the ablity of some MMPs to localise to the cell surface. C-terminally truncated MMP2 can no longer be inhibited by TIMPs (Itoh Y 1998). MT-MMPs can be secreted if they are cleaved at a juxtamembrane site before or after they reach the cell surface (Imai K 1996).

Extracellular MMP levels are also regulated by the direct clearance of intact enzymes. MMPs are bound by α2-macroglobulin and the resulting complex is endocytosed and cleared. Thrombospondin 2 (TS 2) is involved in the clearance of MMPs. Both latent and active forms of MMP 2 are bound by TS2 and the complex is then endocytosed by the low density lipoprotein receptor-related protein LRP (Yang Z 2001). The cellular internalisation of TS2/MMP 2 complexes by the LRP scavenger receptor may well play an important role in regulating MMP 2 levels outside fibroblasts and other cells. MMP 13 is rapidly cleared after it binds to an MMP 13 specific 170 kDa high affinity receptor present on various cell types (Barmina OY 1999). The binding requires calcium and the subsequent internalisation and degradation of MMP 13 requires LRP because LRP-null cells bind MMP 13 but fail to internalise it. Internalisation of both MMP 13 and TS2/MMP 2 complexes is inhibited by the 39 kDa receptor associated protein RAP, which binds and inhibits LRP.

3.10 Association of MMPs/TIMPs with Cancer

As mentioned previously, to allow tumour invasion and metastasis sufficient degraditive enzymatic capacity is needed to breakdown surrounding proteinaceous structural barriers. This can either be provided by the metastatic cells themselves or may be derived from tumour associated host tissues, including adjacent stromal tissue and tumour infiltrating immune cells.

Proteases of all four major subtypes have been linked with the malignant phenotype (Brunner N 1994; Sloane BF 1994). From early work of Liotta et al (Liotta LA 1977, 1980) and Tryggvason et al (Salo T 1982) interest was focussed on type IV

collagenase the enzyme responsible for degradation of type IV collagen, a major structural protein in basement membrane. The enzymes responsible for this activity are now recognised to be either gelatinase A (72 kD type IV collagenase) or gelatinase B (92 kD type IV collagenase). The first member of the MMP family to be cloned was transin, the rat homologue of stromelysin-1, which was identified as an oncogene and growth factor-inducible gene (Matrisian LM 1985). Subsequent work identified the product of this complementary DNA (cDNA) as a protease that was overexpressed in malignant mouse skin tumours (Matrisian LM 1986) and was related to the prototypic member of the MMP family, interstitial collagenase (Goldberg GI 1986; Whitham SE 1986).

Further evidence for MMPs role in cancer progression comes from animal studies. In transplantation assays relatively benign cancer cells acquire malignant properties when MMP expression is upregulated, and highly malignant cells become less aggressive when MMP expression or activity is reduced (Coussens LM 1996). Stromal cells also secrete MMPs that modulate tumour aggressiveness. Fibroblasts that do not express MMP 11 cannot support the in vivo growth of breast cancer cells (Masson R 1998), and colonisation of the lungs of MMP 2 or 9 deficient mice is less marked than wild type mice following intravenous injection of cancer cells (Itoh T 1998, 1999).

In mice tissue specific overexpression of MMP 1 or 7 leads to hyperproliferative disease and increased cancer susceptibility (Sternlicht MD 2001). MMP 3 and MT-MMP 1 (MMP14) expression in the mammary gland of mice leads to spontaneous breast cancers (Sternlicht MD 1999, Ha HY 2001). Mice that lack MMP 2, 7, 9 or 11, or over produce TIMP 1 develop fewer cancers than wild type mice (Sternlicht MD 2001). The development of squamous cell carcinomas in the MMP 9 null mice is restored by transplanting MMP 9 expressing bone marrow cells, showing the importance of the inflammatory cells in carcinogenesis (Coussens LM 2000).

3.11 Role of MMPs and TIMPs in cancer progression

Cancer development is characterised by six alterations in normal cell physiology.

1) Self support in growth signals
2) Insensitivity to growth inhibitory signals
3) Escape from apoptosis
4) Infinite replication
5) Sustained angiogenesis
6) Tissue invasion and metastasis

Initially MMPs and TIMPs were only considered important in invasion and metastasis, but recent developments have shown they are important in most of the above alterations in normal cell physiology.

3.11a Growth regulation

As mentioned earlier, cancer cell proliferation is decreased in tumours from MMP 9 deficient mice compared with wild type mice (Coussens LM 2000). There are 3 ways in which MMPs can promote cancer cell proliferation. MMPs can release the cell-membrane bound precursors of some growth factors, such as TGF-α (Peschon JJ 1998). Peptide growth factors that are sequestered by ECM proteins become bioavailable once these proteins are degraded by MMPs, such as IGF release from MMP cleavage of IGF-BPs (Manes S 1997, 1999). Thirdly, through their effects on the ECM composition, MMPs can regulate proliferative signals through integrins (Agrez M 1994). MMPs may also negatively regulate cancer-cell growth by activation of TGF-β (Derynck R 2001) or generation of proapoptotic molecules such as FAS ligand or TNF-α.

3.11b Apoptosis regulation

Evasion of apoptosis permits survival in the presence of genetic instability, low levels of oxygen and nutrients, attacks from the immune system, anticancer treatment, and detachment from the ECM a prerequesit for metastasis (Reed JC 1999). MMPs have both

apoptotic and anti-apoptotic actions. MMP 3,7,9 and 11 regulate apoptosis. MMP 3 induces apoptosis when over expressed in mammary epithelial cells (Alexander CM 1996, Witty JP 1995), possibly by degrading laminin (Sympson CJ 1994). MMP 7 releases membrane bound FASL, a transmembrane stimulator of the death receptor FAS (Powell WC 1999, Mitsiades N 2001). Released FASL induces apoptosis of neighbouring cells (Powell WC 1999), or decreases cancer cell apoptosis (Mitsiades N 2001), depending on the system. MMP 7 also inhibits apoptosis by cleaving pro-heparin-binding epidermal growth factor (pro-HB-EGF) to generate mature HB-EGF, which promotes cell survival by stimulating the ERBB4 receptor tyrosine kinase (Yu W-H 2002). Overexpression of MMP 11 decreases spontaneous apoptosis in tumour xenografts (Wu E 2001). Cancer cells injected into MMP 11-null mice have a higher rate of apoptosis than wild-type mice (Boulay A 2001). MMP 11 may inhibit apoptosis by releasing IGFs (Manes S 1997), which can act as survival factors (Baserga R 2000). MMPs are also part of the apoptotic process. They cleave VE-cadherin (Herren B 1998), PECAM-1 (Ilan N 2001), and E-cadherin (Steinhusen U 2001) during apoptosis of endothelial or epithelial cells. Shedding of these adhesion molecules might contribute to the typical rounding up of apoptotic cells.

3.11c Angiogenesis regulation

Angiogenesis, or new vessel formation is essential for tumour growth (Hanahan D 1996). The endogenous MMP inhibitors TIMP 1 (Martin DC 1999), TIMP 2 (Li H 2001), thrombospondin 1 (Rodriguez-Manzaneque JC 2001) and RECK (Oh J 2001) all reduce tumour angiogenesis in animal experiments, as does the synthetic MMP inhibitor BAY 12-9566 (Gatto C 1999). This indicates that MMPs are important positive regulators of angiogenesis and they achieve this by a number of mechanisms. One mechanism is the degradation of the ECM, and indeed cleavage of collagen type I is required for endothelial-cell invasion of the ECM and for vessel formation (Seandel M 2001). MMP 2, 9 and 14 directly regulate angiogenesis. Down regulation of MMP 2 expression in cancer

cells decreases angiogenesis in a chicken chorioallantoic membrane model (Fang J 2000). Tumour angiogenesis and growth is reduced in MMP 2 deficient mice compared with wild-type mice (Itoh T 1998). Cleavage of collagen type IV by MMP 2 exposes a cryptic, αvβ3 integrin binding site within the collagen. Blockage of this new site with an antibody decreases migration of endothelial cells and in vitro angiogenesis, and reduces tumour growth in animal experiments (Xu J 2001). MMP 9 has been shown to be important for angiogenesis in 2 transgenic mouse models of human progression: the K14-HPV16 skin cancer model (Coussens LM 2000), and the RIP1-Tag2 insulinoma model (Bergers G 2000). MMP 9 acts by increasing the bioavailability of the pro-angiogenic factor VEGF (Bergers G 2000) by an unknown mechanism. MMP 14 (MT-MMP 1) degrades the fibrin matrix surrounding newly formed vessels (Hiraoka N 1998) potentially allowing the endothelial cells to invade further into the tumour tissue, and antibodies directed against the catalytic domain of MMP 14 block endothelial cell migration, invasion and capillary tube formation in vitro (Galvez BG 2001).

MMPs also produce fragments that have the opposite effect and are angiogenesis inhibitors. Cleavage of plasminogen by MMP 2, 3, 7, 9 and 12 generates angiostatin (Dong Z 1997, Cornelius LA 1998, Gorrin Rivas MJ 2000), and MMP 3, 9, 12, 13 and 20 might be involved in the generation of endostatin, a C-terminal fragment of the basement membrane collagen type XVIII (Ferreras M 2000). Angiostatin and endostatin reduce endothelial cell proliferation (O'Reilly MS 1994, 1997) and endostatin may inhibit endothelial-cell invasion by acting as an inhibitor of MMP 14 and MMP 2 (Kim YM 2000). MMP 12 may also inhibit tumour angiogenesis by cleavage and shedding of cell surface bound urokinase-type plasminogen-activator receptor, which is required for endothelial cell invasion into fibrin (Koolwijk P 2001).

3.11d Invasion and metastasis

Experimental evidence for the role of MMPs in metastasis comes from in vitro invasion assays and in vivo xenogratft metastasis assays. From the invasion assays over expression of TIMPs inhibits (Ahonen M 1998), and MMPs 2, 3, 13 and 14 promote the invasion of cell lines through either collagen type I, optic nerve explants or matrigel (Lochter A 1997, Belien AT 1999, Deryugina EI 1997, Ala-Aho R 2002). In experimental metastasis assays, the number of colonies formed in the lungs of mice is reduced by down regulation of MMP 9 in cancer cells (Hua J 1996) and is also reduced in the MMP 2 and 9 null mice compared with wild type mice (Itoh T 1998, 1999). Migration is one of the first steps in invasion. Cleavage of laminin-5 by MMP 2 and MT-MMP 14 reveals a cryptic site that triggers cell motility (Giannelli G 1997, Koshikawa N 2000). This cleaved form is found in experimental tumours (Giannelli G 1997), and MMP 14 is co-localised with laminin-5 in human cancer (Koshikawa N 2000). Migration is regulated by cycles of MMP activity, the interaction between CD 44 and MMP14, causing cleavage of CD44 (Kajita M 2001) being vital, as is the binding of MMP 9 to CD44, localising MMP 9 to the cell surface to allow tumour invasion and angiogenesis (Yu Q 2000). Cleavage of the cell adhesion molecule E-cadherin by MMP 3 or 7 (Noe V 2001) leads to the promotion of tumour cell invasion in a paracrine manner, probably by binding to and interfering with the function of other full-length E-cadherin molecules. Cleavage of E-cadherin also triggers the epithelial to mesenchymal transition (Sternlicht MD 1999, Lochter A 1997) which is associated with aggressive malignant behaviour (Birchmeier C 1996). Specialised cell surface protrusions, or invadopodia localise the MMPs during invasion. MMP 14 is recruited to invadopodia via its transmembrane and cytoplasmic domains (Nakahara H 1997). MMP 9 is recruited to invadopodia by binding to CD44 as antibodies against CD44 inhibit both invadopodia formation and tumour cell migration (Bourguignon LY 1998).

MMP 2 localise to the invadopodia either by binding to αvβ3 integrin (Brooks PC 1996) or to MMP 14.

MMPs also participate in the late events in the metastatic process, when cancer cells enter, survive and exit the blood vessels or lymphatics. Bernhard et al have demonstrated that MMP 9 expression is strongly associated with the metastatic ability of rat embryo fibroblasts (Bernhard EJ 1990), and that its overexpression results in increased metastatic potential following injection into nude mice (Bernhard EJ 1994). MMP 9 is required in an intravasation model (Kim J 1998), and overexpression of MMP 14 increases the number of cancer cells that survive intravenous injection in an experimental metastasis assay (Tsunezuka Y 1996). In vitro studies measuring invasion through amnion basement membrane, smooth muscle cell-generated basement membrane, or reconstituted basement membrane have generally supported the idea that the effect of MMP activity in metastasis is degradation of the basement membrane and extracellular matrix. An inhibition of in vitro invasion has been observed following the addition of recombinant or transfected TIMP 1 or TIMP 2 (Schultz RM 1988; Albini A 1991; Khokha R 1992), and targetted disruption of the TIMP 1 gene resulted in an increase in in vitro invasion (Alexander CM 1992). However, following transfection studies of various MMP family members, positive effects on in vitro invasion have been documented (Matrisian LM 1990), but there are also examples of a lack of a consistent effect in these assays (Witty JP 1994; Noel AC 1996). Also no change in in vitro invasion was detected in loss of function studies in which expression of stromelysin 3 (Noel AC 1996) and matrilysin (Witty JP 1994) were ablated using antisense technology. This raises the possibility that at least some MMPs may affect steps in metastasis other than extravasation.

IVVM work has also shown that MMP activity may not be important for extravasation, as TIMP 1 over expressing cancer cells exit the vasculature as well as control cells, but they produce fewer and smaller metastases due to diminished cancer cell

growth after they leave the bloodstream (Koop S 1994). Therefore proliferation at the secondary site to establish a metastasis requires MMP activity. Spontaneous and experimental metastasis to the liver is reduced in TIMP 1 over expressing mice, and is increased in mice that express antisense TIMP 1 (Kruger A 1997).

Further studies have backed up the potential effects of MMPs on growth of metastatic lesions. Martin et al examined the effect of TIMP 1 on the initiation and growth of liver tumours in transgenic mice expressing either sense or antisense TIMP 1 constructs (Martin DC 1996). They showed TIMP 1 overexpression inhibited SV40 T-antigen-induced tumour initiation, growth and angiogenesis, while TIMP 1 reduction resulted in more rapid tumour initiation and progression. Khokha et al showed TIMP 1 transfection in B16F10 melanoma cells resulted in a decline in primary tumour growth following a subcutaneous injection as well as a reduction in lung colonisation following an intravenous injection (Khokha R 1994). Soloway et al used co-isogenic cells and genetically manipulated mice varying in expression of TIMP 1 to show that lung colonisation is influenced by the TIMP 1 genotype of the tumour but not that of the host (Soloway PD 1996).

TIMP 2 has also been shown to reduce tumour cell growth as well as metastases. Retroviral introduction of TIMP 2 into transformed rat embryo fibroblasts reduced primary tumour growth as well as haematogenous metastasis (Imran S 1996). TIMP 2 overexpression reduced the growth of metastatic human melanoma cells injected subcutaneously in immunocompromised mice, although it did not prevent metastasis in this study (Montgomery AM 1994). The growth-inhibitory effect of TIMP 2 was shown to require a three-dimensional collagen matrix and was not observed in gelatin-coated dishes; in the presence of matrix, TIMP 2 expressing melanoma cells demonstrated a reduction in growth rate and assumed a differentiated morphology.

Studies with synthetic inhibitors of MMPs further support the requirement for MMP activity in the establishment of metastases. These low molecular weight compounds such as batimastat (BB-94) have been shown to reduce metastasis of melanoma, mammary carcinoma and colorectal tumour cells in experimental metastasis assays (Chirivi RG 1994; Watson SA 1995; Eccles SA 1996) and of human colon (Wang X 1994) and breast (Sledge GW 1995) tumour cells injected orthotopically in nude mice (see Chapter 4).

3.12 MMPs and the immune response to cancer

Mechanisms by which cancer cells escape immune system destruction involve MMPs. Tumour-specific cytotoxic T lymphocytes, natural killer cells, neutrophils and macrophages all infiltrate tumours allowing immune system destruction of tumours. T lymphocyte proliferation is regulated by cytokine signalling via the interleukin-2 receptor-α (IL-2Rα). MMPs including MMP 9 can cleave IL-2Rα and so suppress the proliferation of the T lymphocytes (Sheu BC 2001). MMP 9 also activates TGF-β (Yu Q 2000) an important inhibitor of the T-lymphocyte response against tumours (Gorelik L 2001). MMP 11 generates a cleavage product from α1-proteinase-inhibitor which decreases the sensitivity of tumour cells to natural killer cells (Kataoka H 1999). MMP 11-null mice have an increased neutrophil and macrophage infiltrate in their tumours compared with wild-type mice due to inhibition of a chemoattractant (Boulay A 2001).

Chemokines are also targets of MMPs. MMP 9 cleaves IL-8 (a neutrophil chemoattractant) increasing its activity ten times. It inactivates the CXCL 7 precursor, CXCL 4 (platelet factor 4) and CXCL 1 (Opdenakker G 2001). MMP 2 cleaves CCL 7 inactivating it and antagonising its receptor (McQuibban GA 2000). CXCL 12 is cleaved and inactivated by MMP 1, 3, 9, 13 and 14 (McQuibban GA 2001). Although the immune system reacts against the cancer cells and probably delays tumour progression, in animal models mast cells, neutrophils and macrophages are contributors to the progression of

cancer (Coussens LM 2001). Inflammatory cells produce several MMPs, including MMP 9, 12 and 14 which might stimulate cancer progression.

3.13 New view of the contribution of MMPs and TIMPs to metastasis

MMPs have long been associated with metastasis, and there is no doubt that they are major functional contributors to the metastatic process. The nature of their contribution was assumed originally to be primarily facilitation of the breakdown of the physical barriers between a primary tumour and distant sites for metastasis. Recent evidence, summarised above, suggest that the action of MMPs at steps both before and after the breakdown of the apparent physical barriers to metastasis may in fact be of greater importance. MMPs and their inhibitors appear to be important regulators of the growth of tumours, both at the primary site and as metastases.

Chapter 4: MMPs/TIMPs IN COLORECTAL CANCER

4.1 MMPs in primary colorectal cancer

MMPs are expressed in a small number of colorectal cancer cell lines (Della Porta P 1995) and in ascites produced by mice inoculated with a human colorectal xenograft (Watson SA 1996).

4.1a Collagenases and primary colorectal cancer

MMP 1 activity in colorectal tumours has been investigated using immunohistochemistry (Hewitt RE 1991). They found increased staining for collagenase in the connective tissue stroma of carcinomas, as compared with adenomas and normal mucosa. Little evidence of epithelial cell staining for collagenase was seen in any tissue.

MMP 1 expression in colonic adenoma and carcinoma has been studied using in situ hybridisation (Gray S 1993). They showed MMP 1 expression in eosinophils and fibroblasts in the tumour stroma whereas the adjacent normal mucosa showed no detectable expression.

Urbanski et al (Urbanski SJ 1993) examined mRNA expression of metalloproteinases with the progression of sporadic colorectal neoplasia and found MMPs 1 and 11 more frequently expressed by colonic carcinomas and malignant polyps than by adenomas or non neoplastic tissue.

Otani et al (Otani Y 1994) used in situ hybridisation to look at MMP 1 expression by gastrointestinal tract tumours, including colorectal cancers, and found MMP 1 mRNA in the stroma surrounding invasive tumour nodules of malignant epithelium. They also found strong but scattered signals in stromal cells within cancer nests at the margin of invasion. There was no hybridisation signal in cancer cells or in epithelial cells with adenomatous structure.

Newell et al (Newell KJ 1994) showed expression of MMP 1 in occasional adenomas and carcinomas but Gallegos et al (Gallegos NC 1995) failed to identify any MMP 1 in 40 specimens of colorectal cancer.

However the occurrence of MMP 1 in colorectal cancer has again been studied using immunohistochemistry (Murray GI 1996). Although only 10 (16%) of the tumours showed positive immunoreactivity for MMP 1, in those that did more than 90% of tumour cells were positive, and the presence of MMP 1 was associated with a poor prognosis and had a prognostic value independent of Dukes' stage.

Although work has been done measuring MMP 8 (neutrophil collagenase) in breast carcinoma and correlating it with MMP 9, uPA and oestrogen receptor status (Duffy MJ 1995), at present no work has been performed looking at MMP 8 in colorectal cancer. MMP 13 (collagenase 3) was originally identified in breast carcinomas (Freije JMP 1994) and subsequent work has shown that it is produced by a variety of malignant tumours including head and neck carcinomas, chondrosarcomas and basal cell carcinomas of the skin. In all cases the expression of MMP 13 is associated with aggressive tumours (Balbin M 1999). MMP 13 expression in colorectal cancer has been studied by Bodey et al (Bodey B 2000). They studied the histological expression of MMP 13 in 19 colorectal carcinomas using an indirect alkaline phosphatase conjugated antigen technique. They found no expression of collagenase 3 (MMP 13) in these thirteen colorectal cancer cases.

4.1b Gelatinases in primary colorectal cancer

Immunohistochemical, in situ hybridisation and zymographic techniques have all been used to examine the expression of gelatinases (MMPs 2 and 9) in colorectal cancer. Levy et al (Levy AT 1991) used blot hybridisations of total RNA to examine the MMP 2 expression in 19 cases of colorectal carcinoma and found it to be higher than the surrounding mucosa in 72% of cases. They confirmed this finding using

immunohistochemical techniques in 70 cases of colon tumours and demonstrated a significant correlation with Dukes classification.

Using in situ hybridisation techniques Poulsom et al detected MMP 2 mRNA in 10 of 12 colon carcinomas and in 2 of 3 adenomas, and the expression was localised to mesenchymal cells in the stroma as opposed to tumour epithelial cells (Poulsom R 1992). This work was confirmed by Pyke et al who found MMP 2 in fibroblasts in the stroma surrounding 18 colon carcinomas, and MMP 9 in tissue macrophages especially surrounding invading malignant epithelium. Again no hybridisation signals for either gelatinase was detected in the cancer cells (Pyke C 1993).

Using immunohistochemical techniques Gallegos et al studied 40 specimens of colorectal cancer and found MMP 2 (gelatinase A) expressed by peritumoural fibroblast-like cells. MMP 9 (gelatinase B) was confined to polymorphonuclear leucocytes. Again there was no expression by tumour cells (Gallegos NC 1995). Tomita et al confirmed these results and showed a gradual increase in staining intensity from hyperplastic polyps to tubovillous adenomas, with a marked increase in staining for both gelatinases in in-situ carcinoma and adenocarcinoma (Tomita T 1996).

Zeng et al have studied the expression and localisation of MMP 9 protein and mRNA in human colorectal carcinoma. For 26 patients, colorectal cancer specimens and matched normal mucosa specimens showed a nine fold increase, tumour to normal mucosa (T/N) for MMP 9 RNA and a seven fold increase for MMP 9 protein. Both MMP 9 mRNA and protein signals were strongest in the population of stromal cells concentrated at the tumour-stroma interface of an invading tumour, and these cells were identified as macrophages (Zeng ZS 1996). They have also shown that a high T/N MMP 9 mRNA expression is associated with a significantly shorter disease free and overall survival duration in colorectal carcinoma, and that this was an independent prognostic factor for

disease free survival (Zeng ZS 1996). However other groups (Liabakk N-B 1996) have failed to show MMP 2 or 9 activity to be related to disease free or 5 year survival. More recently Zeng et al have studied the relationship between MMP 2 and 9 activities and the pattern of type IV collagen expression during human colorectal tumourigenesis. They showed a loss of basement membrane type IV collagen along with elevations in MMP 2 and 9 expression, especially the activated forms, occur during colorectal tumourigenesis (Zeng ZS 1999).

Zymographic studies have demonstrated overexpression of MMP 2 and MMP 9 in colorectal cancer compared with normal mucosa. Parsons et al studied 53 colorectal cancers and their corresponding normal mucosa. They found significant overexpression of pro-MMP 9, pro-MMP 2 and active MMP 2 in colorectal cancers compared with corresponding normal mucosa. Pro-MMP 2 was expressed in normal mucosa but there was no expression of the active MMP 2 enzyme. Pro-MMP 9 was overexpressed in all 15 colorectal adenomas compared with normal mucosa but expression was less than in the carcinomas. There was no overexpression of MMP 2 in adenomas compared with normal mucosa either for the pro-MMP 2 or the active form. No statistically significant correlations between Dukes' stage and gelatinase expression were seen (Parsons SL 1998).

4.1c Stromelysins in primary colorectal cancer

MMP 7 (Matrilysin) has been detected in colorectal cancer using RT-PCR techniques while being absent in normal mucosa. Yoshimoto et al examined 10 cases of colorectal cancer and found MMP 7 mRNA in 9 out of 10 cancerous tissues whereas none was detected in adjacent normal colon tissue (Yoshimoto M 1993).

Newell et al used in situ hybridisation and immunohistochemistry to study MMP 7 expression in colorectal adenomas and carcinomas. They found 50% of adenomas and 90% of carcinomas expressed MMP 7, whereas normal colonic mucosa did not. The MMP 7 was expressed by epithelial tumour cells, as opposed to the adjacent stroma where most

MMPs are localised in colorectal cancer (Newell KJ 1994). Yamamoto et al confirmed that MMP 7 mRNA was located in colorectal adenomas but not hyperplastic polyps, inflamed regions of ulcerative colitis or normal colon tissues (Yamamoto H 1994). The expression of matrilysin in colorectal adenomas suggests its expression is an early event in colorectal tumourogenesis.

MMP 7 expression has been shown to be significantly correlated with Dukes' stage. Mori et al looked at 47 cases of colorectal carcinoma and nine cases of liver metastases from colorectal carcinoma. They found the mRNA signal was greater in the colorectal carcinoma than in paired adjacent normal colonic or rectal mucosa in 39 of 47 cases. The expression of MMP 7 mRNA in tumour tissues increased with increasing Dukes' stage and was greatest in the metastatic liver lesions (Mori M 1995).

These findings were confirmed by Ishikawa et al (Ishikawa T 1996) and the same group went on to show MMP 7 expression in sporadic colorectal adenomas correlated with the degree of dysplasia and the size of the mass, whereas most of the adenomas in patients with familial adenomatous polyposis coli expressed MMP 7 mRNA irrespective of adenoma size or degree of dysplasia. They concluded that as MMP 7 is more likely to be expressed in adenomas with a potential for malignancy, this enzyme may play a role in the malignant conversion of colorectal adenomas (Takeuchi N 1997).

The differential expression of MMP 7 and cyclooxygenase-2 in colorectal neoplasms has been studied by Shattuck-Brandt et al. Both MMP 7 and the prostaglandin H synthase cyclooxygenase-2 (Cox-2) are thought to play key roles in colorectal carcinogenesis. Although over 80% of colorectal cancers expressed both MMP 7 and Cox-2 in the neoplastic epithelium the levels and localisation were distinct. Cox-2 expression was strongest in well-differentiated areas, and matrilysin immunostaining was strongest in the more dysplastic and invasive regions of the tumour (Shattuck-Brandt RL 1999).

MMP 3 (Stromelysin 1) has been shown to be expressed in the stroma of colorectal carcinomas (Newell KJ 1994; Gallegos NC 1995) and was not present in adenomas (Newell KJ 1994).

MMP 11 (Stromelysin 3) has been detected in colorectal cancers, especially in the advanced stages (Dukes B and C) and in liver metastases, while little is expressed in Dukes A disease, adenomas or normal mucosa (Porte H 1995).

4.2 TIMPs and Primary colorectal cancer

Immunohistochemical and in situ hybridisation studies have shown that TIMP 1 and 2 are also expressed by colorectal carcinomas, primarily in surrounding stromal tissue. Hewitt et al showed TIMP 1 immunostaining for 20 colorectal carcinomas in the connective tissue stroma of the carcinomas, the pattern being similar to MMP 1 staining (Hewitt RE 1991) and Poulsom et al, using in situ hybridisation, showed TIMP 2 mRNA in the mesenchymal cells of the stroma (Poulsom R 1992).

Using in situ hybridisation and immunohistochemistry Newell et al showed TIMP 1 expression was widespread and observed in both epithelial and stromal cells of adenomas and carcinomas (Newell KJ 1994).

Zeng et al examined 56 cases of colorectal carcinoma and 10 cases of liver metastasis from colorectal carcinoma by northern-blot hybridisation and found that TIMP 1 RNA was significantly elevated in both primary CRC an liver metastasis. No relationship was noted between TIMP 1 expression and tumour size, location or differentiation (Zeng ZS 1995). In situ hybridisation localised TIMP 1 mRNA predominantly within spindle-shaped fibroblast-like cells rather than in cancer cells themselves (Zeng ZS 1995).

Tomita et al used immunocytochemistry to examine the expression of TIMPs 1 and 2 by hyperplastic polyps, tubular adenomas, tubovillous adenomas, villous adenomas

and adenocarcinomas of the colorectum. They found increasing levels of TIMP 1 and 2 in stromal cells as they passed from tubular adenomas to adenocarcinomas (Tomita T 1996). Murashige et al used Northern and dot-blot hybridisation to examine the expression of MMPs 2 and 9, and TIMPs 1 and 2 in 66 cases of colorectal carcinoma and 10 liver metastases. They found the levels of MMPs 2 and 9, and TIMP 1 mRNA were significantly higher in primary colorectal cancers than in their adjacent normal tissues, and the levels of all four mRNAs were significantly higher in liver metastases than in normal colorectal tissues. They also found that higher levels of TIMP 1 mRNA were positively correlated with lymph node metastasis and the five year survival, and higher levels of TIMP 1 and 2 mRNA were positively correlated with the Dukes' classification (Murashige M 1996).

Ring et al used immunohistochemical techniques to look at the staining patterns of TIMP 2 in CRC. They found significantly more positive expression of TIMP 2 in basement membranes, diffusely in stroma and subglandularly, in localised tumours than in tumours with regional or distant metastases (Ring P 1997).

The correlation noted in these studies between increased TIMP 1 and 2 mRNA level and advanced CRC stage reflects a possible growth-promoting function for the TIMPs in human CRC. This is consistent with in vitro data showing that TIMP 1 has erythroid-potentiating activity and increases the growth of a variety of both malignant and nonmalignant cells (Hayakawa T 1994).

4.3 MMPs and TIMPs in CRC liver metastases

Less work has been done on MMPs and TIMPs in CRC liver metastases, and most focuses on the gelatinases.

4.3a Gelatinases in CRC liver metastases

Murashige et al studied the expression of MMPs 2 and 9, and TIMPs 1 and 2 in 10 liver metastases from colorectal cancer using Northern and dot-blot hybridisation. They

found that mRNAs for all four genes were significantly higher in liver metastases than in normal colorectal tissues (Murashige M 1996).

Karakiulakis et al using gelatin zymography found that activity of MMPs 2 and 9 was significantly enhanced in liver metastases compared with the primary tumour (Karakiulakis G 1997).

Musso et al used in situ hybridisation and zymography to investigate the cellular source of MMP 2 and TIMP 2 in seven liver metastases. They found that MMP 2 and TIMP 2 mRNA were expressed by anti-alpha-smooth muscle actin-positive cells at the invasive front. Intratumour microvessels showed a strong labelling for MMP 2 but weak for TIMP 2 mRNA. Direct zymography of samples from the invasive front revealed both active and latent forms of MMP 2 (Musso O 1997).

Brand et al evaluated the role of adenoviral delivery of tissue inhibitor of MMP (TIMP 2) for inhibiting metastatic colorectal carcinoma to the liver. They used a highly metastatic cell line (LS174T) that was shown to secrete MMP 2 primarily. Transfection of TIMP 2 into the liver before innoculation of tumour or after tumour was established decreased tumour burden by 95% and 77% respectively. Reductions in proliferation and apoptosis were also seen (Brand K 2000). Work with a COX-2 inhibitor (JTE-522) in mice using a highly metastasizible colon carcinoma cell line, LM-H3 showed a reduction in the number of metastatic nodules on the surface of nude mouse livers. Using gelatin zymography, JTE-522 was shown to inhibit MMP 2 secretion by LM-H3 giving a potential mechanism of action for its reduction in metastatic nodules in the mouse model (Nagatsuka I 2002).

Zeng and Guillem studied MMP 9 and TIMP 1 mRNA expression in 10 liver metastases from colorectal cancer, using in situ hybridisation. Both MMP 9 and TIMP 1 mRNA were expressed in all 10 metastases, with MMP 9 localised within peritumour stroma or at the interface between the tumour stroma and normal liver. TIMP 1 mRNA on

the other hand was located throughout the malignant tumour stroma (Zeng ZS 1995). The same group then looked at the cellular enzymatic expression of MMP 9 in 18 human colorectal cancer liver metastasis specimens using ELISA and zymography. ELISA revealed that the latent form of MMP 9 is present in both liver metastasis and paired adjacent normal liver tissue. Gelatin zymography showed that although the latent form of MMP 9 was present in both metastasis and normal liver, the active form of MMP 9 was seen only in liver metastasis (Zeng ZS 1998).

4.3b Stromelysins in CRC liver metastases

MMP 7 mRNA expression has been shown to increase moving from colorectal adenomas to carcinomas and the same holds true for liver metastases. Both Mori et al (Mori M 1995) and Ishikawa et al (Ishikawa T 1996) have shown MMP 7 mRNA expression to be greater in liver metastases compared with the primary colorectal carcinoma.

This important role of MMP 7 in colorectal liver metastasis has been confirmed in vitro. An MMP 7-specific antisense oligonucleotide inhibited liver metastasis of human colon cancer cells in a nude mouse model. (Hasegawa S 1998).

MMP 11 has also been shown to be expressed in CRC liver metastases. Using northern blot hybridisation, Porte et al found 10 out of 13 liver metastases expressed MMP 11 (Porte H 1992).

Chapter 5: SYNTHETIC MATRIX METALLOPROTEINASE INHIBITORS (MMPIs)

Inhibition of MMPs can occur at a number of levels including blocking the synthesis of MMPs, preventing their interaction with molecules that direct their activities to the cell surface, or inhibiting their enzymatic activity.

5.1 Inhibition of MMP synthesis

MMP synthesis can be inhibited directly by transfecting cells with antisense mRNA or oligonucleotides, or by targeting mRNA with ribozymes. Down regulation of MMP 7 and 9 by these mechanisms results in reduced tumour burden or metastasis in mouse models (Hua J 1996, Yonemura Y 2001, Kondraganti S 2000). MMP expression can also be reduced indirectly by inhibition of signal transduction pathways, such as tyrosine kinase receptor signalling (Noonberg SB 2000).

5.2 Inhibiting interactions between MMP and other proteins

MMPs can be inhibited by blocking their interactions with other proteins. A compound has been developed that inhibits MMP 2 binding to αvβ3 integrin thereby inhibiting tumour growth in animal experiments but not affecting other MMP 2 activity (Silletti S 2001).

5.3 Exploiting MMP activity

Recombinant proteins have been developed that are cytotoxic agents activated by MMPs. One such contains anthrax toxin fused to an MMP cleavage site which is cleaved at the cell surface, leading to internalisation of the anthrax toxin and cell death (Liu S 2000).

5.4 Blocking MMP activity

Three categories of synthetic MMP inhibitors have been developed and are being used in clinical trials:

1) Collagen peptidomimetics: mimic the cleavage sites of MMP substrates
2) Collagen non-peptidomimetics: conform to the MMP active site
3) Tetracycline derivatives: inhibit activity and synthesis of MMPs

A new class of MMP inhibitors, small peptides, have recently been developed that have high specificity for individual MMPs. Although these are not yet in clinical trials, one such molecule that inhibits MMP 2 and 9 has shown inhibitory effects (Koivunen E 1999).

5.5 Collagen Peptidomimetic MMP inhibitors

5.5a Batimastat

This MMPI has potent activity against most of the major MMPs (see table 6) by mimicking the substrate of the MMP. It therefore works by competitive, potent but reversible inhibition. Batimastat is almost completely insoluble and consequently has a very poor bioavailability when administered orally. Therefore batimastat can only be administered by direct injection into various body cavities (peritoneal or pleural).Intraperitoneal injection of batimastat gives rise to sustained plasma concentrations, with a half-life in humans of up to 28 days. Although far from being suitable for human studies, this is a convenient administration schedule for rodent studies.

5.5b Marimastat

Marimastat has an enzyme inhibitory profile similar to that of batimastat, but it is absorbed almost completely after oral administration with a high and predictable bioavailability and a half life of approximately 15 hours. Thus it is more useful than batimastat in clinical trials using twice daily oral dosing.

However, preclinical data on marimastat is hard to obtain as it is rapidly metabolised in

rodents and so sustained plasma concentrations in rats are difficult to obtain. Therefore most of the preclinical antitumour data has been generated with batimastat whereas marimastat is being used in clinical trials, an approach justified by their similar enzyme inhibitory activity.

Table 6: IC_{50} values for Batimastat and Marimastat against MMPs they inhibit

MMP		IC_{50} Batimastat	IC_{50} Marimastat
Interstitial collagenase	(MMP 1)	3 nM	5 nM
Stromelysin	(MMP 3)	20 nM	230nM
Matrilysin	(MMP 7)	6 nM	16 nM
Gelatinase A	(MMP 2)	4 nM	6 nM
Gelatinase B	(MMP 9)	4 nM	3 nM

5.5c Antitumour activity: Preclinical studies

Batimastat has tested in a number of animal models of various human tumours including:

Murine melanoma model	(Chirivi RG 1994)
Murine haemangioma model	(Taraboletti G 1995)
Xenograft model in ovarian cancer	(Davies B 1993)
Xenograft model in breast cancer	(Sledge GW 1995)
Xenograft model in pancreatic cancer	(Zervos EE 2000)

All these showed a reduction in tumour mass and/or metastases for mice treated with batimastat compared to saline.

Batimastat has also been tested in an orthotopic model of human colorectal carcinoma cells in which tumour cells were implanted into the intestinal wall of nude mice (Wang X 1994). Batimastat, at a dose of 30mg/kg, or saline was administered on day 7 after tumour transplantation. Batimastat resulted in a significant reduction in tumour growth (50% reduction as compared with control animals, $P< 0.05$), a reduction in the

incidence of local and regional invasion, and a reduction in distant metastases. These changes resulted in a significant increase in survival (from 110 to 140 days, $P<0.05$) (Wang X 1994).

5.5d Antitumour activity: Clinical studies

The fact that MMPIs are not cytotoxic agents makes the design of suitable clinical trials complex. Traditionally when developing a new cytotoxic agent the Phase I trial is designed to find the maximum tolerated dose. However for an enzyme inhibitor this is not relevant as following complete enzyme inhibition no further activity would be expected, and it would be inappropriate to continue escalating the dose simply to induce toxicity. Therefore the purpose of the Phase I-II programme with these drugs should be to identify the "optimal biological dose" rather than the "maximum tolerated dose". Also as MMPIs do not kill tumour cells, but work by prevention or reduction of further growth, the standard responses used to evaluate efficacy in cytotoxic agents (complete response, partial response, stable disease, and progressive disease) are inappropriate.

The only way activity can be demonstrated for this class of agents is by randomised trials, demonstrating increases in survival and/or time to disease progression. This requires large studies, including significant numbers of patients being treated for a significant period of time, and is unsuitable for a traditional Phase II programme. One potential way around this is to identify biological markers which could be used during the Phase II programme to get an initial indication about the therapeutic potential of the drug, as well as being able to identify the "optimum biological dose".

5.5e Batimastat: Clinical studies

Batimastat has been tested in Phase I studies in patients with malignant ascites, using intraperitoneal injections (Beattie GJ 1998), and in patients with malignant pleural effusions, using intrapleural injections (Macaulay VM 1995). Intraperitoneal injections of batimastat gave rise to high and sustained plasma concentrations of batimastat (100-200

ng/ml), detectable for upto 28 days after a single injection of batimastat (150-1350mg/m^2). In patients with malignant ascites, batimastat was well tolerated, although some local pain was experienced (Beattie GJ 1998). Following this a further Phase I trial was performed in patients with advanced lung cancer using intraperitoneal administration, as sustained plasma concentrations can be achieved using this method (Wojtowicz-Praga S 1996). In the patients without ascites, however, the injections of batimastat resulted in very substantial dose-related local pain and irritation. No systemic toxicity was noted.

In the second Phase I study patients with malignant pleural effusion received single doses of batimastat (15-135 mg/m^2) given intrapleurally in 50mls 5% dextrose after aspiration of the existing effusion (Macaulay VM 1995). Batimastat was well tolerated and a significant reduction in the number of aspirations was observed, from an average of 2.33 aspirations in the month before treatment to 0.22 in the month after treatment ($P<0.01$).

However, as noted previously batimastat is virtually insoluble and can only be administered by direct injection into various body cavities, making its clinical use limited.

5.5f Marimastat: Clinical studies

As marimastat is not a cytotoxic agent the initial pharmacokinetic work was done in healthy volunteers. This showed a linear dose-plasma concentration relationship, a half-life of approximately 10 hours, and a balanced excretion, as 75% was metabolised in the liver and the remaining 25% excreted unchanged via the kidney. Plasma concentrations at all dose levels studied were well in excess of IC_{90} concentrations, indicating that oral adiministration of marimastat produces pharmacologically active blood levels (Drummond AH 1995).

Marimastat has been tested in more than 400 patients in Phase I/II studies in a number of solid tumours in North America and Europe. In most of these studies cancer specific antigens (CSAs) were measured as surrogate markers for biological activity (CEA

in colorectal cancer (CRC), CA 19-9 in pancreatic cancer, CA 125 in ovarian cancer and prostate specific antigen in prostate cancer). Meta-analysis of all these studies (Millar A 1996), as well as analysis of the individual studies, indicated that marimastat treatment significantly reduced all four CSA rates of rise in a dose dependent fashion.

In the CRC trial, Primrose et al performed an escalating-dose study of oral marimastat in patients with recurrent colorectal cancer, in whom evaluation of serological response was made by measurement of carcinoembryonic antigen (CEA) (Primrose JN 1999). Patients were recruited with a serum CEA level greater than 5 ng/ml, and rising by more than 25% over a four week screening period. Patients were treated for 28 days and entered into a continuation protocol if a serological or clinical benefit was observed. Various treatment regimens were used, either once or twice daily. A biological effect (BE) was defined as a CEA value on day 28 no greater than on day 0; a partial biological effect (PBE) was defined as a rise in CEA over the 28 day treatment period of less than 25%. Of 70 patients recruited, 63 completed the 28 day treatment period, and 55 were eligible for cancer antigen analysis. Examination of the dose-effect relationships provides evidence for a causal relationship between marimastat and biological effects: the proportion of patients with BE or PBE was higher with twice daily dosing (16 out of 25, 64%) than with once daily dosing (11 out of 30, 37%) ($p=0.043$). The median rates of rise of CEA fell markedly during treatment compared with the screening period for patients receiving twice daily marimastat ($P<0.0001$), but not for patients receiving marimastat once daily ($P=0.25$). Musculoskeletal side-effects emerged as the principal drug-related toxicity of marimastat, occuring in a dose and time dependent fashion. 25mg twice daily was defined as the upper limit of the dose range for continuous use in further studies. Primrose et al conclude that marimastat was associated with dose-dependent biological effects in cancer patients.

Bramhall et al have reported the results of using marimastat as first-line therapy for

patients with unresectable pancreatic cancer (Bramhall SR 2001). This showed no increased survival for the marimastat treated group but it was as effective as conventional therapy.

The results of all these studies were achieved in patients with advanced, rapidly progressive, treatment refractory cancer, a group that is traditionally hard to treat. Phase III trials are underway to demonstrate the efficacy of marimastat in terms of conventional clinical end points, such as survival and disease progression and when completed should provide the first step towards identifying the potential clinical use of this class of agents.

5.6 Collagen non-peptidopmimetic MMP inhibitors

This group includes BAY 12-9566, Prinomastat/AG3340, BMS 275291 and CGS 27023A/MMI270. Phase I trials had shown some encouragement but Phase III trials with BAY 12-9566 were terminated when trials on advanced pancreatic cancer showed significantly poorer survival for groups treated with the drug than for placebo-treated groups (Moore MJ 2003).

5.7 Other MMP inhibitors

Tetracyclines and the synthetic forms, doxycycline and minocycline are weak inhibitors of MMPs, independent of their antimicrobial activity (Lee 1991). Tetracyclines can inhibit MMP1 in vivo and in vitro, as well as MMP 2 and MMP 12 in vitro (Golub 1991). Synthetic tetracyclines are more efficient than the parent compound (IC_{50} 15mM for doxycycline compared to 350mM for tetracycline). Despite encouraging preclinical data, clinical trials have shown no improvement in clinical symptoms or radiological signs in patients with arthritic disorders (Vincenti 1994).

The anti-neoplastic anthracycline drugs, daunorubicin, doxorubicin and epirubicin inhibit MMP 9 activity and basement membrane collagen degrading activity in a reversible, non-competitive manner (Karakiulakis 1990). These compounds probably

function like tetracycline by chelating the metal ion.

Bryostatin-1 is a naturally occurring macrocyclic lactone derived from marine bryozoans. In culture this drug induced the differentiation of and halted the growth of several malignant cell lines (Hornung 1992). The exact mechanism of action is unknown but one effect is the down regulated production of MMPs 1, 3, 9, 10, 11 with no other MMPs affected. The role of Bryostatin in the treatment of malignant disease is under investigation.

Galardin (Glycomed) is a synthetic MMPI, not orally bioavailable which is being developed as a topical agent for the treatment of corneal ulceration (Galardy 1994).

Chapter 6: HYPOTHESIS, MATERIALS AND METHODS

6.1 Hypothesis

In metastatic colorectal carcinoma it is my hypothesis that there is induced variation in the expression of MMPs and/or TIMPs.

I propose to look at fresh tissue samples from 30 people undergoing hepatectomy for metastases from colorectal carcinoma. These samples consist of matched sets of metastasis, the immediately adjacent liver (< 1 cm distant from the metastasis) and distal liver (>5cm away from the site of metastasis), snap frozen in liquid nitrogen and stored at –80 °C. I will examine the mRNA expression of these samples for each of the three subgroups of MMPs: Collagenases (MMP 1,8,13); Gelatinases (MMP 2,9) and Stromelysins (MMP 3,7); as well as the Tissue inhibitors of metalloproteinases (TIMPs 1 and 2).

Relative levels of expression will be analysed by comparing the intensity of products against that of β-actin (a ubiquitously expressed gene). All products will be cloned and sequenced to ensure gene specificity. Subsequently the clones will be used to generate sense and antisense riboprobes for future in situ hybridisation work.

Paraffin sections are available from the 30 hepatectomies and immunohistochemical studies will be performed on these using specific antibodies for the nine MMPs and TIMPs. This will confirm the presence of the protein corresponding to the mRNA and identify the cells which are producing it.

Immunohistochemistry studies will also be performed on paraffin sections from the primary tumours giving rise to these liver metastases, allowing comparison of their MMP and TIMP profiles.

6.2 Materials

6.2a General

All solutions, media and glassware were sterilised by autoclaving at 120°C, 15lbs/inch^2 for 20 mins. ddH_2O was used as standard for all methods unless stated otherwise.

6.2b Biochemicals

General chemicals were obtained from BDH-Merck and Sigma. Restriction enzymes were from Promega and New England BioLabs. Taq Polythermase Gold and buffers were obtained from Biogene. The radiochemicals were purchased from Amersham International.

6.2c Stains and Vectors

The pGEM^{TM}-T Easy vector was obtained from Promega and the pCR^{TM}II vector was purchased from InVitrogen. Both vectors contain ampicillin resistance and the B-Galactosidase gene as selection methods. The bacterial strain Epicurian Coli® XL-1 Blue supercompetent cells were used as the host for these two vector systems and were purchased from Stratagene.

6.2d Solutions

All solutions were sterilised by autoclaving and stored at room temperature unless stated otherwise. The TAE and TBE buffers used for gel electrophoresis were made with dH2O and not generally sterilised. Solutions used specifically for the production and analysis of RNA were prepared RNase free as described in Sambrook et al (1989) by the use of baked glassware and ddH2O treated with 0.1% diethylpyrocarbonate (DEPC) for 24 hours prior to autoclaving.

The recipes for all the solutions used are shown in Appendix i.

6.2e Subjects

30 patients undergoing hepatectomy for metastases from colorectal carcinoma at the Southampton General Hospital and the North Hampshire Hospital, Basingstoke and 3 patients undergoing hepatectomy for benign liver lesions were used for this study. Ethical committee approval was obtained before starting the collection.

6.2f Sample Collection

Fresh tissue samples from these 33 patients, consisting of matched sets of the metastasis (from the edge avoiding the necrotic centre), the immediately adjacent liver (< 1 cm distant from the metastasis) and distal liver (>5cm away from the site of metastasis), were snap frozen immediately in liquid nitrogen and stored at -80°C prior to DNA and RNA extraction. A history was recorded for each patient including sex, date of birth and tumour histology (primary and secondary).

Paraffin sections from the 30 hepatectomies were retrieved as well as paraffin sections from the corresponding primary (where this was available). Paraffin sections from the 3 benign lesions were also studied.

6.3 Methods

6.3a RNA Extraction

RNA was extracted from 30-150mg of tissue using the Hybaid Ribolyser ™ Kit Green Protocol as supplied by Hybaid and used as per the manufacturer's instructions (Appendix ii).

Briefly, 150-300mg of tissue was excised from the specimen and added to a pre-chilled (10 minutes on ice) HYBAID Ribolyser ™ matrix tube containing 500µl of reagent A, 500µl of reagent B and 100µl of reagent C. The tubes were placed in the HYBAID ribolyser ™ and processed for 20 seconds at a setting of 4.0. This was repeated four times to allow a complete homogenization of the tissue sample after which the tubes were placed

on ice for 10 minutes. The two phases were separated by centrifugation at 13,000rpm for 15 minutes. The top phase was aliquoted into a fresh eppendorf and 300µl of reagent C added. The solution was vortexed for 10 seconds, placed on ice for 5 minutes and then centrifuged at 13,000 rpm for 2 minutes. The top phase was transferred to a new eppendorf and 500µl of reagent D added, mixed by inversion and incubated at room temperature for 2 minutes before centrifugation at 13,000rpm for 5 minutes to pellet the RNA. The RNA pellet was washed twice with 250µl of reagent E, air-dried and resuspended in 100µl of DEPC-treated dH_2O and stored at –80°C.

6.3b cDNA Synthesis

The Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) enzyme supplied by Promega and a $pd(T)_{12-18}$ primer (Pharmacia Biotech) were used to generate cDNA sequences using the mRNA extracted above as a template.

1µg of total RNA dissolved in DEPC-treated dH_2O (5µl) was incubated at 70°C for 5 minutes and placed on ice prior to the addition of a reaction mixture containing 1x M-MLV RT Buffer (Promega), 0.06Units of pd $(T)_{12-18}$ primer (Pharmacia Biotech), 200mM of each dNTP and 150 units of M-MLV RT to a final volume of 40µl. The solution was mixed, briefly centrifuged before an incubation of 1 hour at 37°C and 5 minutes at 94°C. All cDNA's were stored at -20°C until required for PCR analysis.

6.3c Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was developed in 1984 as a DNA amplification procedure based on an in vitro process. This method allows the generation of large amounts of a specific DNA fragment from a mixed DNA template in a simple enzymatic reaction (Arnheim 1992, Saiki 1985). Two primers are required usually 20-30 bases in length and designed to anneal specifically to the gene of interest. The PCR reaction is performed in a series of cycles to allow amplification. The cycle begins with a

denaturation step to render the DNA single stranded. This is followed by an annealing step at a temperature calculated by the primer pair to allow them to bind to their complementary sequences so that their 3'hydroxyl ends face the target. The cycle is completed by an extension step for the extension from the primer through the target gene to generate two double stranded copies of the target gene by Taq Polythermase. This cycle is repeated several times resulting in large amounts of the target gene by exponential amplification.

6.3d General PCR conditions.

All PCR reactions were carried out in a 0.5ml PCR tube using 4µl of genomic DNA or cDNA, 2µl of forward and reverse primers (British Biotech, Oswell, MWG), 10x reaction buffer A (Biogene), 200µM dNTP's (Pharmacia Biotech), 0.5 units Taq Polymerase (Taq Polythermase Gold, Biogene), 1-3mM magnesium chloride and 0-4% DMSO in a final volume of 50µl.

The PCR reactions were carried out under the following conditions using a Techne Progene PCR machine:

1 cycle	94°C	3 minutes (initial denaturation step)
35 cycles	94°C	30 seconds (denaturation step)
	x°C	30 seconds (annealing step)
	72°C	20-45 seconds (elongation step)
1 cycle	72°C	2 minutes (final elongation step)

The melting temperature (Tm) of each individual primer as shown by the following equation determined the annealing step of the PCR cycling reaction.

$$Tm\ (°C) = 4\ (G+C) + 2\ (A+T) - (2/4)$$

This Tm value determines the annealing temperature for the PCR. x °C is calculated as the lowest Tm of the two primers used in the reaction.

Ten microlitres of PCR products combined with 3µl of bromophenol blue loading dye were analysed by non-denaturing polyacrylamide gel electrophoresis. A DNA molecular weight marker was loaded to allow the determination of the DNA fragment size and for quantification purposes (3-5µl of Lambda Hae III marker, Promega).

6.3e Optimisation of PCR with Magnesium Chloride and Dimethylsulphoxide.

Each separate PCR was titrated with various concentrations of magnesium chloride and DMSO to obtain optimal reaction conditions, using the titration square in Table 7.

Table 7: PCR Optimisation of Magnesium Chloride and DMSO Concentrations

Magnesium Chloride Concentration (mM)	**% DMSO**		
	0	**1**	**2**
1	1	2	3
1.5	4	5	6
2	7	8	9
2.5	10	11	12
3	13	14	15

A reaction mix for 16 PCR was set up in 1x PCR buffer, 150ng of forward and reverse primer, 200mM dNTP, 0.5 unit of Taq polymerase and 50ng DNA or cDNA. The solution was then mixed and aliquotted into 0.5ml PCR tubes before the addition of the appropriate volume of magnesium chloride and DMSO. Each mix was then adjusted to a final volume of 50µl before being subjected to the PCR cycling determined for the individual set of primers. 10µl of PCR mix was analysed by non-denaturing polyacrylamide gel electrophoresis and studied under UV transillumination. The optimal conditions were deduced from the resultant gel. The lane with the cleanest/brightest

fragment of the correct size was taken to be the best conditions for the PCR and were used for all subsequent reactions. The target cDNA used for these optimisation PCR's was tissue known to express that MMP/TIMP (positive controls Table 8).

Table 8: Primer sequences, conditions, product size and positive controls used for polymerase chain reactions.

	Primer sequence	$MgCl_2$ (Mm)	DMSO (%)	Product size(bp)	Anneal T (°C)	+ control
MMP 1	F 5' AATGTGCTACACGGATACCC 3'	2.5	1	214	58	Tonsil
	R 5' CTTTGTGGCCAATTCCAGGA 3'					
MMP 8	F 5' AAACTATGGCTTCCCCAGCA 3'	3.0	0	223	58	Tonsil
	R 5' TTGGTCCACTGAAGACATGG 3'					
MMP13	F 5' ATACAGGCAAGACTCTCCTG 3'	2.5	0	214	58	Colon
	R 5' GCGAACAATACGGTTACTCC 3'					
MMP 2	F 5' AGCGTGAAGTTTGGAAGCAT 3'	1.5	0	253	60	Stomach
	R 5' GCCTGGGAGGAGTACAGTCA 3'					
MMP 9	F 5' GAAGATGCTGCTGTTCAGCG 3'	2.0	2	221	60	Stomach
	R 5' ACTTGGTCCACCTGGTTCAA 3'					
MMP 3	F 5' GAGGAAAATCGATGCAGCCA3'	2.0	2	216	58	Tumour
	R 5' CTCCAACTGTGAAGATCCAG 3'					
MMP 7	F 5' TTTGATGGGCCAGGAAACAC 3'	2.5	0	220	60	Uterus
	R 5' GGGGATCTCCATTTCCATAG 3'					
TIMP 1	F 5' CGGGGCTTCACCAAGAC 3'	1.0	1	216	58	Nerve
	R 5' TCAGGCTATCTGGGACCGC 3'					
TIMP 2	F 5' GAAGAAGAGCCTGAACCACA3'	2.0	0	222	58	Tonsil
	R 5' GTCCTCGATGTCGAGAAACT 3'					
β actin	F 5'TGACGGGGTCACCCACACTGTG CCCATCTA 3'	3.0	0	200	64	Mixed RT
	R 5' CTAGAAGCATTTGCGGTGGAC GATGGAGGG 3'					

The optimal concentration of primers and cycling protocol for each PCR had previously been established by British Biotech (primer producer and supplier). The primers used for each PCR and concentrations used are shown in Table 8. The optimised magnesium chloride, DMSO concentrations and annealing temperatures are shown in Table 8. The PCRs were otherwise performed as described before. 10µl of PCR product was analysed by non-denaturing polyacrylamide gel electrophoresis.

6.4 Semi-quantitative analysis of RT-PCR

The levels of mRNA expression of the various MMPs and TIMPs was studied by semi-quantitative RT-PCR, using a ubiquitously expressed gene, β-actin, as a control. The RT-PCR's were performed in total volume of 50μl in 0.5ml PCR tubes. All the cDNA used in the experiments was diluted to approximately the same concentration before RT-PCR was performed. Four microlitres of cDNA's was used as the template. For each experiment, cDNA from tissue known to express the appropriate MMP or TIMP was used as a positive control and a reaction set up with no template as a negative control. The primers, optimised conditions and PCR cycling parameters used for the RT-PCR's are shown (Table 8). The optimisation PCR reactions were cycled 35 times to obtain a strong signal observed by gel electrophoresis.

For the semi-quantitative analysis of the RT-PCR product, a cycle titration was performed to reduce the likelihood of band saturation on the polyacrylamide gels. To determine this saturation point for the various RT-PCR's used, a cycle titration was carried out. A reaction mix of 50μl was set up for each of the following cycle numbers 10, 15, 20, 25, 30, 35 and 40 containing 1x PCR buffer, magnesium chloride, 200mM dNTP's, forward and reverse primers, 0.5 units of Taq polymerase, DMSO (if required) in ddH_2O. The PCR's were performed using the conditions in Table 8. After 10,15,20,25,30 and 35 cycles, a PCR tube was removed and incubated at 72°C for 2 minutes in a dry block. The 40 cycle tube and negative control were left in the PCR machine. The appropriate positive cDNA for each MMP/TIMP was again used as the template. Ten microlitres of PCR product was analysed by 8% PAGE and the saturation point determined by the Kodak Digital Science™ 1D Image Analysis System. All the RT-PCR reactions were carried out at 35 cycles and the results for the β-actin RT-PCR are shown in the results section.

Each RT-PCR was repeated at least twice. Ten microlitres of RT-PCR product was

analysed by non-denaturing PAGE with three nanograms of Lambda Hae III marker run on each gel to allow determination of the size of the PCR product and analysis to calculate the amount of PCR product in each lane. For each PCR a negative control was run using dH_2O instead of cDNA, and a positive control was run using cDNA from tissue known to express the MMP or TIMP (Table 8). The amount of PCR product was calculated for each sample using the Kodak Digital Science™ 1D Image Analysis System either from scanned polaroid gel photographs or digital images captured using the Kodak DC120 Camera. The amount of β-actin PCR was used to normalise each sample for variations in the PCR quality of the cDNA target. The final amount of PCR product was expressed as the amount of PCR product (ng)/ ng of β-actin.

6.5 Gel Electrophoresis

6.5a Agarose Gel Electrophoresis

All agarose gel electrophoresis of vector preparations was carried out on horizontal gels. Agarose gels were routinely made at 0.6% in 1x TAE buffer, pre-stained in ethidium bromide solution (20μg/ml). Gels were run using 1x TAE buffer at 80 volts for 1-2 hours. After electrophoresis, the samples were visualised by UV excitation and photographed using a Kodak polaroid camera.

For agarose gel electrophoresis, 5ng of Lambda Hind III molecular weight marker (Promega) was used to deduce the size of samples.

6.5b Non-Denaturing Polyacrylamide Gel Electrophorsis

All non-denaturing polyacrylamide gel electrophoresis of PCR and restriction enzyme digest samples was performed on vertical gel systems. 8% polyacrylamide gels were generally used made up to a total volume of 50ml with 1x TBE buffer, 50μl TEMED, 200μl of 25% ammonium persulphate (fresh) and dH2O. Electrophoresis was performed in 1x TBE buffer, at 40mA for 2-3 hours depending on the degree of separation

required. The gel was then stained in ethidium bromide solution (20μg/ml), visualised by ultra-violet transillumination and recorded by Kodak polaroid film or the Kodak KDS 120 system.

For non-denaturing gel electrophoresis, 3-5ng of Lambda Hae III molecular weight marker (Promega) was used to deduce the size and amount of sample.

6.6 Confirmation of PCR specificity

The specificity of the primers used for the cDNA PCR's were confirmed by the cloning and sequencing of the individual PCR products generated in the reactions.

6.6a PCR Ligation

The pGEM® -T Easy Vector System (Promega) was employed for the ligation reactions for the cDNA PCR products due to the requirement of cDNA probe synthesis for *in situ* hybridisation experiments. The pGEM® -T Easy Vector System was used for cDNA probe generation as the vector contains both T7 RNA and SP6 RNA polymerase transcription initiation sites. The kit was used as per the manufacturer's instructions (Appendix iii).

The pGEM™-T Easy Vector system reactions were performed in a final volume of 10μl, using 3μl of cDNA PCR product, 50ng of pGEM™-T Easy vector, 1x ligation buffer and 5 units of T4 DNA ligase. The ligations were mixed, centrifuged and incubated at 4-10°C for 24 hours prior to storage at -20°C until required.

6.6b Transformation of Ligation Constructs into Epicurian Coli® Supercompetent Cells

All PCR/plasmid constructs made above were transformed into Epicurian Coli® XL1-Blue supercompetent cells (Stratagene), following the manufacturer's instructions. For each transformation, a 100μl aliquot of supercompetent cells (previously thawed on ice) was transferred to a pre-chilled 15-ml Falcon 2059 polypropylene tube and β-

mercaptoethanol added to a final concentration of 25mM. The contents were mixed gently by swirling and incubated for 10 minutes on ice, mixing every 2 minutes. 10-50ng of the PCR/vector construct or 0.1ng of pUC18 (control) was added to the cells, mixed gently and incubated for 30 minutes on ice. The Falcon tubes were then incubated at 42°C for exactly 45 seconds and placed on ice for 2 minutes before the addition of 0.9ml of Terrific Broth. The cultures were grown at 37°C for 1 hour shaking at 225rpm and 100µl / 300µl aliquots were spread onto LB-ampicillin agar plates. The agar plates were inverted, incubated overnight at 37°C, and stored at 4°C.

The colonies observed on the LB-ampicillin agar plates were assessed for blue/white colour formation. The blue colonies indicated cells that had been transformed with re-ligated pGEM™-T Easy vector. The white colonies indicated cells that contained a pGEM™-T Easy vector construct. These white colonies were picked and grown in 3-5ml of terrific broth containing 100µg/ml ampicillin in an universal container, at 37°C overnight prior to plasmid DNA extraction.

6.6c Plasmid DNA Extraction by Alkali Lysis

The PCR fragment/vector constructs were extracted from the Epicurian Coli ® cells and purified using the alkali lysis method (Sambrook, 1989).

1.5ml of culture was aliquoted into an eppendorf and centrifuged at 13,000rpm, 3 minutes to pellet the cells. The supernatant was discarded into 5% virkon solution and a further 1.5ml of culture transferred to the eppendorf, recentrifuged and the supernatant removed as before. The pellet was resuspended in 100µl of T.E.pH 8.0 by pipetting before the addition of 150µl of lysis buffer and mixed by inversion. 250µl of 4°C 7M ammonium acetate (Sigma) was then added to the lysate, inverted 5-6 times and incubated at 4°C for 20 minutes. The suspension was centrifuged at 13,000rpm for 15 minutes to pellet the precipitated fraction and the supernatant transferred to a new eppendorf. The plasmid DNA was precipitated using 1ml of -20°C ethanol containing 0.3M sodium acetate. The

contents were mixed by inversion and incubated at -80°C for 1 hour. The DNA was pelleted by centrifugation at 13,000rpm for 30 minutes. The supernatant was discarded and the pellet washed with 100µl of -20°C 70% ethanol, before being dried at 37°C for 10-15 minutes and resuspended in 25µl of T.E.pH 8.0 containing 100µg RNase (Sigma). The samples / minipreps were then stored at -20°C.

These samples were analysed by agarose gel electrophoresis to ascertain the efficiency of the extraction. A mixture of 3µl of miniprep, 3µl bromophenol loading dye and 4µl T.E.pH8.0 was loaded onto a 0.6% agarose gel and separated by electrophoresis.

6.6d Analysis of the Purified Constructs by Restriction Digestion

To analyse for the presence of the correct size DNA fragment, a restriction endonuclease reaction was performed. The pGEM™-T Easy vector contains Eco RI restriction enzyme sites either side of the ligation site, therefore this enzyme was chosen to cleave the vectors releasing the inserted fragment of DNA.

A mixture of 3-5µl of miniprep, 5 units of Eco RI (Promega), 1x Buffer H (Promega) was prepared and adjusted to a final volume of 20µl with ddH_2O in an 0.5ml tube. The contents was mixed, centrifuged and incubated at 37°C for 2-24 hours. 5µl of bromophenol loading dye was added to the digest and analysed by 8% non-denaturing PAGE. All minpreps containing plasmids with the correct size DNA fragment were stored at -20°C until required for sequence analysis.

6.6e DNA Cycle Sequencing

The DNA sequence of each DNA fragment cloned into the pGEM™-T Easy vector was obtained using the Thermo Sequenase radiolabeled terminator cycle sequencing kit and four Redivue™ ^{33}P labeled terminators (Amersham Life Science). This kit was used according to the manufacturer's instructions (Appendix iv).

The reaction mixture was prepared on ice unless stated otherwise. For one sequence reaction, four 0.5ml tubes were labeled A,C,G and T. To each tube, 2µl of dGTP terminator mix (7.5µM dATP, dCTP, dGTP and dTTP) was added. 0.5µl of the appropriate ^{33}P labeled terminator was then added as shown in table 9 and mixed by pipetting.

Table 9: Preparation of terminator reaction mixture

	Tube A	Tube C	Tube G	Tube T
^{33}P labeled ddATP (µl)	0.5	-	-	-
^{33}P labeled ddCTP (µl)	-	0.5	-	-
^{33}P labeled ddGTP (µl)	-	-	0.5	-
^{33}P labeled ddTTP (µl)	-	-	-	0.5
Termination mix (µl)	2	2	2	2

The reaction mixture was prepared by the combination of 1x Reaction buffer, 100-300ng of plasmid DNA, 3pmol of T7 or SP6 primer, 8 units of Thermo Sequenase polymerase, in a final volume of 20µl. 4.5µl of this solution was added to each of the four tubes, mixed and briefly centrifuged. The reaction mixtures were then subjected to the PCR below before the addition of 4µl of Stop solution.

1x	94°C	3 minutes
35x	94°C	30 seconds
	56°C	30 seconds (for both T7 and SP6 primers)
	72°C	30 seconds
1x	72°C	2 minutes

3µl of each sample was heated to 94°C for 2 minutes and placed on ice prior to loading onto a 6% polyacrylamide gel containing 7M urea. The DNA fragments were separated by electrophoresis for 2-5 hours at a constant power of 50W. The DNA bands were fixed in the gel by an incubation of 15-30 minutes in a 10% methanol, 10% acetic

acid solution before being dried under vacuum onto filter paper. The sequence of each DNA fragment was read after the autoradiographic exposure on Kodak Film of the gel over a 24-48 hour period.

6.7 Immunohistochemistry

Immunohistochemical staining using a three stage streptavidin-biotin peroxidase complex technique was performed on paraffin sections mounted on APES coated slides (Mepham BL et al 1990) (Appendix v).

Antigen retrieval by heat-mediated microwave pretreatment using citrate buffer was used (Cattoretti G et al 1993) for all the MMPs and TIMPs except MMP2 which required antigen retrieval by pronase (Appendix vi and vii).

As liver has endogenous avidin binding sites these were blocked using an avidin/biotin blocking kit (Vector, Burlingame, California USA) applied before the primary antibody (Appendix viii).

The primary antibodies were left to incubate overnight at 4°C and the secondary antibodies were either biotinylated sheep anti-mouse Ig for monoclonal antibodies (Amersham Life Science,UK) or biotinylated swine anti-rabbit Ig for polyclonal antibodies (DAKO, Ely, UK). Streptavidin/Biotin peroxidase complexes (DAKO) and liquid DAB (BioGenex, San Ramon, USA) were used to identify the primary antibodies, brown staining confirming their presence (Appendix ix).

Negative controls were run with no primary antibody and also with matching isotype antibodies (IgG_1 and IgG_{2A}).

The primary antibodies against the MMPs were supplied by British Biotech (Oxford, UK) and the TIMP antibodies purchased from Chemicon (Temecula, California, USA), and their characteristics, concentrations used and positive control tissues are given in Table 10. The same MMP antibodies were later available from R&D Systems (Minneapolis, USA). The correct concentration for each antibody was established by

titration using differing concentrations of antibody on a tissue section known to express that MMP/TIMP (Appendix x).

Table 10: Characteristics of antibodies used for immunohistochemistry. There was no cross reactivity between antibodies.

	Isotype	Form of MMP	Type	Dilution	Positive control
MMP 1	IgG_1	Pro & Active	Mouse monoclonal	1:50	Tonsil/Rh art.
MMP 8	-	Pro & Active	Rabbit polyclonal	1:200	Tonsil/Rh art.
MMP 13	IgG_1	Pro & Active	Mouse monoclonal	1:100	Rheumatoid arthritis (Rh art).
MMP 2	IgG_{2A}	Pro & Active	Mouse monoclonal	1:80	Tonsil
MMP 9	IgG_1	Pro & Active	Mouse monoclonal	1:80	Tonsil
MMP 3	-	Pro & Active	Rabbit polyclonal	1:200	Tumour
MMP 7	-	Pro & Active	Rabbit polyclonal	1:200	Rh art/Uterus
TIMP 1	IgG_1	-	Mouse monoclonal	1:50	Nerve
TIMP 2	IgG_1	-	Mouse monoclonal	1:200	Tonsil/melanoma

The MMP 1,2,9 and 13 antibodies were produced from a murine hybridoma elicited from a mouse immunized with purified, CHO cell-derived, recombinant human MMP 1,2,9 and 13. The IgG fraction of ascites fluid was purified by Protein A affinity chromatography for MMP 1 and 9 and Protein G affinity chromatography for MMP 13. The IgG fraction of the tissue culture supernatant was purified by Protein A affinity chromatography for MMP 2. All MMP 1,2,9 and 13 antibodies bound both pro and active forms of the corresponding human MMP. Based on Western blot results MMP 1 antibody showed no cross-reactivity with MMP 2,3 or 9; MMP 2 antibody showed no cross-reactivity with MMP 9,1 or 3; MMP 9 antibody showed no cross-reactivity with MMP 2,1 or 3; and MMP 13 antibody showed no cross-reactivity with recombinant human MMP 1,2,3,7,8 or 9 (Appendix xi).

The MMP 3,7 and 8 antibodies were produced in rabbits immunized with purified, NSO-derived, recombinant Human MMP 3, 7 and 8 respectively. Specific IgG antibodies were purified by human MMP 3, 7 and 8 affinity chromatography. The MMP 3 antibody

bound both pro and active forms of MMP 3 and does not cross-react with MMP 1,2,7,8,9 or 13 The MMP 7 antibody bound both pro and active forms of MMP 7 and does not cross-react with MMP 1,2,3,8,9 or 13. MMP 8 antibody did not cross-react with MMP 1,2,3,7,9 or 13.

TIMP 1 and 2 antibodies specifically reacted with the appropriate TIMP and showed no cross reactivity with any other TIMPs. They are purified mouse monoclonal antibodies to recombinant human TIMP 1 and human TIMP 2 respectively.

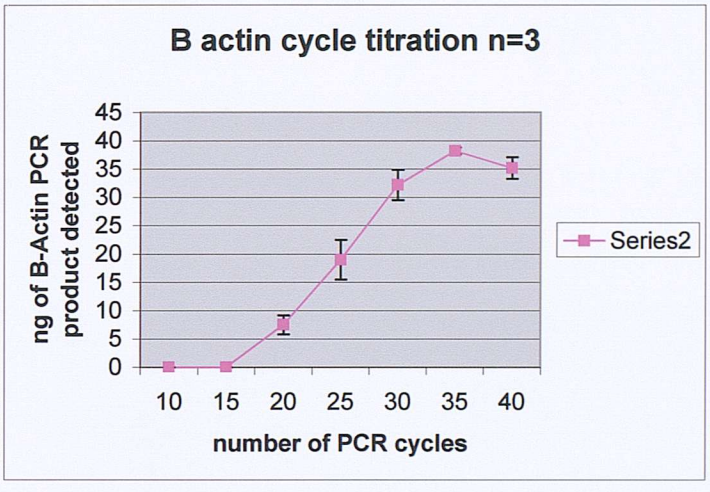
Chapter 7: RESULTS

7.1 PCR results

Table 11: B actin cycle titration data (ng β actin)

cycle number	n=1	n=2	n=3		mean	sd
10	0	0	0		0	0
15	0	0	0		0	0
20	7.139	6.001	9.282		7.5	1.7
25	15.123	19.85	22.04		19	3.5
30	29.77	35.04	31.85		32.2	2.7
35	38.79	37.99	37.67		38.2	0.6
40	34.85	33.52	37.23		35.2	1.9

Figure 4: Graph of B actin cycle titration data



The results confirm that 35 cycles produced the maximum amount of β actin product and so this was used in the β actin PCR allowing semiquantitative analysis.

7.2 Optimisation of PCR conditions with Magnesium Chloride and Dimethylsulphoxide

7.2a β-actin optimisation with $MgCl_2$ and DMSO

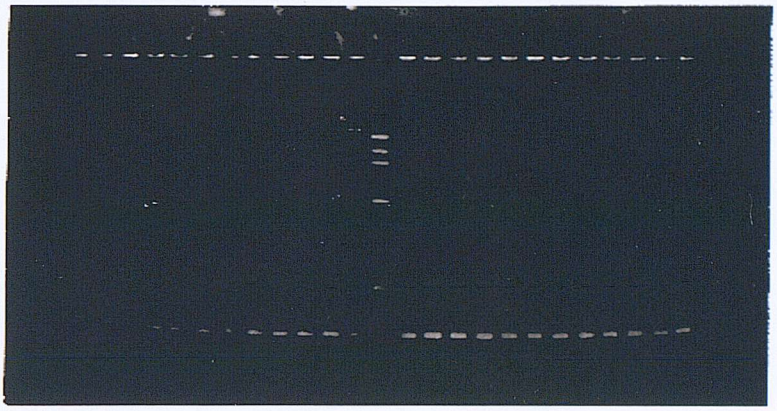
Table 12: Optimisation table of $MgCl_2$ and DMSO for β-actin PCR

Magnesium Chloride Concentration (mM)	% DMSO 0	2	4
1.5	1	2	3
2.0	4	5	6
2.5	7	8	9
3.0	10	11	12

Figure 5: Optimisation PCR for β-actin

5µl PCR product 10µl PCR product

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 10 11 12



Optimum conditions: Tube 10: 3.0 mM $MgCl_2$ and 0% DMSO

7.2b MMP 1 optimisation with $MgCl_2$ and DMSO

Figure 6: Optimisation PCR for MMP 1

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

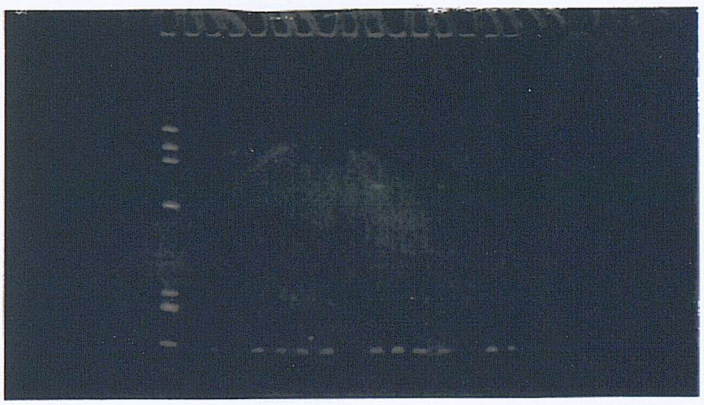


Table 13: Optimisation table of $MgCl_2$ and DMSO for MMP 1 PCR

Magnesium Chloride Concentration (mM)	% DMSO		
	0	1	2
1	1	2	3
1.5	4	5	6
2	7	8	9
2.5	10	11	12
3	13	14	15

Optimum conditions: Tube 11: 2.5 mM $MgCl_2$ and 1% DMSO

7.2c MMP 8 optimisation with $MgCl_2$ and DMSO

Figure 7: Optimisation PCR for MMP 8

Tube No 1 2 3 4 5 6 7 8 9 10 11 12

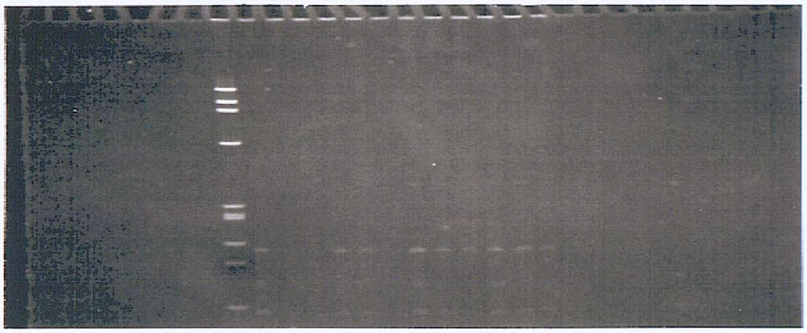


Table 14: Optimisation table of $MgCl_2$ and DMSO for MMP 8 PCR

Magnesium Chloride Concentration (mM)	% DMSO		
	0	1	2
2.5	1	2	3
3.0	4	5	6
3.5	7	8	9
4.0	10	11	12

Optimum conditions: Tube 7: 3.5 mM $MgCl_2$ and 0% DMSO

7.2d MMP 13 optimisation with $MgCl_2$ and DMSO

Figure 8: Optimisation PCR for MMP 8

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

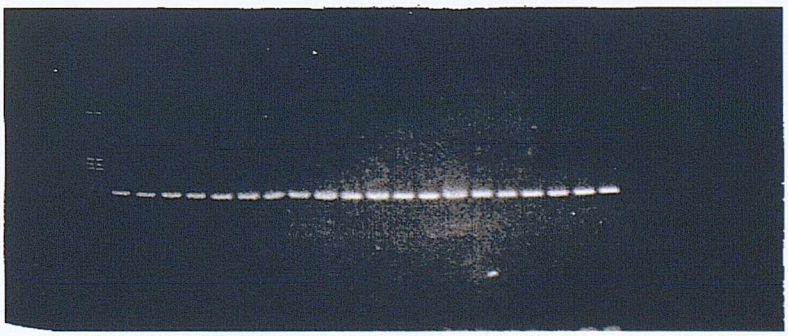


Table 15: Optimisation table of $MgCl_2$ and DMSO for MMP 13 PCR

Magnesium Chloride Concentration (mM)	%DMSO 0	1	2	4
1	1	2	3	4
1.5	5	6	7	8
2	9	10	11	12
2.5	13	14	15	16
3	17	18	19	20

Optimum conditions: Tube 13: 2.5 mM $MgCl_2$ and 0% DMSO

7.2e MMP 2 optimisation with $MgCl_2$ and DMSO

Figure 9: Optimisation PCR for MMP 2

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

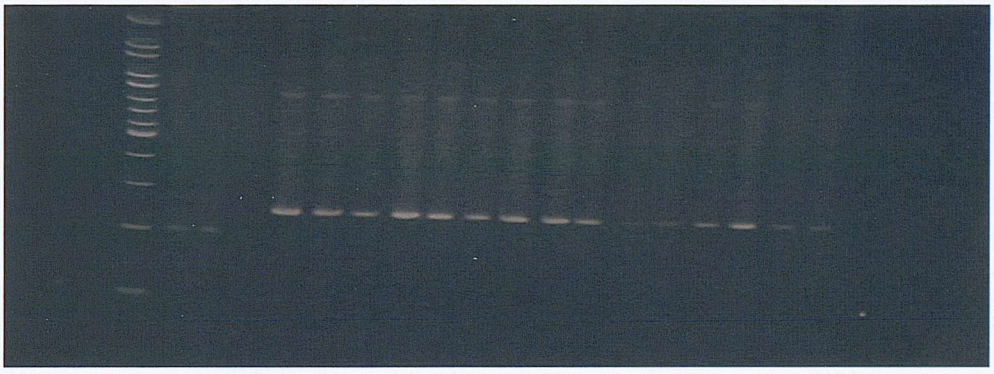


Table 16: Optimisation table of $MgCl_2$ and DMSO for MMP 2 PCR

Magnesium Chloride Concentration (mM)	% DMSO 0	1	2
1	1	2	3
1.5	4	5	6
2	7	8	9
2.5	10	11	12
3	13	14	15
3.5	16	17	18

Optimum conditions: Tube 4: 1.5 mM $MgCl_2$ and 0% DMSO

7.2f MMP 9 optimisation with $MgCl_2$ and DMSO

Figure 10: MMP 9 optimisation with $MgCl_2$ and DMSO

Tube No 1 2 3 4 5 6 7 8 9 10 11 12

Table 17: Optimisation table of $MgCl_2$ and DMSO for MMP 9 PCR

Magnesium Chloride Concentration (mM)	% DMSO 0	1	2
1	1	2	3
1.5	4	5	6
2	7	8	9
2.5	10	11	12

Optimum conditions: Tube 7: 2.0 mM $MgCl_2$ and 0% DMSO

7.2g MMP 3 optimisation with $MgCl_2$ and DMSO

Figure 11: MMP 3 optimisation with $MgCl_2$ and DMSO

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

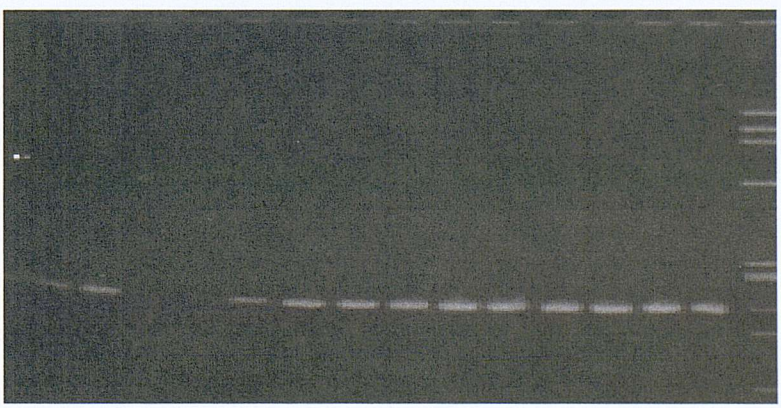


Table 18: Optimisation table of $MgCl_2$ and DMSO for MMP 3 PCR

Magnesium Chloride Concentration (mM)	% DMSO 0	2	4
1	1	2	3
1.5	4	5	6
2	7	8	9
2.5	10	11	12
3	13	14	15

Optimum conditions: Tube 8: 2.0 mM $MgCl_2$ and 2% DMSO

7.2h MMP 7 optimisation with $MgCl_2$ and DMSO

Figure 12: MMP 7 optimisation with $MgCl_2$ and DMSO

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

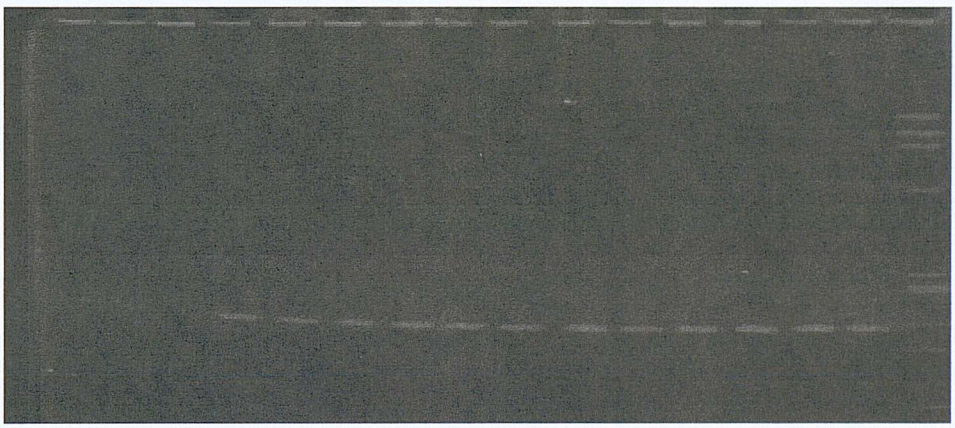


Table 19: Optimisation table of $MgCl_2$ and DMSO for MMP 7 PCR

Magnesium Chloride Concentration (mM)	% DMSO 0	1	2
1	1	2	3
1.5	4	5	6
2	7	8	9
2.5	10	11	12
2.5	13	14	15

Optimum conditions: Tube 10: 2.5 mM $MgCl_2$ and 0% DMSO

7.2i TIMP 1 optimisation with $MgCl_2$ and DMSO

Figure 13: TIMP 1 optimisation with $MgCl_2$ and DMSO

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

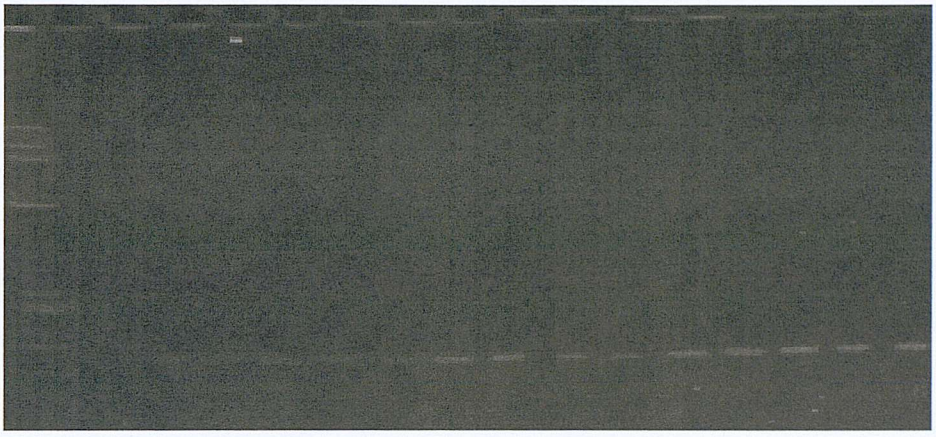


Table 20: Optimisation table of $MgCl_2$ and DMSO for TIMP 1 PCR

Magnesium Chloride Concentration (mM)	% DMSO		
	0	1	2
0	1	2	3
0.5	4	5	6
1	7	8	9
1.5	10	11	12
2	13	14	15

Optimum conditions: Tube 8: 1.0 mM $MgCl_2$ and 1% DMSO

7.2j TIMP 2 optimisation with $MgCl_2$ and DMSO

Figure 14: TIMP 2 optimisation with $MgCl_2$ and DMSO

Tube No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Table 21: Optimisation table of $MgCl_2$ and DMSO for TIMP 2 PCR

Magnesium Chloride Concentration (mM)	% DMSO		
	0	1	2
0.5	1	2	3
1	4	5	6
1.5	7	8	9
2	10	11	12
2.5	13	14	15

Optimum conditions: Tube 10: 2.0 mM $MgCl_2$ and 0% DMSO

7.3 Confirmation of PCR Specificity

The specificity of the primers used for the cDNA PCRs were confirmed by the cloning and sequencing of the individual PCR products generated in the reactions.

Figure 15: Collagenases/Gelatinases sequencing gels

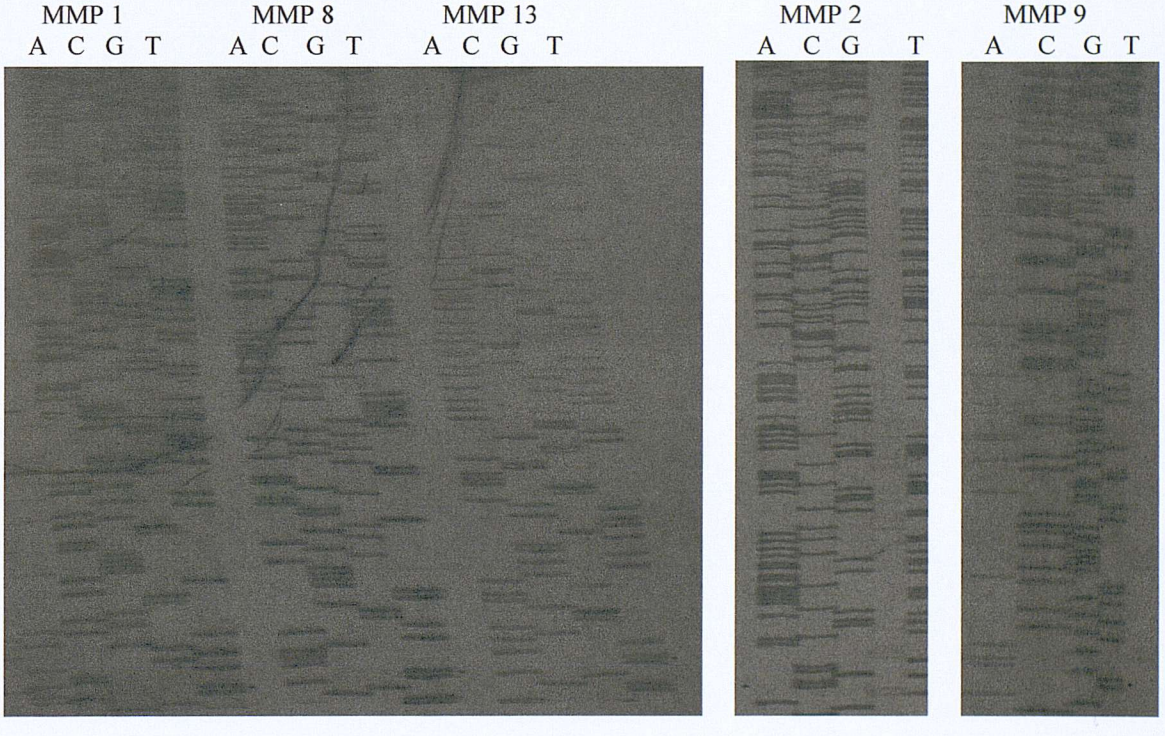
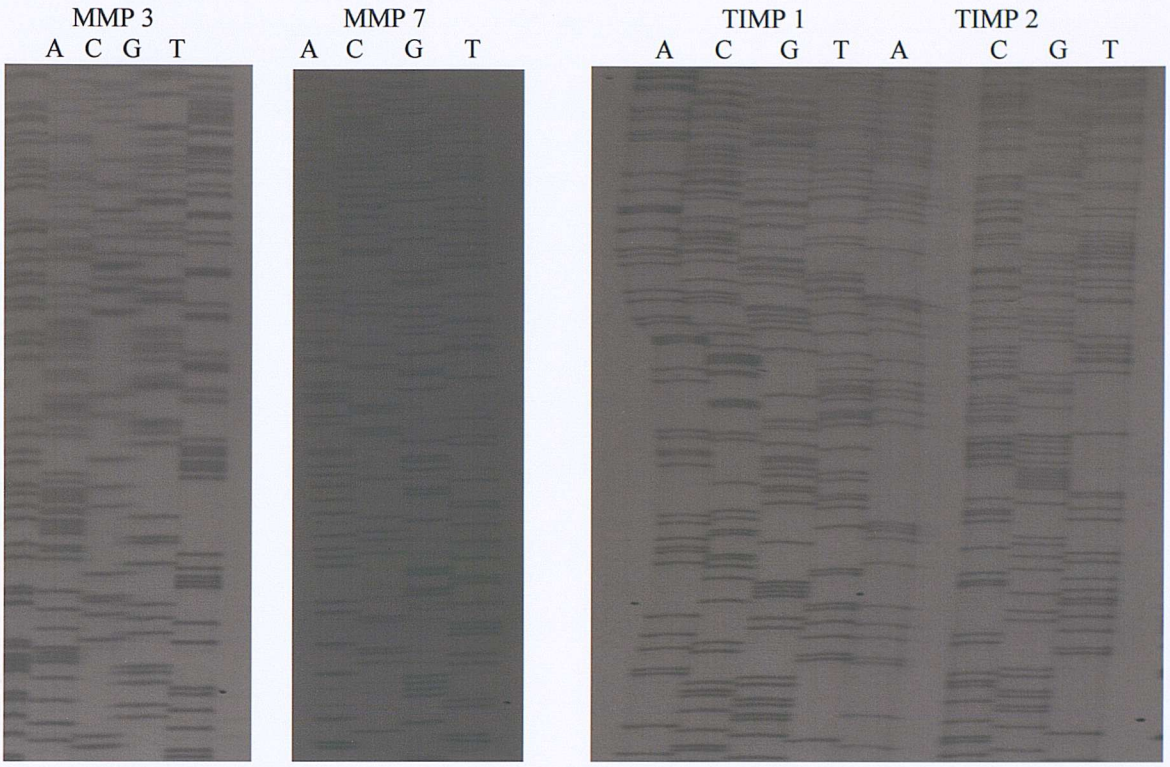


Figure 16: Stromelysins/TIMPs sequencing gels



7.4 Polymerase Chain Reaction

Confirmation of RNA integrity was carried out by RT-PCR amplification of the ubiquitously expressed β-actin gene. Amplifiable cDNA was obtained from all 30 matched sets of metastasis, adjacent liver and distal liver from hepatectomies for colorectal cancer and all 3 benign liver lesions.

Representative polyacrylamide gels are shown followed by graphical and tabular presentation of the amount of PCR product. This was semi-quantified for each sample using the Kodak Digital Science^{TM} 1D Image Analysis System from scanned polaroid gel photographs. The amount of β-actin PCR was used to normalise each sample for variations in the PCR quality of the cDNA target. The final amount of PCR product was expressed as the amount of PCR product(ng)/ng of β-actin.The polyacrylamide gels for each of MMPs, TIMPs and β-actin have the same layout except for MMP 2. All of the former have 18 lanes on them. The first lane has a standardised marker Hae III. This has

11 fragments of the following size base pairs: 1353 1078 872 603 310 281 271 234 194 118 72 and allows the size of the PCR product to be calculated. The next lane is a negative control in which dH_2O was used instead of cDNA. Then follow five sets of three lanes which have c DNA from the metastasis, adjacent liver and distal liver from five of the thirty patients. The final lane is a positive control using cDNA from tissue known to express that respective MMP or TIMP (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

The polyacrylamide gels for MMP 2 have a different configuration. The gels have a double band as β actin (the lower band) was run with each specimen. The first lane again has the Hae III marker followed by the sets of three lanes with metastases, adjacent liver and distal liver. In groups A,B,C and E there are 5 sets that correspond to the samples in the other MMPs and TIMPs. In gel D there are six sets, F1 having been included in the gel D. Two lanes are then left free followed by the negative lane and then the positive lane. In gel D the final lane (F1 distal) is run twice and in gel E one further negative and two positive lanes are run along with a further Hae III marker.

The initial table shows the total amount of PCR product in ng. For all of the MMPs and TIMPs there is a second table and a graph which gives the ratio of PCR product to β-actin. The median, and ranges, for each MMP and TIMP are given at the end of each results section, and it is this semiquantitative value which is used subsequently for the expression of that MMP or TIMP.

The final amount of PCR product for each specimen (90 in total) expressed as amount of PCR product(ηg)/ηg of β-actin was calculated and the median value, and ranges, for each MMP and TIMP is given in table 11.

7.4a β-actin results

Figure 17: Polyacrylamide gels for β-actin PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have c DNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

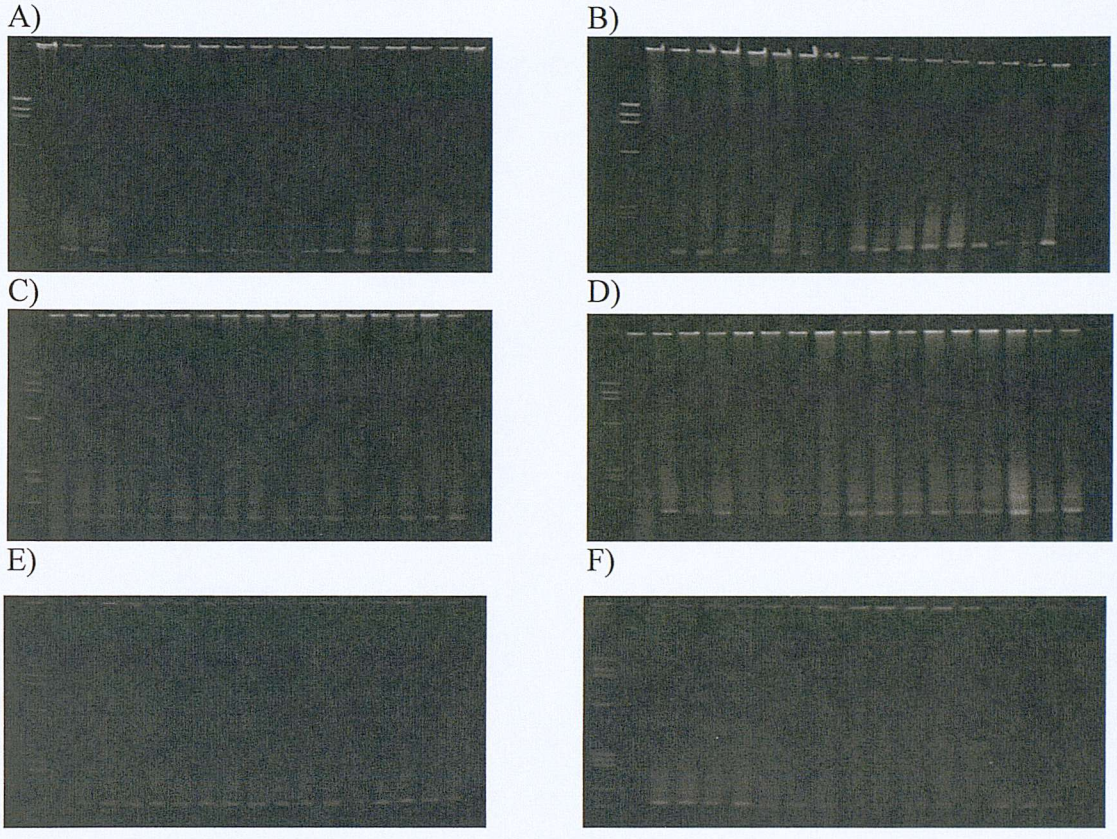


Table 22: β actin PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	20.16	24.41	30.6	19.68	26.45	64.3
A	18.3	21.62	22.22	23.96	41.66	79.66
D	17.32	30.55	23.54	16.18	30.51	51.89
M	11.65	16.73	21.17	21.03	44.05	62.82
A	17.13	20.36	30.13	15.28	32.32	9.118
D	15.18	9.321	25.85	17.39	46.17	20.41
M	11.67	18.29	18.08	18.44	44.66	5.44
A	16.49	50.77	9.839	20.47	39.4	8.199
D	14.33	36.43	9.7	21.71	32.94	10.36
M	14.73	45.34	12.92	18.06	32.88	5.951
A	20.18	52.28	19	13.76	43.13	10.05
D	14.34	15.64	18.42	18.2	27.03	12.46
M	17.53	42.45	17.21	18.13	46.39	10.15
A	17.6	74.19	17.6	30.95	48.83	39.93
D	17.5	48.2	24.7	20.17	39.79	33.35
Pos	20.66	76.84	15.17	19.62	27.99	32.28

7.4b MMP 1 results

Figure 18: Polyacrylamide gels for MMP 1 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

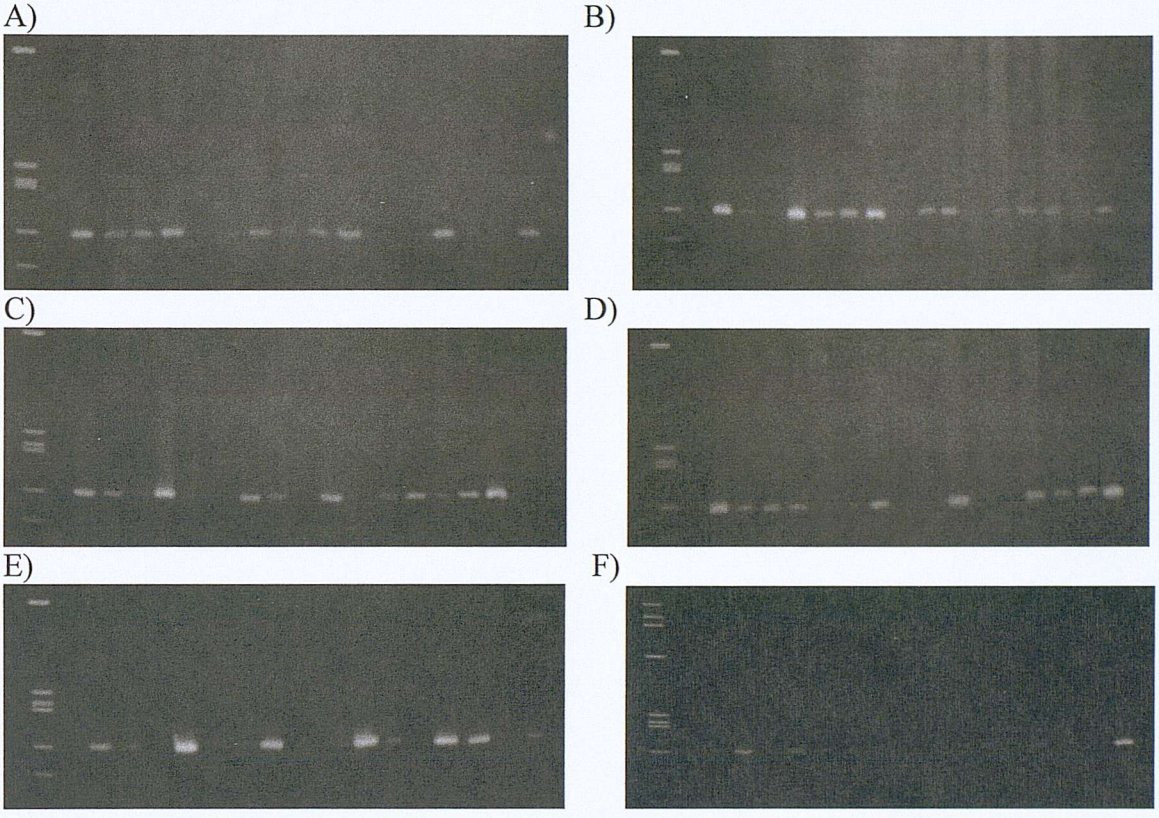


Table 23: MMP 1 PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	29.28	35.11	21.82	45.8	23.97	12.02
A	1.608	12.5	11.62	20.46	7.56	18.59
D	13.29	0	4.382	27.6	0	0
M	28.41	36.92	36.67	22.15	78.64	16.2
A	0	16.78	1.771	3.08	4.89	12.81
D	0.3158	15.13	0.4711	7.776	5.15	12.16
M	13.72	20.48	18.2	35.05	42.59	11.84
A	1.844	8.092	9.634	0	0	0
D	11.6	11.84	0	0	0	0
M	19.44	15.45	21.19	38.68	59.38	12.85
A	0	7.525	0	0	8.22	0
D	0	8.426	8.179	0	0	0
M	22.14	15.02	19.36	34.7	45.39	14.41
A	0	12.7	5.891	16.66	33.1	0
D	0	0	18.52	28.64	0	0
Pos	13.27	16.12	43.72	55.52	5.054	24.62

Table 24: MMP 1 PCR/β actin (median value and range at foot of table)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	1.45	1.48	0.71	2.33	0.91	0.19
A	0.08	0.58	0.52	0.85	0.18	0.23
D	0.77	0	0.19	1.71	0	0
M	2.44	2.21	1.73	1.05	1.78	0.26
A	0	0.82	0.06	0.2	0.15	1.4
D	0.02	1.62	0.02	0.45	0.11	0.6
M	1.17	1.12	1	1.9	0.95	2.18
A	0.11	0.16	0.98	0	0	0
D	0.81	0.33	0	0	0	0
M	1.32	0.34	1.64	2.14	1.81	2.15
A	0	0.14	0	0	0.19	0
D	0	0.54	0.44	0	0	0
M	1.26	0.35	1.13	1.91	0.98	1.42
A	0	0.17	0.33	0.54	0.68	0
D	0	0	0.75	1.42	0	0
Pos	0.64	0.21	2.88	2.83	0.18	0.76

Metastasis (M)	Adjacent liver (A)	Distal liver (D)
1.42 (0.19-2.44)	**0.16** (0-1.4)	**0.02** (0-1.71)

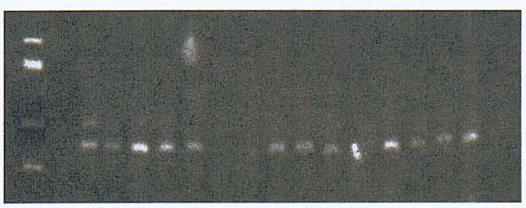
7.4c MMP 8 results

Figure 19: Polyacrylamide gels for MMP 8 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

A)

B)

C)



D)

E)

F)

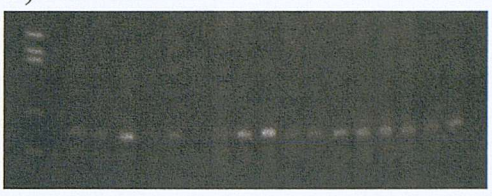


Table 25: MMP 8 PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	11.88	1.745	12.73	12.21	0	10.74
A	9.563	4.65	8.038	11.3	21.46	11.23
D	14.01	16.86	17.07	6.219	12.38	20.51
M	4.379	1.237	16.04	13.59	15.41	5.76
A	4.014	7.894	10.15	11.76	9.899	13.92
D	0.4593	5.045	1.974	17.75	21.23	4.87
M	3.299	4.867	4.755	2.47	0	12.85
A	4.119	12.83	7.043	10.38	15.23	18.69
D	4.201	15.44	13.08	12.5	0	22.36
M	7.479	2.733	7.694	0.9484	11.48	9.95
A	9.436	8.52	13.75	4.414	10.46	10.84
D	14.62	11.34	11.11	3.522	0	14.21
M	12.24	10.45	15.34	14.26	15.37	14.95
A	10.73	8.076	14.95	12.71	0	15.81
D	16.78	5.705	13.35	19.13	0	13.76
Pos	16.99	6.093	8.339	3.861	23.01	17.54

Table 26: MMP 8 PCR/β actin (median value and range at foot of table)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	0.59	0.07	0.42	0.62	0	0.16
A	0.52	0.22	0.36	0.47	0.52	0.14
D	0.81	0.55	0.73	0.38	0.41	0.39
M	0.38	0.07	0.76	0.64	0.35	0.09
A	0.23	0.39	0.34	0.77	0.31	1.53
D	0.03	0.54	0.08	1.02	0.46	0.17
M	0.28	0.27	0.26	0.13	0	2.36
A	0.1	0.25	0.72	0.51	0.39	2.28
D	0.29	0.42	1.35	0.58	0	2.16
M	0.51	0.06	0.59	0.05	0.35	1.67
A	0.47	0.16	0.72	0.32	0.24	1.08
D	1.02	0.73	0.6	0.19	0	1.14
M	0.7	0.25	0.89	0.79	0.33	1.47
A	0.61	0.11	0.85	0.41	0	0.39
D	0.96	0.12	0.54	0.95	0	0.41
Pos	0.82	0.08	0.55	0.2	0.82	0.54

Metastasis (M)	Adjacent liver (A)	Distal liver (D)
0.35 (0-0.89)	**0.39** (0-0.85)	**0.02** (0-1.35)

7.4d MMP 13 results

Figure 20: Polyacrylamide gels for MMP 13 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

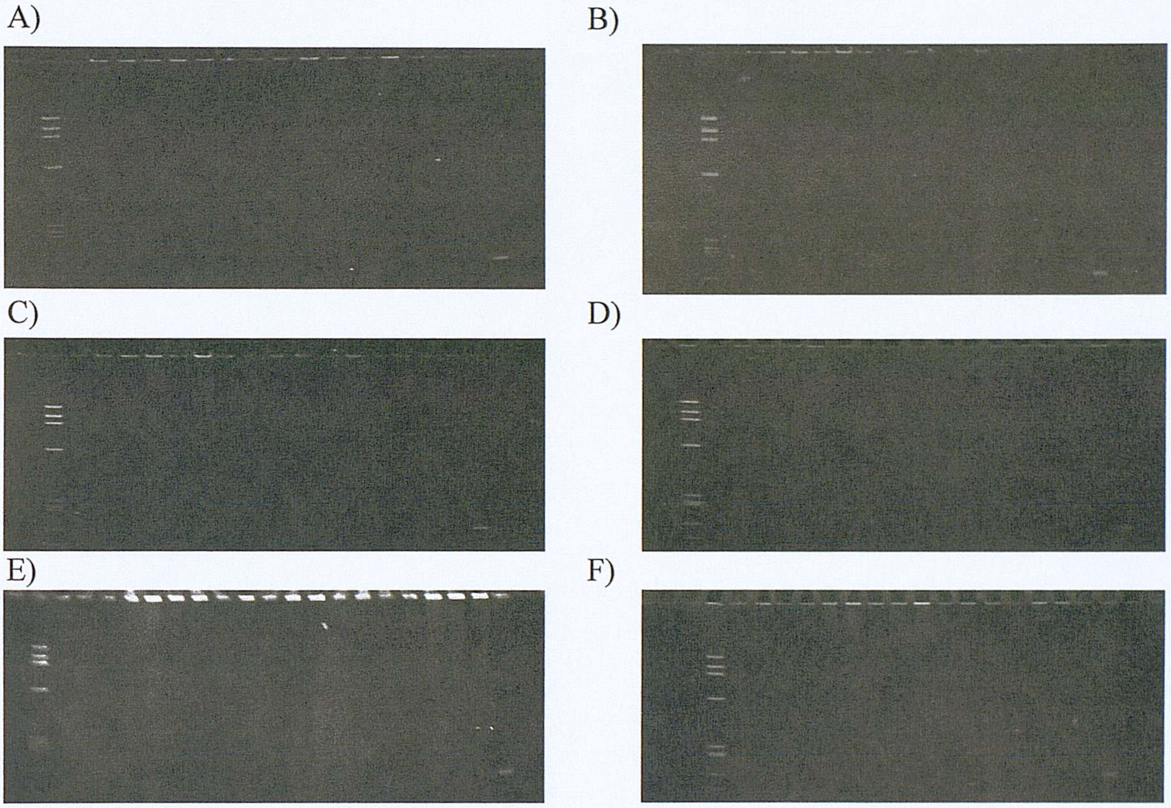


Table 27: MMP 13 PCR/β actin

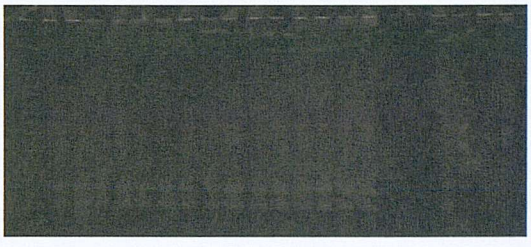
	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	0	0	0	0	0	0
A	0	0	0	0	0	0
D	0	0	0	0	0	0
M	0	0	0	0	0	0
A	0	0	0	0	0	0
D	0	0	0	0	0	0
M	0	0	0	0	0	0
A	0	0	0	0	0	0
D	0	0	0	0	0	0
M	0	0	0	0	0	0
A	0	0	0	0	0	0
D	0	0	0	0	0	0
M	0	0	0	0	0	0
A	0	0	0	0	0	0
D	0	0	0	0	0	0
Pos	1.64	0.25	1.74	1.19	0.55	0.11

7.4e MMP 2 results

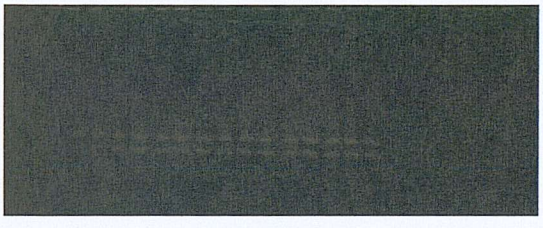
Figure 21: Polyacrylamide gels for MMP 2 PCRs. The gels have a double band as β actin (the lower band) was run with each specimen. The first lane again has the Hae III marker followed by the sets of three lanes with metastases, adjacent liver and distal liver. In groups A,B,C and E there are 5 sets that correspond to the samples in the other MMPs and TIMPs. In gel D there are six sets, F1 having been included in the gel D and only 4 sets in F. Two lanes are then left free followed by the negative lane and then the positive lane. In gel D the final lane (F1 distal) is run twice and in gel E one further negative and two positive lanes are run along with a further Hae III marker.

a)

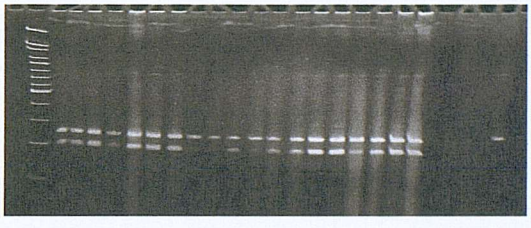
B)



C)



D)



E)

F)

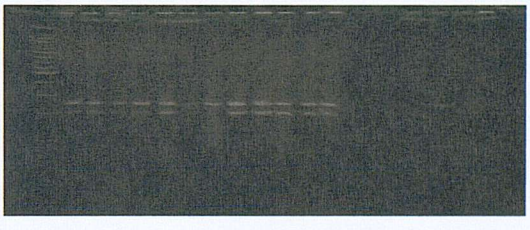


Table 28: MMP 2 PCR (ηg)

	A	B	C	D	E	F
M	39.3	30.1	28.6	20.7	17.2	20.7
A	32.2	12.8	30.8	22.4	14.9	25.9
D	43.9	14.9	40.4	26.4	25.6	41.5
M	53.9	20.9	33.6	15.4	13.8	39.3
A	64.4	22.4	46.3	28.7	22.4	50
D	41	23.1	51.2	32.2	3	38.9
M	73.4	22.1	54.2	30.5	9.5	77.3
A	52.3	9.6	35.7	17.5	17.7	71.8
D	68.2	29.7	58.7	11.7	6.7	15.2
M	61.1	26.2	61.8	14.2	32.1	50.9
A	20.1	41	58.9	18.9	32.9	91.5
D	39.1	38.3	72.6	17.3	41.1	84.5
M	27.9	37.1	50.4	26.1	48.4	46.7
A	27.2	45.4	54.2	38.4	67.9	60.8
D	22.2	41.2	51.4	34.3	43	72.3
Neg	0	0	0	0	0	0
Pos	33.97	17.4	22.87	46.29	17.23	15.08

Table 29: MMP 2 PCR/β-actin (median value and range at foot of table)

	A	B	C	D	E	F
M	1.67	0.8	0.88	1.42	0.46	1.96
A	4.13	0.67	0.96	1.55	0.5	1.2
D	2.55	0.8	1.51	1.15	0.67	1.36
M	1.75	0.66	1.22	1.76	0.32	2.04
A	1.95	0.79	1.5	1.19	0.67	1.58
D	3.39	0.95	1.11	1.23	0.26	2.38
M	1.75	1.01	1.26	1.27	0.46	2.57
A	1.64	0.53	1.41	5.75	0.58	1.27
D	2.61	1.05	1.42	6.29	0.37	1.64
M	2.28	0.89	1.18	1.19	0.67	5.06
A	0.82	0.94	1.15	5.27	0.63	1.35
D	2.6	1.3	1.39	1.15	0.63	1.19
M	1.72	1.09	1.14	1.3	1.01	1.08
A	1.65	1.1	1.06	1.09	0.76	1.13
D	3.47	1.09	1.21	0.98	0.52	1.29
Neg	0	0	0	0	0	0
Pos	1.64	0.94	1.51	2.36	0.62	0.47

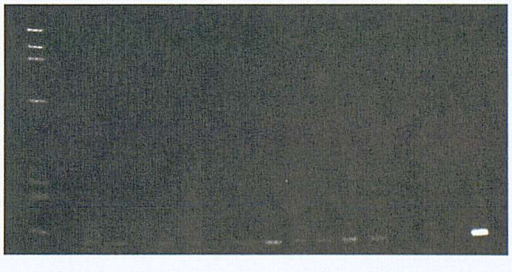
Metastasis (M)	Adjacent liver (A)	Distal liver (D)
1.22 (0.32-5.06)	**1.15** (0.5-5.75)	**1.23** (0.26-6.29)

7.4f MMP 9 results

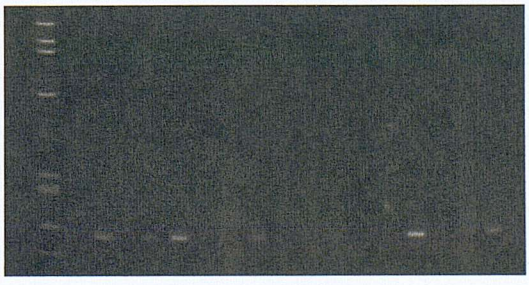
Figure 22: Polyacrylamide gels for MMP 9 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

A)

B)

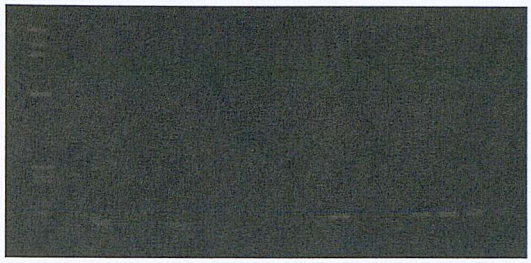


C)



D)

E)



F)

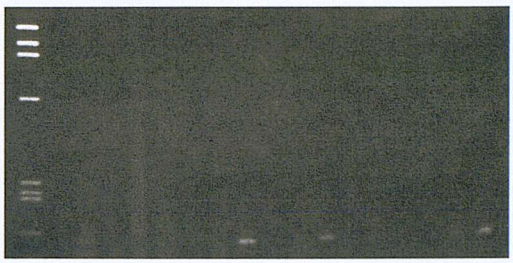


Table 30: MMP 9 PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	8.245	17.72	19.07	0	9.477	20.81
A	10.73	19.3	0	0	0	0
D	0	0	2.9	0	0	0
M	26.23	20.61	33.34	17.81	0	23.18
A	0	10.35	0	20.84	4.537	0
D	0	8.766	0	17.79	0	0
M	17.2	21.79	9.98	17.83	17.32	0
A	0	24.18	0	0	0	0
D	0	6.496	1.72	17.82	0	0
M	13.34	13.37	6.849	17.81	14.39	32.82
A	0	9.905	1.9	17.76	0	7.52
D	7.583	8.442	1.8	17.71	0	0
M	13.03	0	41.41	0	0	25.17
A	9.549	0	2.1	0	0	35.63
D	0	5.529	2.545	17.79	5.185	18.93
Pos	19.1	26.44	20.3	17.4	16.04	18.26

Table 31: MMP 9 PCR/β actin (median value and range at foot of table)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	0.41	0.73	0.62	0	0.36	0.32
A	0.59	0.89	0	0	0	0
D	0	0	0.12	0	0	0
M	2.25	1.23	1.57	0.85	0	0.37
A	0	0.51	0	1.36	0.14	0
D	0	0.94	0	1.02	0	0
M	1.47	1.19	0.55	0.97	0.39	0
A	0	0.48	0	0	0	0
D	0	0.18	0.18	0.82	0	0
M	0.91	0.29	0.53	0.99	0.44	5.52
A	0	0.19	0.1	1.29	0	0.75
D	0.53	0.54	0.1	0.97	0	0
M	0.74	0	2.41	0	0	2.48
A	0.54	0	0.12	0	0	0.89
D	0	0.11	0.1	0.88	0.13	0.57
Pos	0.92	0.34	1.34	0.89	0.57	0.56

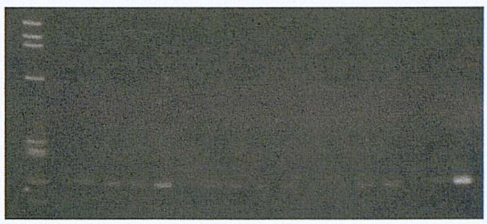
Metastasis (M)	Adjacent liver (A)	Distal liver (D)
0.62 (0-5.52)	**0** (0-1.36)	**0.1** (0-1.02)

7.4g MMP 3 results

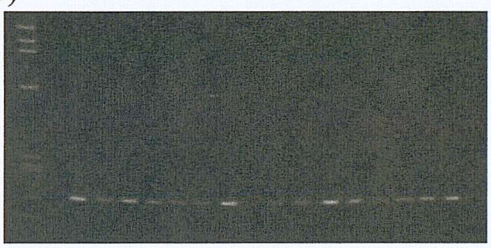
Figure 23: Polyacrylamide gels for MMP 3 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

A)

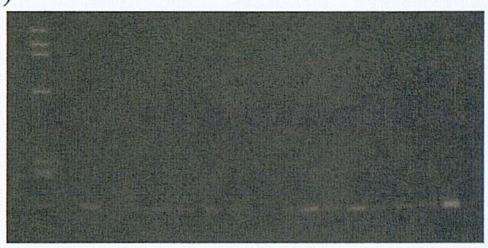
B)



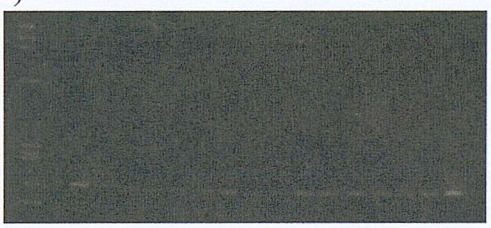
C)



D)



E)



F)

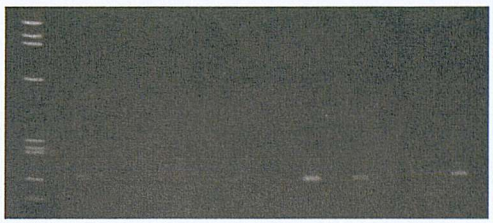


Table 32: MMP 3 PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	26.15	6.118	22.41	19.07	31.72	25.76
A	2.886	8.09	16.36	5.918	12.93	10.39
D	33.72	5.312	19.19	4.641	10.27	8.18
M	59.61	18.39	16.51	8.832	10.94	11.59
A	57.24	0	14.51	8.427	15.92	3.27
D	17.89	4.911	14.48	8.073	12.96	8.94
M	58.01	5.111	20.54	9.894	29.14	9.44
A	31.79	1.751	10.47	0	12.52	3.06
D	29.96	2.515	16.06	9.503	10.34	7.13
M	54.08	0	14.06	28.95	20.57	31.41
A	38.25	2.161	20.1	13.7	18.6	14.64
D	45.92	5.432	17.35	24.83	19.72	28.33
M	64.31	7.404	14.45	8.517	19.64	8.01
A	42.39	0	16.75	13.68	13.59	11.9
D	26.94	6.175	17.19	12.02	26.35	11.67
Pos	61.46	37.8	19.88	38.5	41.36	31.25

Table 33: MMP 3 PCR/β actin (median value and range at foot of table)

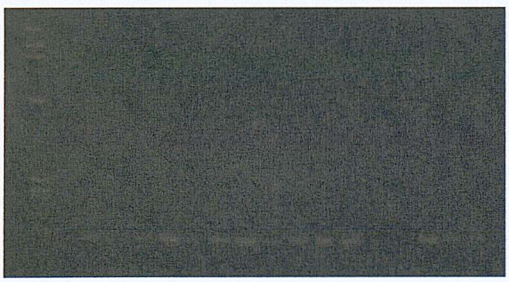
	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	1.3	0.25	0.73	0.97	1.2	0.4
A	0.16	0.37	0.74	0.25	0.31	0.13
D	1.95	0.17	0.82	0.29	0.34	0.16
M	5.12	1.1	0.78	0.42	0.25	0.18
A	3.34	0	0.48	0.55	0.49	0.36
D	1.18	0.53	0.56	0.46	0.28	0.44
M	4.97	0.28	1.14	0.54	0.65	1.73
A	1.93	0.03	1.06	0	0.32	0.37
D	2.09	0.07	1.65	0.44	0.31	0.69
M	3.67	0	1.09	1.6	0.63	5.77
A	1.89	0.04	1.06	0.99	0.43	1.46
D	3.2	0.35	0.94	1.36	0.73	2.27
M	3.67	0.17	0.84	0.47	0.42	0.79
A	2.41	0	0.95	0.44	0.28	0.3
D	1.54	0.13	0.69	0.6	0.66	0.35
Pos	2.97	0.49	1.31	1.96	1.48	0.97

Metastasis (M)	Adjacent liver (A)	Distal liver (D)
0.78 (0-5.12)	**0.44** (0-3.34)	**0.6** (0.07-3.2)

7.4h MMP 7 results

Figure 24: Polyacrylamide gels for MMP 7 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

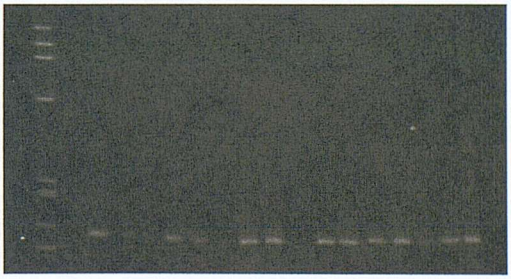
A)



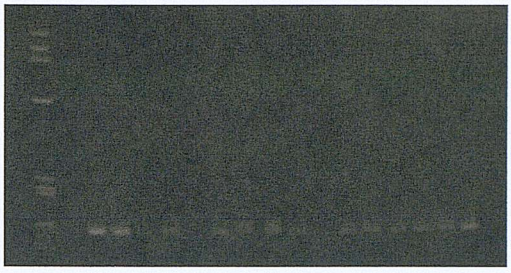
B)

C)

D)



E)



F)

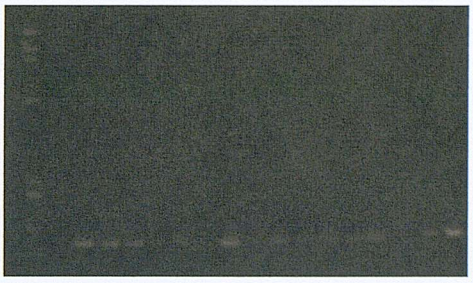


Table 34: MMP 7 PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	25.89	27.4	20.64	25.76	29.57	27.65
A	0	26.03	19	10.3	27.09	20.34
D	18.43	29.63	17.92	10.18	0	21.83
M	59.04	22.87	16.32	21.3	17.28	15.41
A	8.719	29.51	17.48	13.45	0	16.99
D	35.41	26.99	0	8.716	17.96	0
M	51.28	35.16	23.9	25.53	17.19	29.26
A	19.54	21.2	0	27.63	24	6.72
D	37.28	25.18	17.9	0	15.79	21.47
M	49.21	34.76	16.08	24.31	0	14.8
A	53.45	15.28	15.04	26.75	15.22	12.31
D	12.1	0	16.13	28.49	20.85	17.57
M	0	29.31	16.26	28.14	16.24	21.3
A	42.54	19.54	0	11.46	17.99	0
D	10.23	0	16.17	24.91	20.41	20.77
Pos	12.22	38.28	21.08	31.02	24.51	32.59

Table 35: MMP 7 PCR/β actin (median value and range at foot of table)

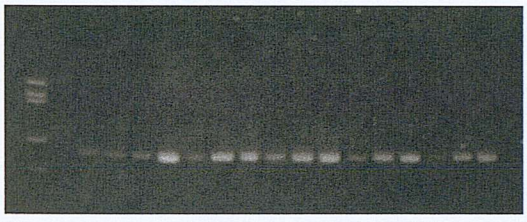
	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	1.28	1.12	0.67	1.31	1.12	0.43
A	0	1.2	0.86	0.43	0.65	0.26
D	1.06	0.97	0.76	0.63	0	0.42
M	5.07	1.37	0.77	1.01	0.39	0.25
A	0.51	1.45	0.58	0.88	0	1.86
D	2.33	2.89	0	0.5	0.39	0
M	4.39	1.92	1.32	1.38	0.38	5.37
A	1.18	0.42	0	1.35	0.61	0.82
D	2.6	0.69	1.84	0	0.48	2.07
M	3.34	0.77	1.24	1.35	0	2.49
A	2.65	0.29	0.79	1.9	0.35	1.22
D	0.84	0	0.87	1.56	0.77	1.41
M	0	0.69	0.95	1.55	0.35	2.1
A	2.42	0.26	0	0.37	0.36	0
D	0.58	0	0.65	1.24	0.51	0.62
Pos	0.59	0.5	1.39	1.58	0.88	1.01

Metastasis (M)	Adjacent liver (A)	Distal liver (D)
1.12 (0-5.07)	**0.58** (0-2.65)	**0.69** (0-2.89)

7.4i TIMP 1 results

Figure 25: Polyacrylamide gels for TIMP 1 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

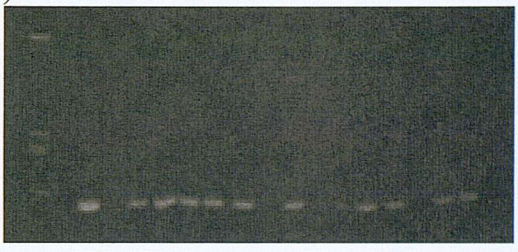
A)



B)

C)

D)



E)

F)

Table 36: TIMP 1 PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	5.58	32.13	18.86	32.42	28.46	32.56
A	8.972	7.518	0	0	9.442	0
D	14.41	23.88	15.44	19.22	26.34	29.81
M	51.97	7.758	8.886	22.78	21.88	12.7
A	9.188	0	19.09	23.92	21.72	0
D	41.97	10.87	13.36	22.07	22.26	12.7
M	34.82	32.02	20.81	20.79	36.28	24.06
A	22.14	22.01	0	0	19.11	13.02
D	39.86	22.86	12.14	20.76	13.24	15.91
M	42.58	10.22	14.12	10.28	38.03	30.14
A	10.19	0	16.23	13.47	25.04	16.22
D	31.96	19.73	7.838	21.51	28.38	30.33
M	36.22	0	19.06	18.9	32.34	22.96
A	2.328	14.81	0	0	23.67	44.23
D	31.02	15.29	19.21	15.93	21.29	24.83
Pos	42.38	28.43	11.77	18.61	26.86	31.65

Table 37: TIMP 1 PCR/β actin (median value and range at foot of table)

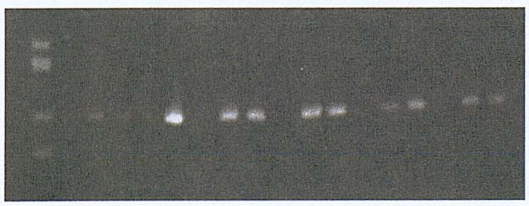
	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	0.28	1.32	0.62	1.65	1.08	0.51
A	0.49	0.35	0	0	0.23	0
D	0.83	0.78	0.66	1.19	0.86	0.57
M	4.46	0.46	0.42	1.08	0.5	0.2
A	0.54	0	0.63	1.57	0.67	0
D	2.76	1.17	0.52	1.27	0.48	0.62
M	2.98	1.75	1.15	1.13	0.81	4.42
A	1.34	0.43	0	0	0.49	1.59
D	2.78	0.63	1.25	0.96	0.4	1.54
M	2.89	0.23	1.09	0.57	1.16	5.06
A	0.5	0	0.85	0.98	0.58	1.61
D	2.23	1.26	0.43	1.18	1.05	2.43
M	2.07	0	1.12	1.04	0.69	2.26
A	0.13	0.2	0	0	0.48	1.11
D	1.77	0.32	0.78	0.79	0.53	0.74
Pos	2.05	0.36	0.78	0.95	0.96	0.98

Metastasis (M)	Adjacent liver (A)	Distal liver (D)
1.09 (0-0.89)	**0.48** (0-1.61)	**0.86** (0.32-2.78)

7.4j TIMP 2 results

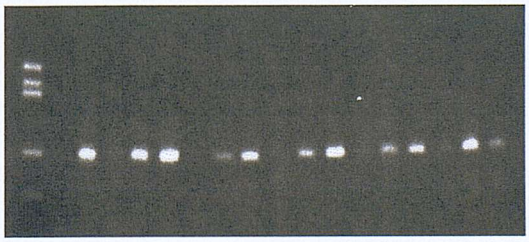
Figure 26: Polyacrylamide gels for TIMP 2 PCRs 18 lanes: the first lane has a standardised marker Hae III, the next lane is a negative control (dH_2O),then five sets of three lanes which have cDNA from the metastasis(M), adjacent liver(A) and distal liver(D) from 5 of the 30 patients. The final lane is a positive control using cDNA (see table 8). The thirty patients are therefore numbered A1-5, B1-5, C1-5, D1-5, E1-5, F1-5.

A)

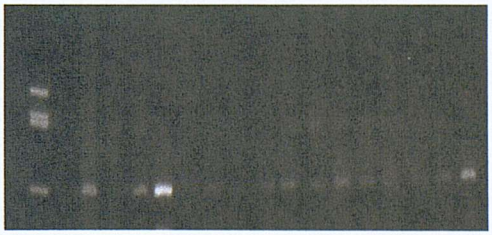


B)

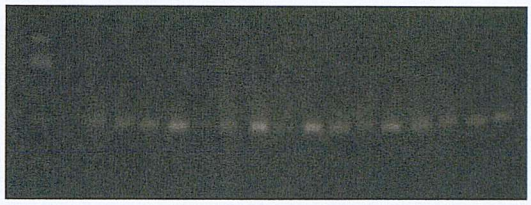
C)



D)



E)



F)

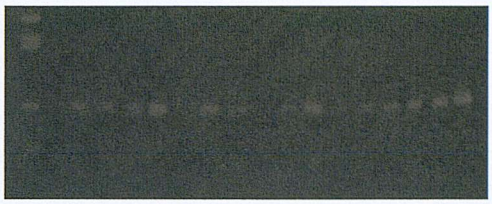


Table 38: TIMP 2 PCR (ηg)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	13.13	16.71	28.37	11.5	18.86	8.024
A	4.374	0	0	0	18.88	8.208
D	3.524	4.271	19.96	12.67	18.83	8.015
M	51.77	4.52	30.9	29.24	18.49	16.76
A	0	0	0	0	0	0
D	37.7	3.011	12.64	5.008	18.9	11.7
M	30.32	24.68	18.18	3.749	18.39	5.445
A	0	0	0	3.257	0	0
D	30.83	11.4	16.14	7.498	18.5	4.794
M	29.4	8.86	24.3	5.266	18.62	15.23
A	0	0.9489	3.189	10.38	0	0
D	14.77	6.009	17.79	7.836	18.56	6.475
M	24.88	14.52	20.15	6.746	18.55	9.139
A	0	0	0.9594	3.458	18.8	13.45
D	20.2	7.601	19.63	6.914	18.84	15.13
Pos	18.19	28.13	11.14	15.83	18.82	18.56

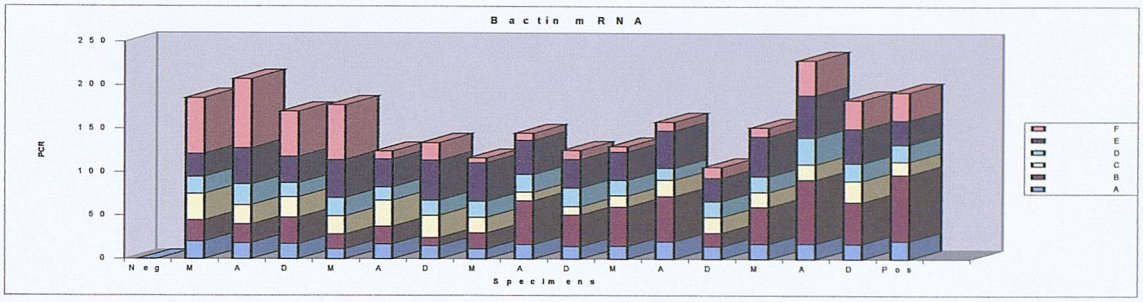
Table 39: TIMP 2 PCR/β actin (median value and range at foot of table)

	A	B	C	D	E	F
Neg	0	0	0	0	0	0
M	0.65	0.68	0.93	0.58	0.71	0.12
A	0.24	0	0	0	0.45	0.1
D	0.2	0.14	0.85	0.78	0.62	0.15
M	4.44	0.27	1.46	1.39	0.42	0.27
A	0	0	0	0	0	0
D	2.48	0.32	0.49	0.29	0.41	0.57
M	2.6	1.35	1.01	0.2	0.41	1
A	0	0	0	0.16	0	0
D	2.15	0.31	1.66	0.35	0.56	0.46
M	2	0.2	1.88	0.29	0.57	2.56
A	0	0.02	0.17	0.75	0	0
D	1.03	0.38	0.97	0.43	0.69	0.52
M	1.42	0.34	1.17	0.37	0.4	0.9
A	0	0	0.05	0.11	0.38	0.34
D	1.15	0.16	0.79	0.34	0.47	0.45
Pos	0.88	0.37	0.73	0.81	0.67	0.57

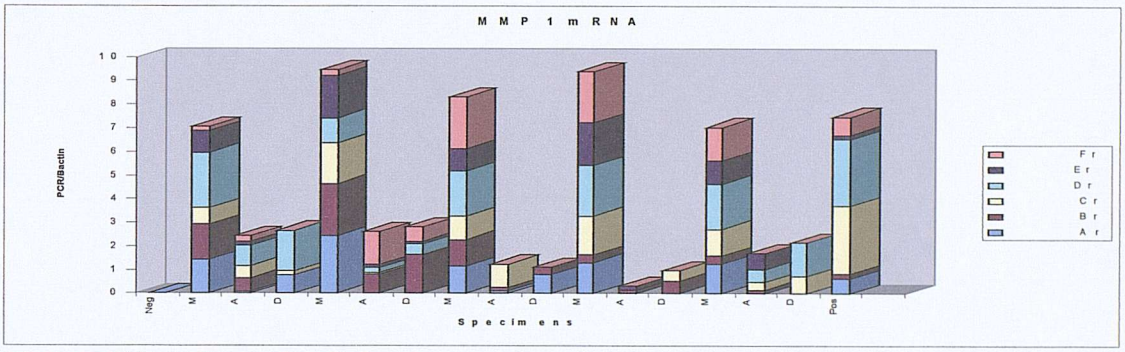
Metastasis	Adjacent liver	Distal liver
0.71 (0.12-4.44)	**0** (0-0.75)	**0.49** (0.14-2.48)

Figure 27: Graphical representation of β actin results, MMP/ β actin results and TIMP/ β actin results given in proceeding tables

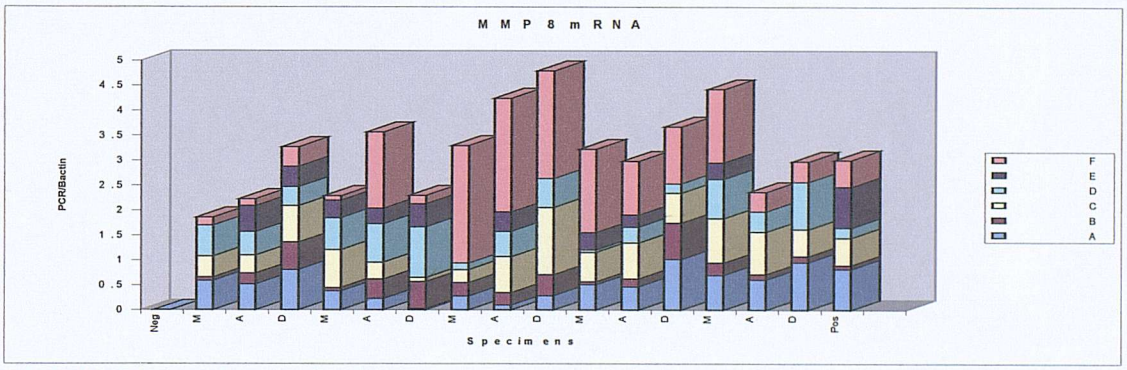
a) β actin mRNA results (below)



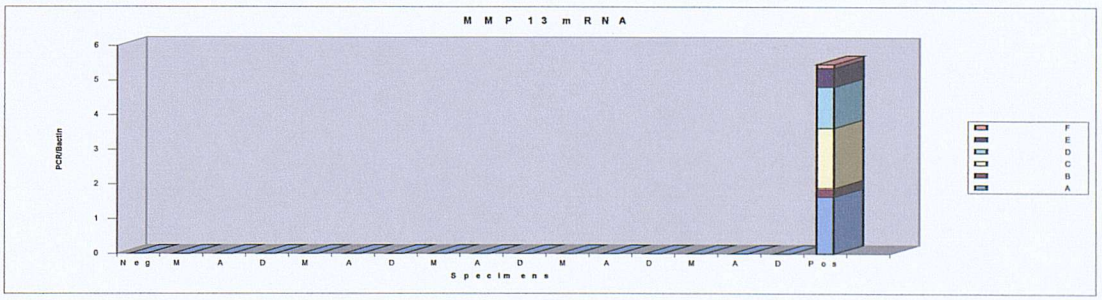
b) MMP1/ β actin results (below)



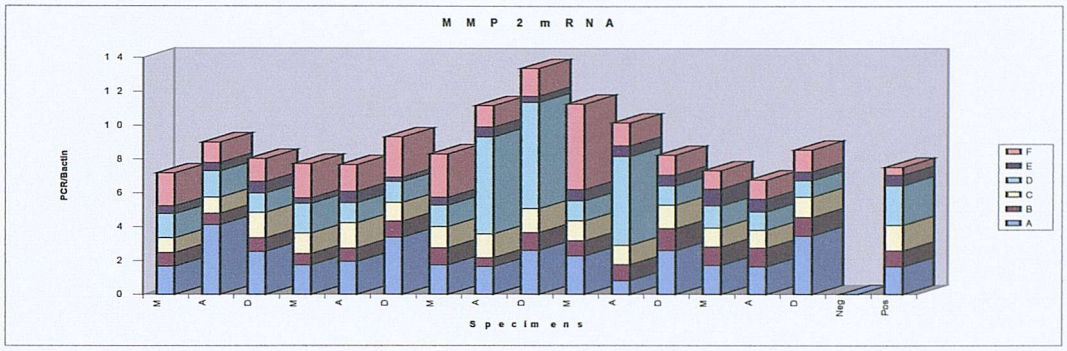
c) MMP8/ β actin results (below)



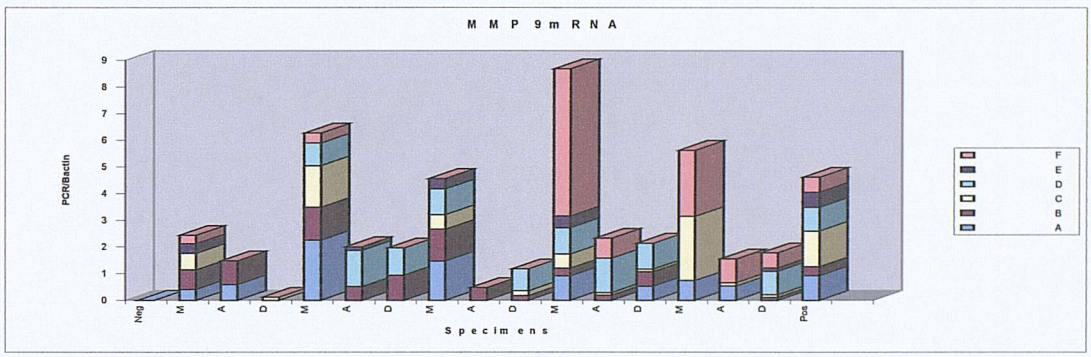
d) MMP 13/ β actin results (below)



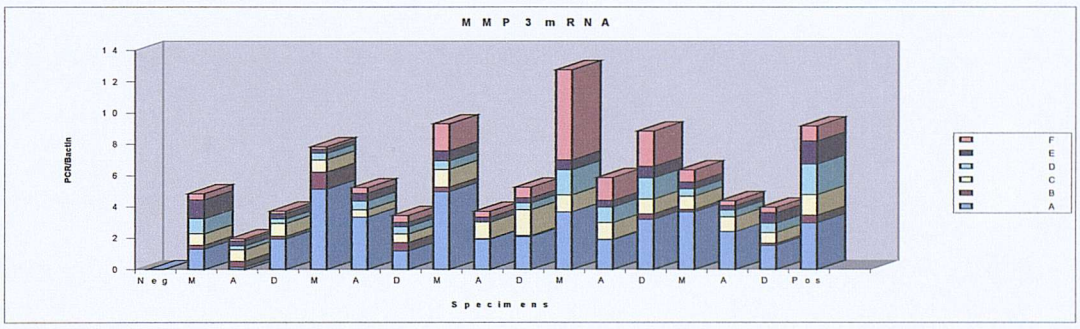
e) MMP 2/ β actin results (below)



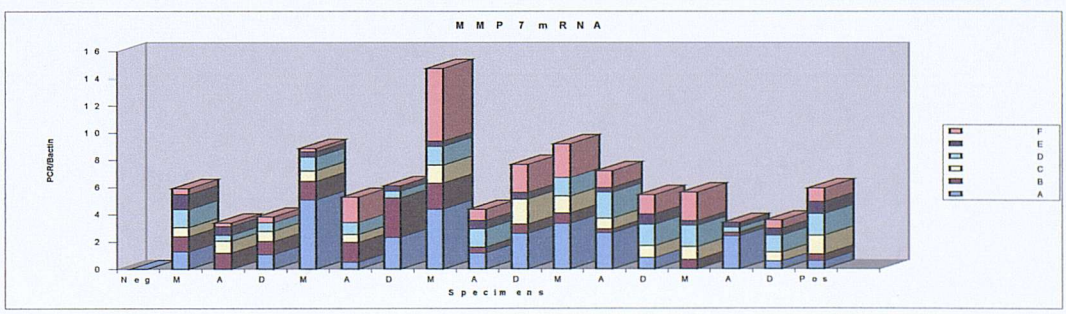
f) MMP 9/ β actin results (below)



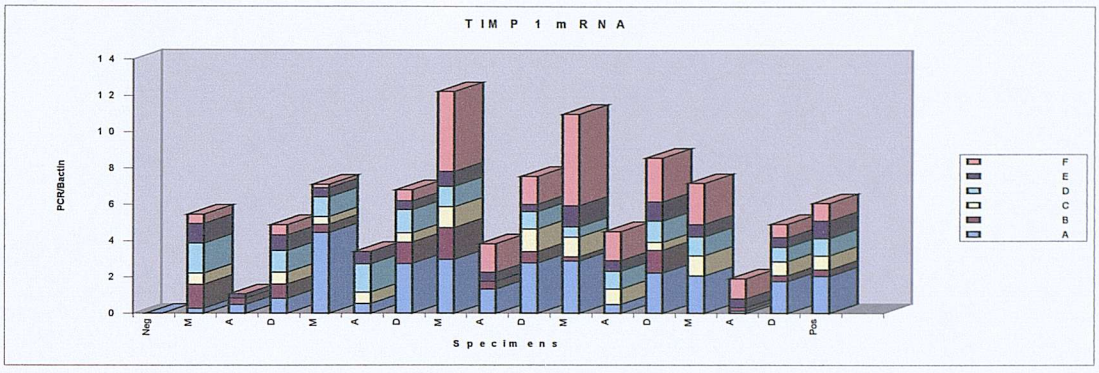
g) MMP 3/ β actin results (below)



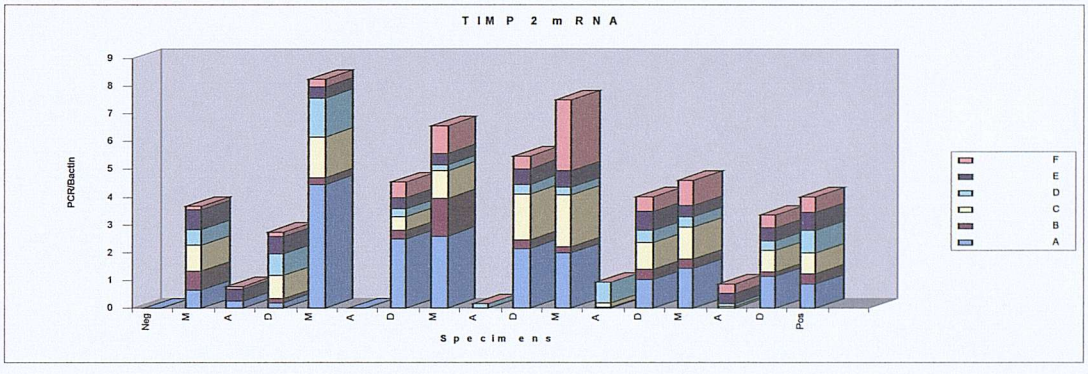
h) MMP 7/ β actin results (below)



i) TIMP 1/β actin results (below)



j) TIMP 2/β actin results (below)



7.5 Summary of PCR results

7.5a Collagenases

MMP 1 mRNA was expressed at a significant higher level in colorectal cancer liver metastases than in the immediately adjacent liver and distal liver ($p < 0.002$, Wilcoxon signed-rank test). There was no expression of MMP 1 mRNA in or adjacent to the benign lesions. MMP8 mRNA expression was the same in metastasis, adjacent liver and distal liver from the 30 hepatectomies for colorectal cancer, and there was no difference in the expression in the benign lesions compared to the adjacent and normal liver. There was no expression of MMP 13 mRNA by any tissue sample collected.

Table 40: Median values (and ranges) for MMP/β actin and TIMP/β actin ratios for 30 colorectal carcinoma metastases and 3 benign liver lesions. p values for the 30 CRC metastases calculated using the Wilcoxon signed-rank test. p values were not calculated for the benign lesions due to low numbers (The PCR gels for the benign lesions are shown later in this chapter).

		Metastasis/ Benign (M/B)	Adjacent liver (A)	Distal liver (D)
MMP 1	Met	**1.42** (0.19-2.44)	**0.16** (0-1.4)	**0.02** (0-1.71)
	Benign	**0**	**0**	**0**
MMP 8	Met	**0.35** (0-0.89)	**0.39** (0-0.85)	**0.54** (0-1.35)
	Benign	**0.39** (0.27-0.65)	**0.32** (0-0.63)	**0.71** (0.25-0.83)
MMP 13	Met	**0**	**0**	**0**
	Benign	**0**	**0**	**0**
MMP 2	Met	**1.22** (0.32-5.06)	**1.15** (0.5-5.75)	**1.23** (0.26-6.29)
	Benign	**0.6** (0.5-0.7)	**0.5** (0.4-0.6)	**0.6** (0.45-0.75)
MMP 9	Met	**0.62** (0-5.52)	**0** (0-1.36)	**0.1** (0-1.02)
	Benign	**0** (0-0.51)	**0.2** (0-0.39)	**0** (0-0.27)
MMP 3	Met	**0.78** (0-5.12)	**0.44** (0-3.34)	**0.6** (0.07-3.2)
	Benign	**0.53** (0.11-0.62)	**0.44** (0.35-0.53)	**0.42** (0.22-0.7)
MMP 7	Met	**1.12** (0-5.07)	**0.58** (0-2.65)	**0.69** (0-2.89)
	Benign	**0** (0-0.55)	**0.29** (0-0.58)	**0**
TIMP 1	Met	**1.09** (0-5.06)	**0.48** (0-1.61)	**0.86** (0.32-2.78)
	Benign	**1.48** (0.45-1.85)	**1.57** (1.45-1.69)	**1.31** (0.84-1.4)
TIMP 2	Met	**0.71** (0.12-4.44)	**0** (0-0.75)	**0.49** (0.14-2.48)
	Benign	**0.18** (0.08-0.49)	**0.42** (0.42-0.42)	**0.29** (0.17-0.32)
p values				
		MvA	MvD	AvD
MMP 1	Met	**<0.002**	**<0.002**	0.98
MMP 8	Met	0.67	0.10	0.08
MMP 2	Met	0.3	0.13	0.28
MMP 9	Met	**<0.002**	**<0.002**	0.35
MMP 3	Met	**<0.002**	0.056	0.13
MMP 7	Met	**0.01**	**0.026**	0.60
		MvA	MvD	DvA
TIMP 1	Met	**<0.002**	**0.023**	**<0.002**
TIMP 2	Met	**<0.002**	**0.015**	**<0.002**

7.5b Gelatinases

MMP 2 mRNA was expressed at a similar level in metastasis, adjacent liver and distal liver and there was no difference in the expression in the benign lesions compared to the adjacent and normal liver. MMP 9 mRNA was expressed at significantly higher levels in the metastases than the immediately adjacent liver or the distal liver ($p < 0.002$) and the levels in the adjacent liver were the same as those in the distal liver. MMP 9 mRNA was expressed by only one benign lesion.

7.5c Stromelysins

MMP 3 mRNA was expressed at significantly higher levels by the metastases compared to the adjacent liver ($p<0.002$). There was no significant difference in expression between the adjacent and distal liver. MMP 3 mRNA expression was similar in the benign lesions and their adjacent and distal liver. MMP 7 mRNA was expressed at significantly higher levels in the metastases than the immediately adjacent liver and the distal liver ($p=0.01$). There was expression, at low levels, by only one benign lesion.

7.5d Tissue inhibitors of metalloproteinases (TIMPs)

TIMP 1 mRNA was expressed by both colorectal carcinoma metastases and distal liver, but at significantly higher levels in the metastases ($p=0.02$). However the expression of TIMP 1 mRNA by the immediately adjacent liver was markedly lower than both metastases and distal liver ($p<0.002$), being absent in 10 of the 30 patients. In the benign lesions TIMP 1 mRNA expression was equal throughout.TIMP 2 mRNA was expressed by both colorectal carcinoma metastases and distal liver, but at significantly higher level in the metastases ($p=0.02$). Again the expression of TIMP 2 mRNA by the immediately adjacent liver was significantly lower than both metastases and distal liver ($p<0.002$), being absent in 19 of the 30 patients. In the benign lesions TIMP 2 mRNA expression was equal throughout.

7.6 Positive and Negative controls for Immunohistochemistry

The tissues used as positive controls were as follows:

MMP 1: infected tonsil/Rheumatoid synovium

MMP 8: infected tonsil/Rheumatoid synovium

MMP 2: tonsil/inflammatory bowel disease

MMP 9: tonsil/inflammatory bowel disease

MMP 3: tumour

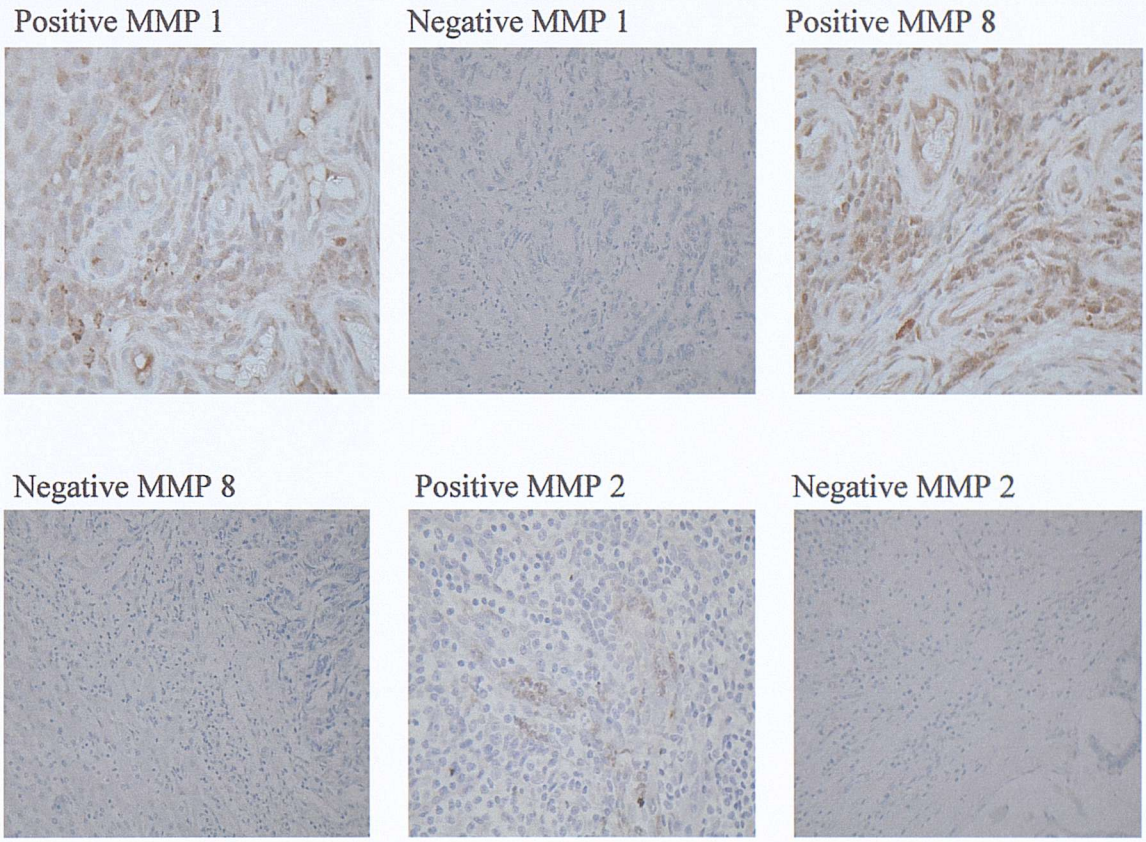
MMP 7: Rheumatoid synovium

TIMP 1: foreskin/nerve

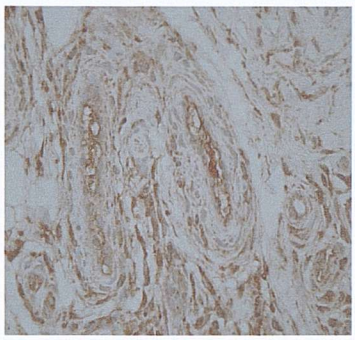
TIMP 2: melanoma/tonsil

Negative controls were run as omission of antibody (not shown) and matching isotype controls (as given in the methods section) for each antibody (shown).

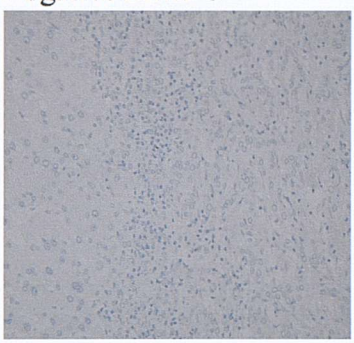
Figure 28: Positive and Negative controls for Immunohistochemistry. Brown staining indicates positive result for that particular MMP/TIMP



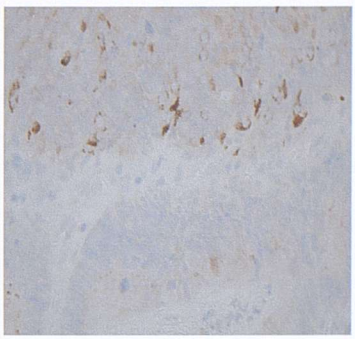
Positive MMP 9



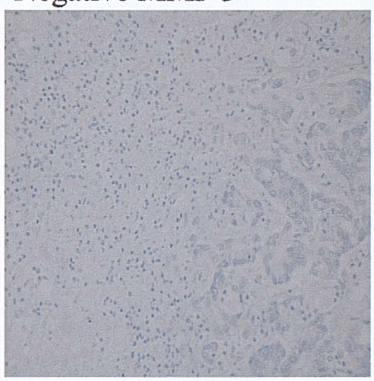
Negative MMP 9



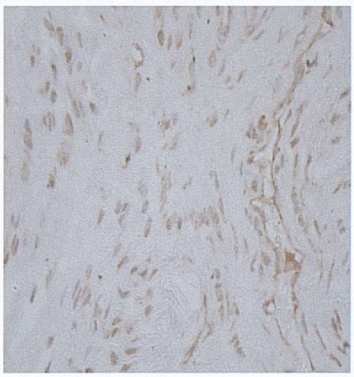
Positive MMP 3



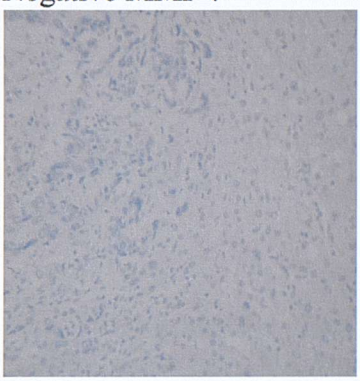
Negative MMP 3



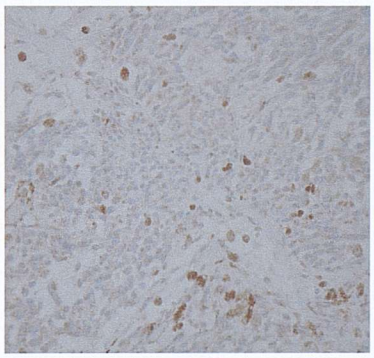
Positive MMP 7



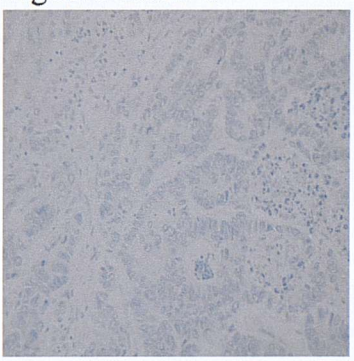
Negative MMP 7



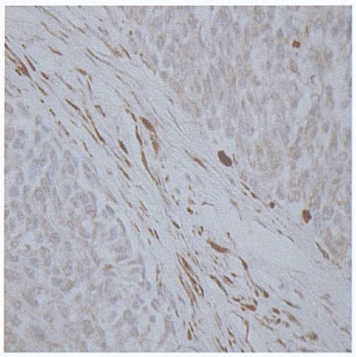
Positive TIMP 1



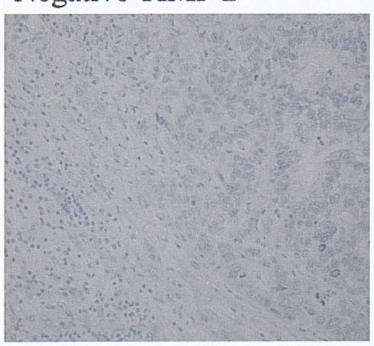
Negative TIMP 1



Positive TIMP 2



Negative TIMP 2



7.7 Immunohistochemistry (IHC) results

Immunohistochemistry was performed on all thirty patients with metastases, adjacent livers and distal livers. These were then examined under a light powered microscope to identify MMP and TIMP expression. For ten of these patients the resultant images on light microscopy were captured with digital imaging and are shown below. The expression for each MMP and TIMP is shown for all ten patients together. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous).

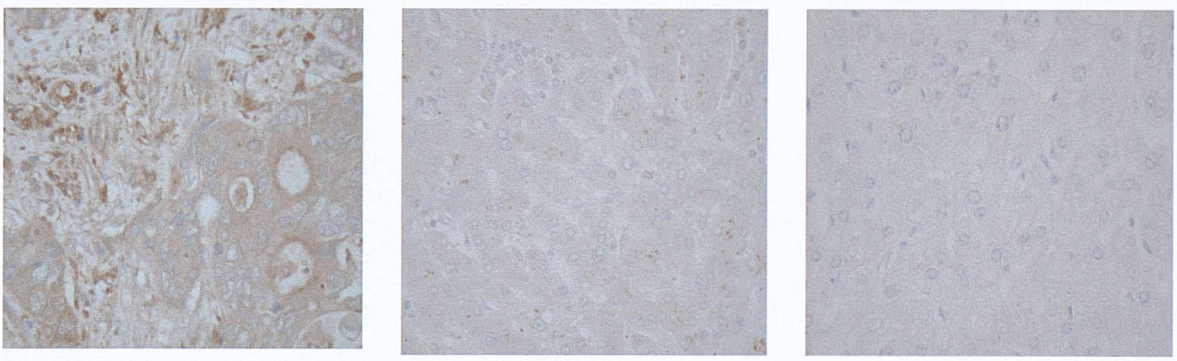
7.8 Secondary tumour: Metastasis/Adjacent liver/Distal liver

7.8a MMP 1 results

Figure 29: IHC results for MMP 1 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. MMP 1 expressed strongly by stromal macrophages and fibroblasts in metastases but only minimally by the adjacent and distal liver.

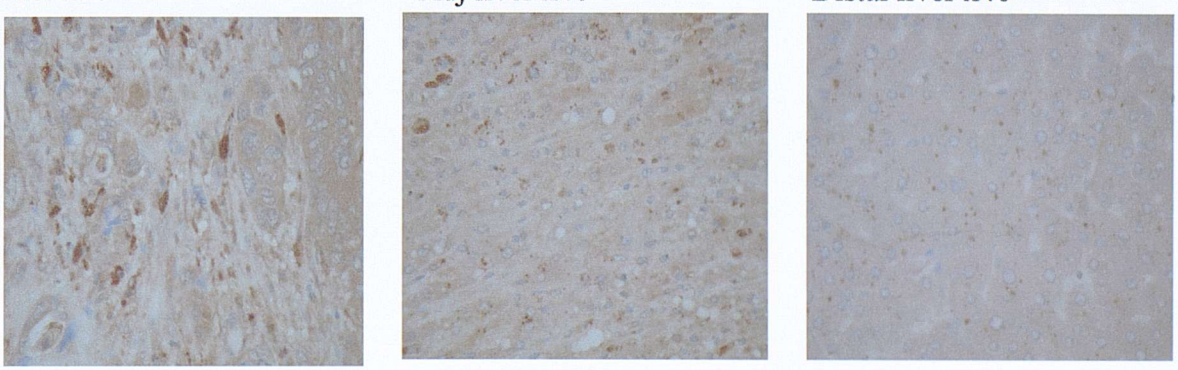
B1

Met x40 | Adj liver x40 | Distal liver x40



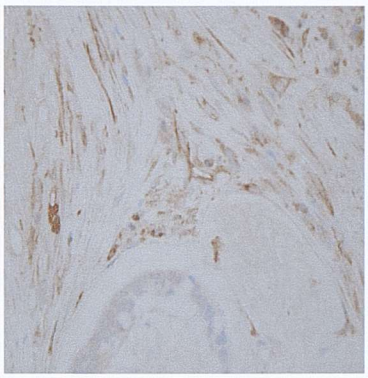
B2

Met x40 | Adj liver x40 | Distal liver x40

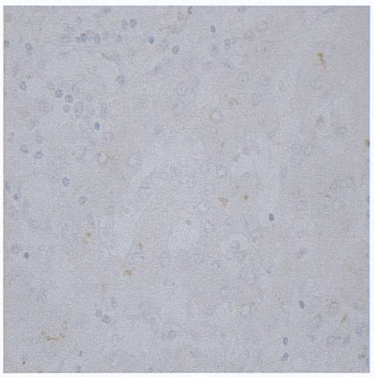


B3

Met x40



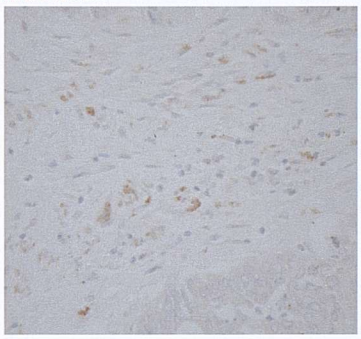
Adj liver x40



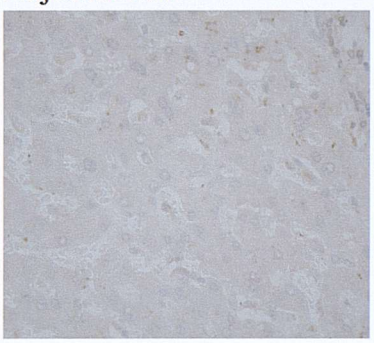
Distal liver x40

C4

Met x40



Adj liver x40

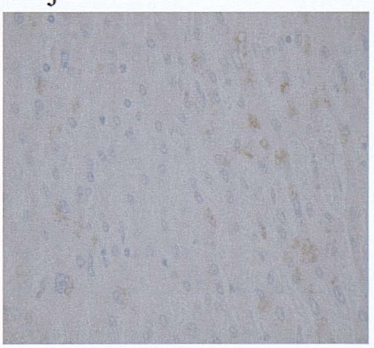


Distal liver x40

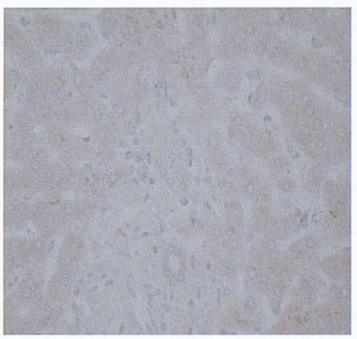
C5

Met x40

Adj liver x40

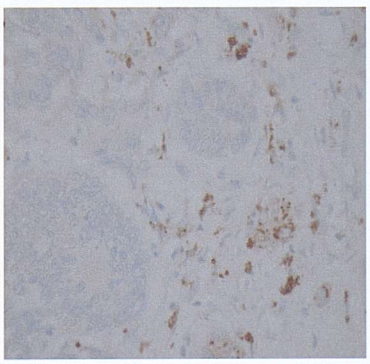


Distal liver x40

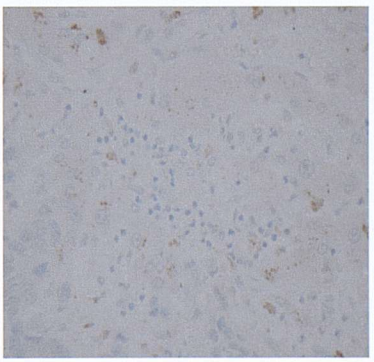


E2

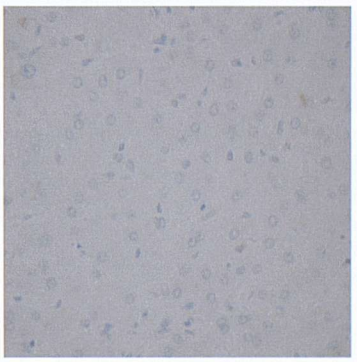
Met x40



Adj liver x40

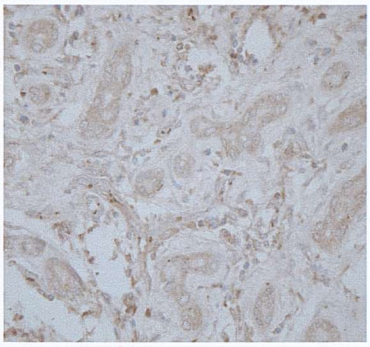


Distal liver x40

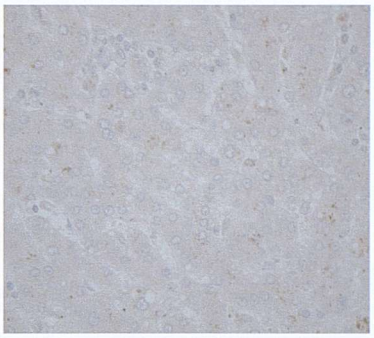


E3

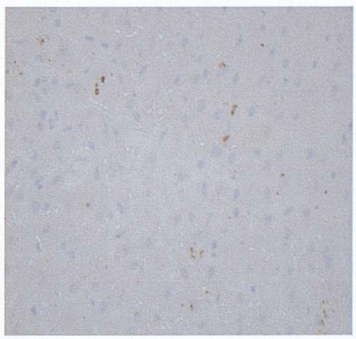
Met x40



Adj liver x40

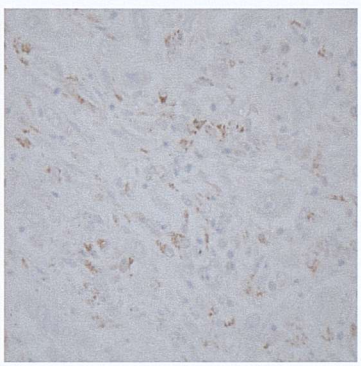


Distal liver x40

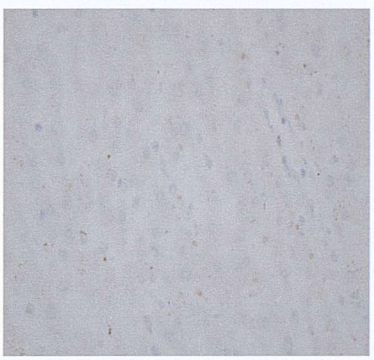


E4

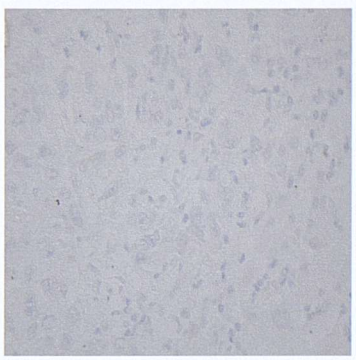
Met x40



Adj liver x40

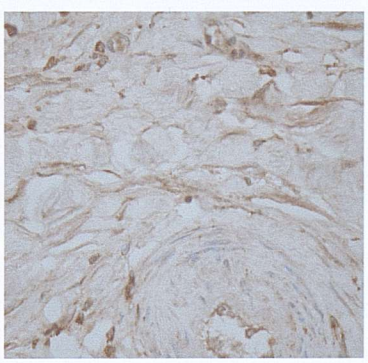


Distal liver x40

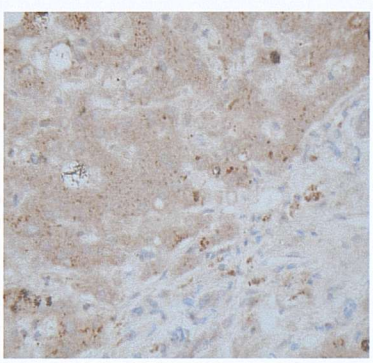


F2

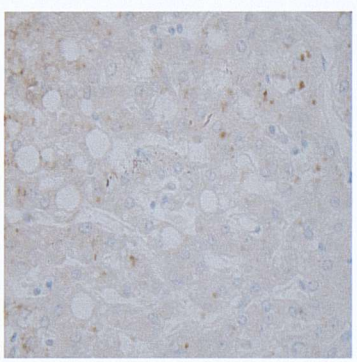
Met x40



Adj liver x40

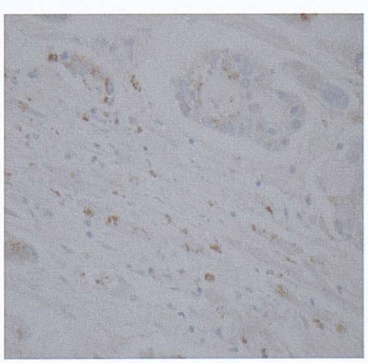


Distal liver x40

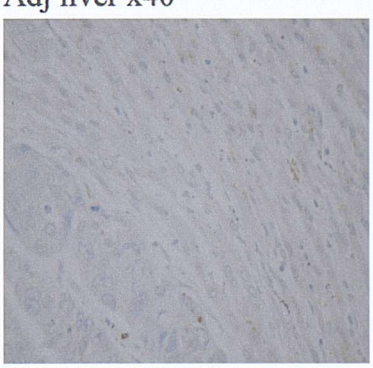


F5

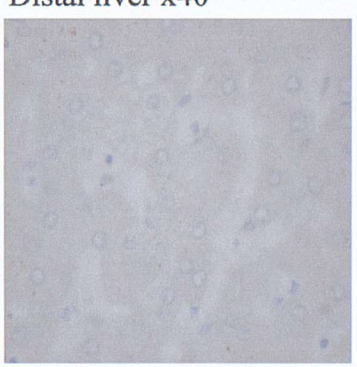
Met x40



Adj liver x40



Distal liver x40

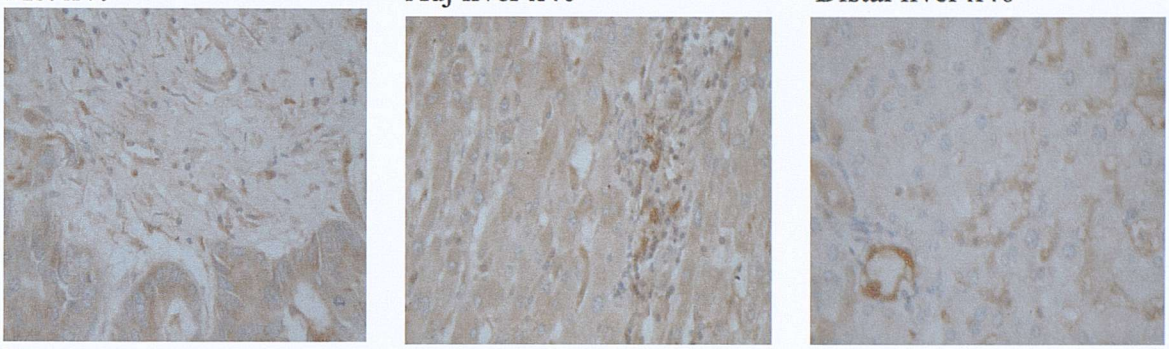


7.8b MMP 8 results

Figure 30: IHC results for MMP 8 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. MMP 8 expressed by fibroblasts in tumour stroma and by both the adjacent and distal liver.

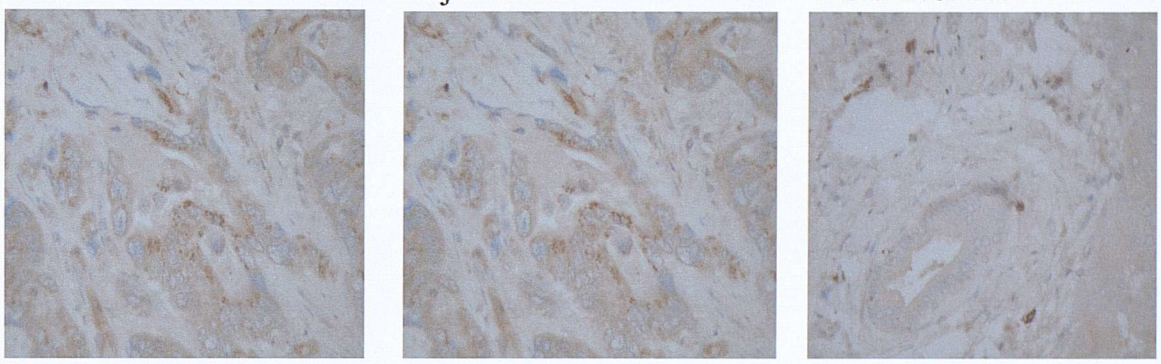
B1

Met x40 | Adj liver x40 | Distal liver x40



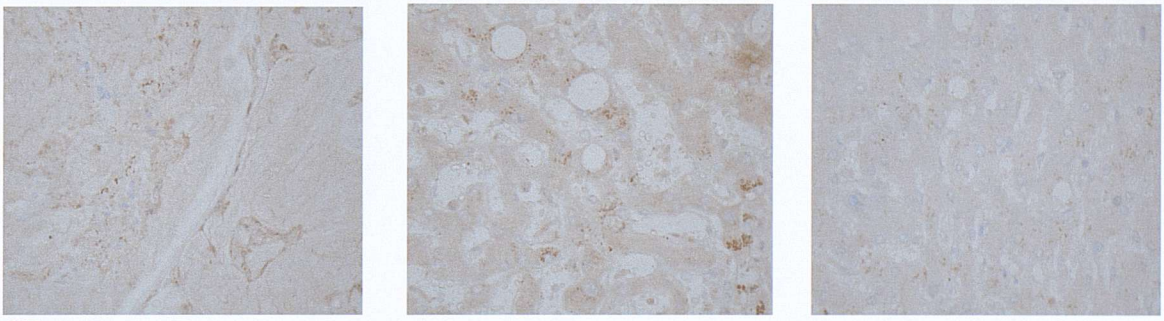
B2

Met x40 | Adj liver x40 | Distal liver x40



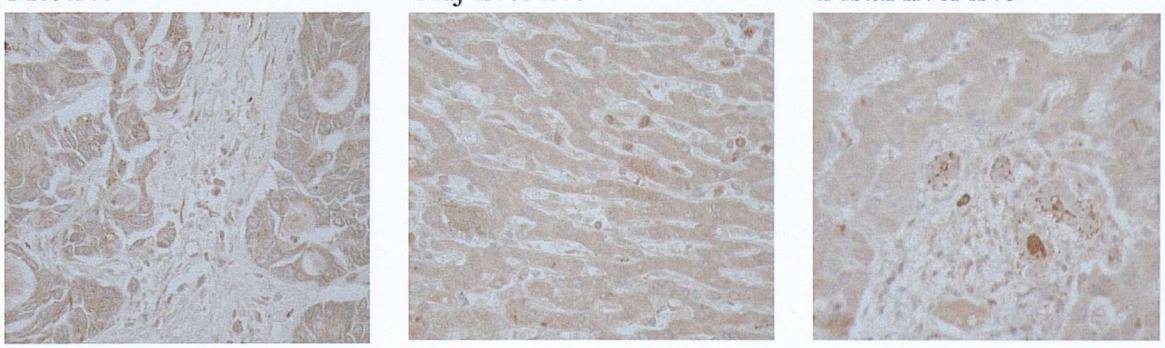
B3

Met x40 | Adj liver x40 | Distal liver x40



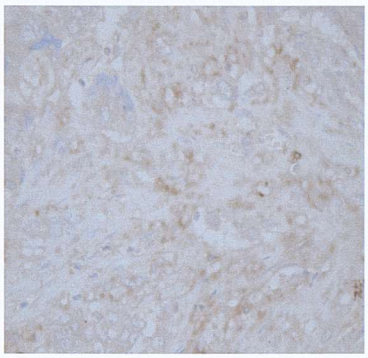
C4

Met x40 | Adj liver x40 | Distal liver x40

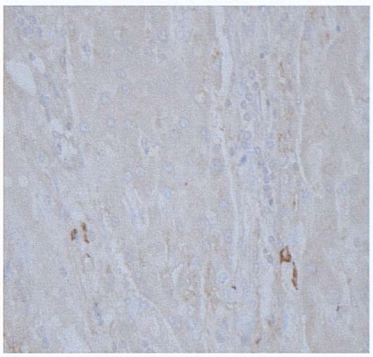


C5

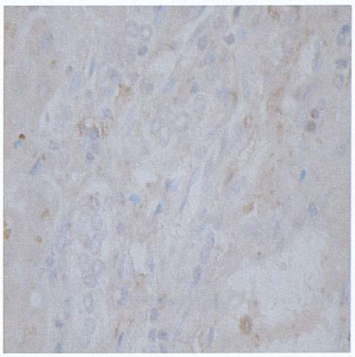
Met x40



Adj liver x40

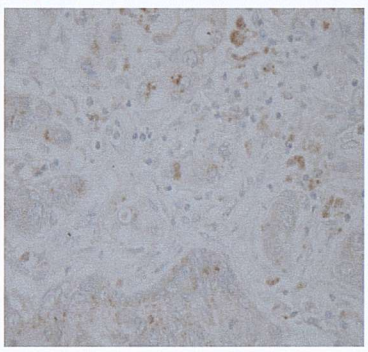


Distal liver x40

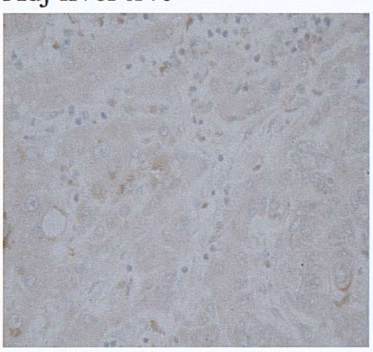


E2

Met x40



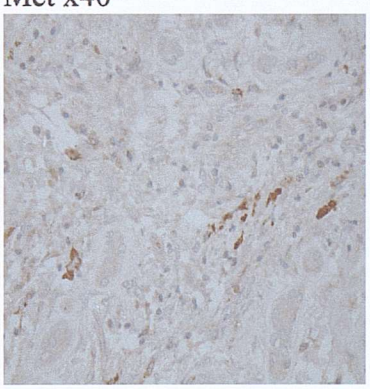
Adj liver x40



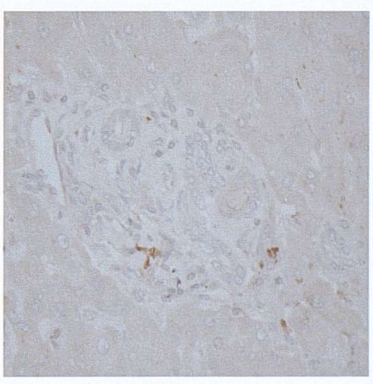
Distal liver x40

E3

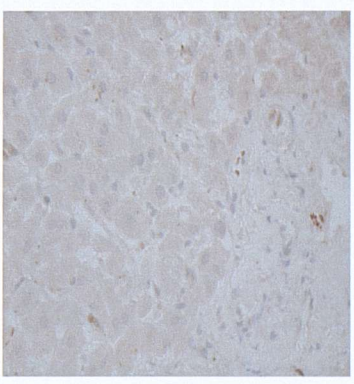
Met x40



Adj liver x40

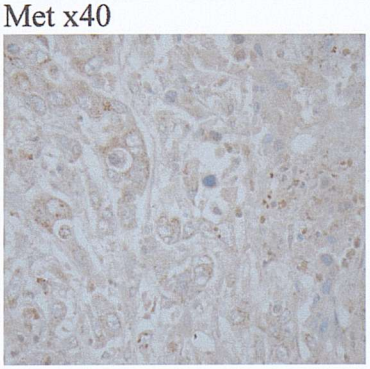


Distal liver x40



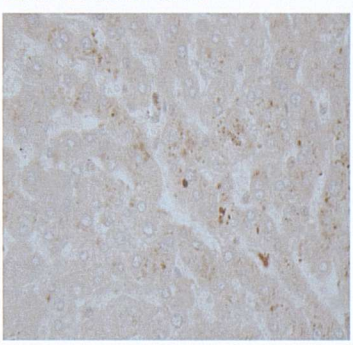
E4

Met x40



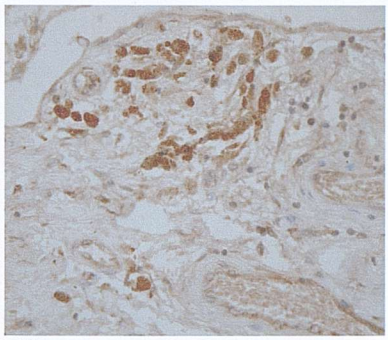
Adj liver x40

Distal liver x40

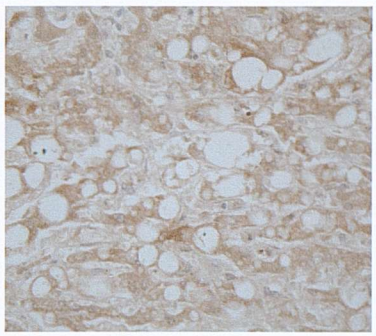


F2

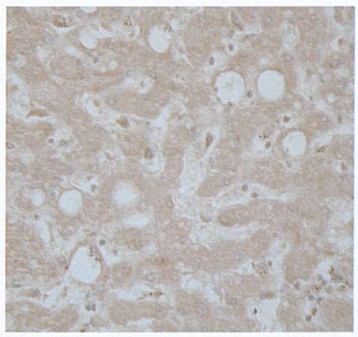
Met x40



Adj liver x40

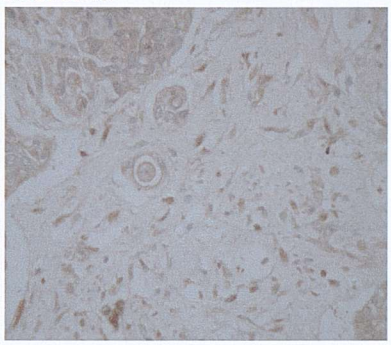


Distal liver x40

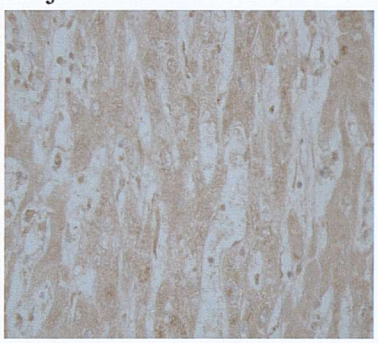


F5

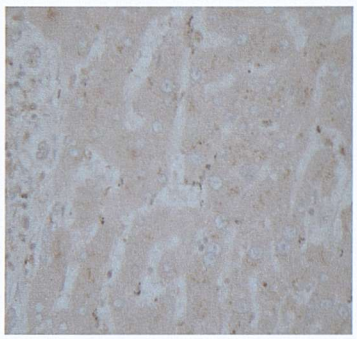
Met x40



Adj liver x40



Distal liver x40

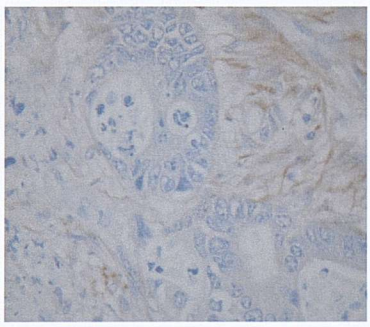


7.8c MMP 2 results

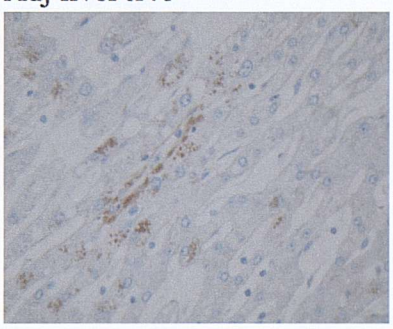
Figure 31: IHC results for MMP 2 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. MMP 2 expressed by fibroblasts in tumour stroma, and by hepatocytes in both adjacent and distal liver.

B1

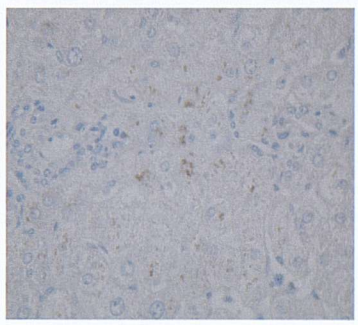
Met x40



Adj liver x40

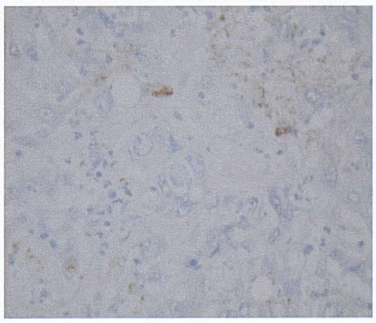


Distal liver x40

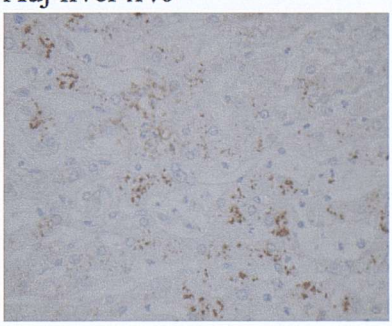


B2

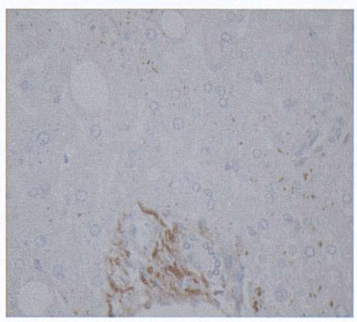
Met x40



Adj liver x40

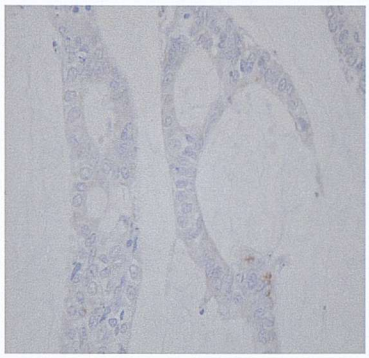


Distal liver x40

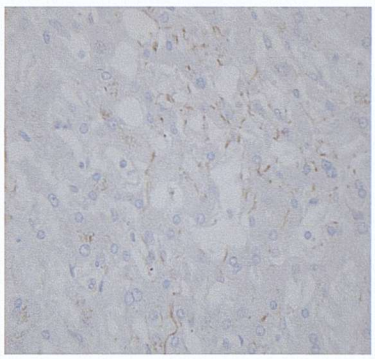


B3

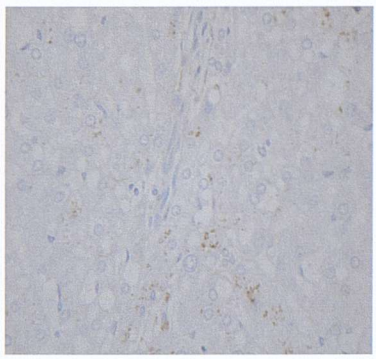
Met x40



Adj liver x40

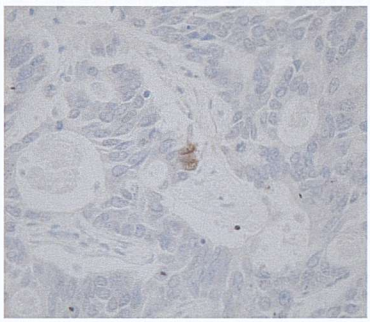


Distal liver x40

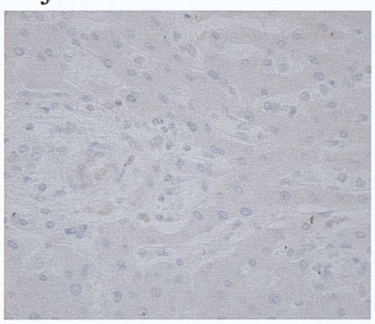


C4

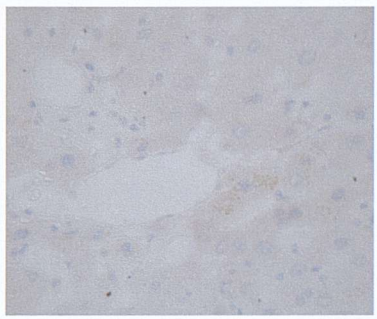
Met x40



Adj liver x40

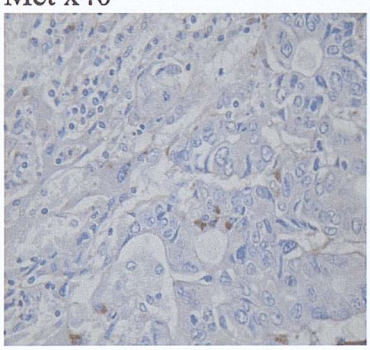


Distal liver x40

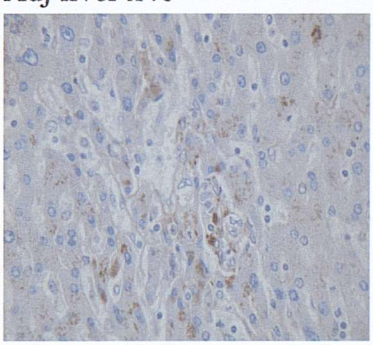


C5

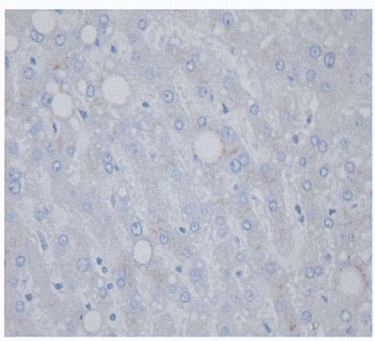
Met x40



Adj liver x40

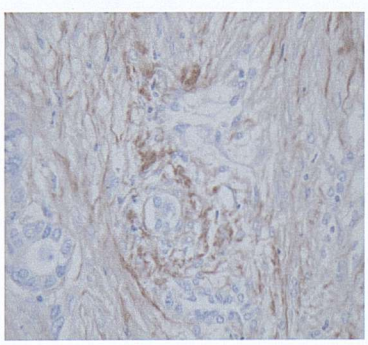


Distal liver x40

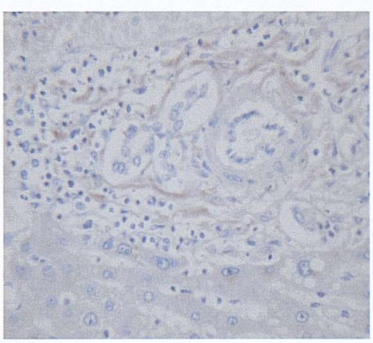


E2

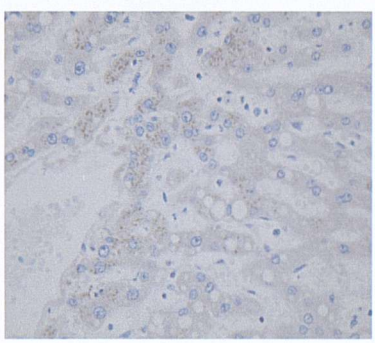
Met x40



Adj liver x40

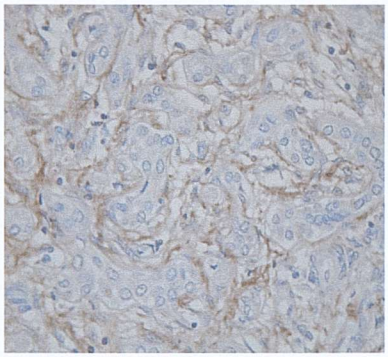


Distal liver x40

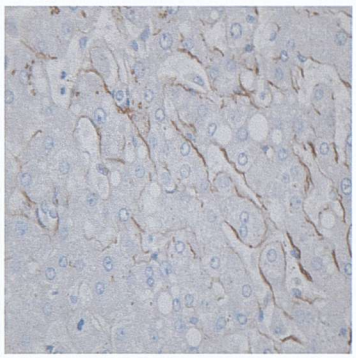


E3

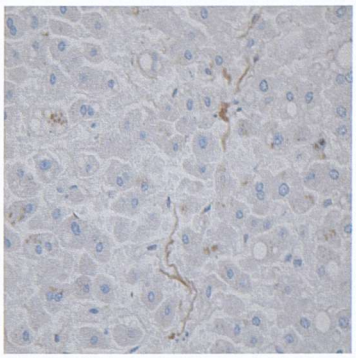
Met x40



Adj liver x40

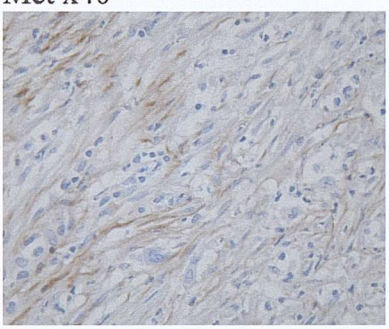


Distal liver x40

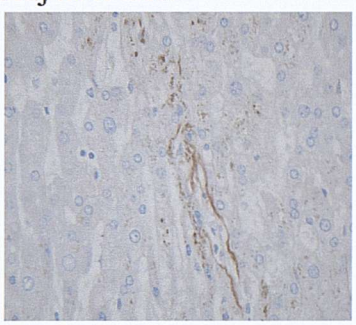


E4

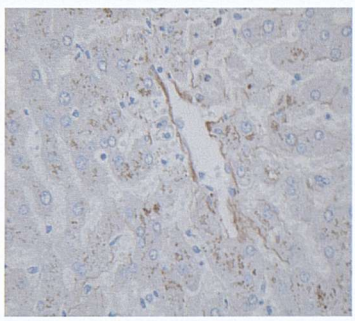
Met x40



Adj liver x40

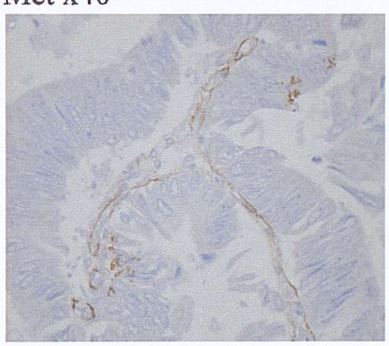


Distal liver x40

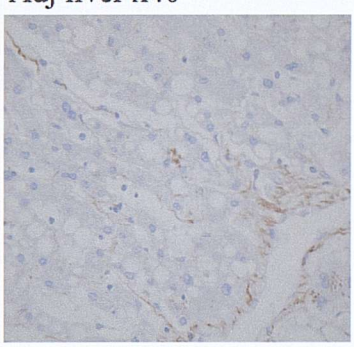


F2

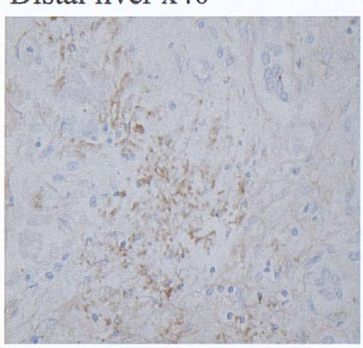
Met x40



Adj liver x40

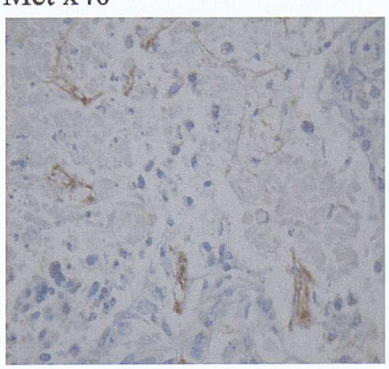


Distal liver x40

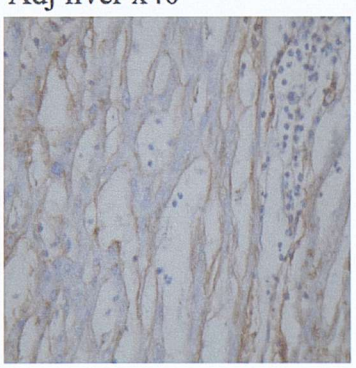


F5

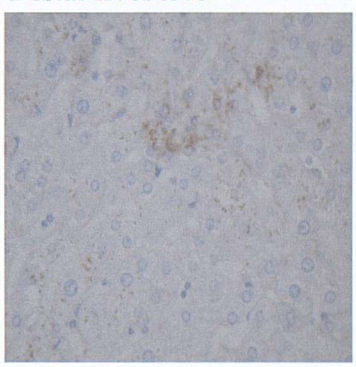
Met x40



Adj liver x40



Distal liver x40

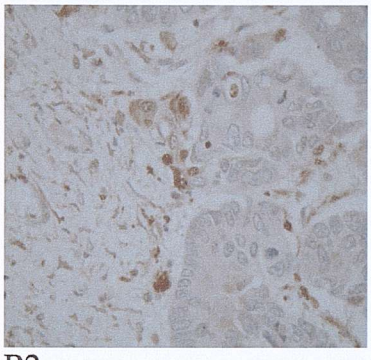


7.8d MMP 9 results

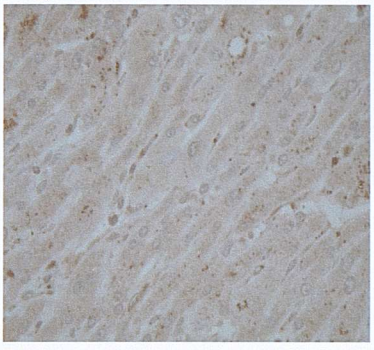
Figure 32: IHC results for MMP 9 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. MMP 9 expressed by macrophages in tumour stroma with little expression in adjacent or distal liver.

B1

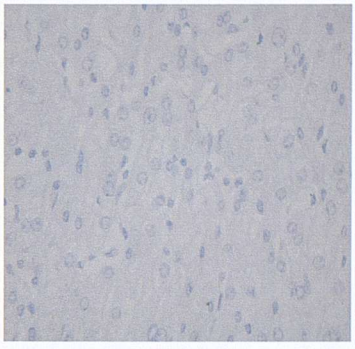
Met x40



Adj liver x40

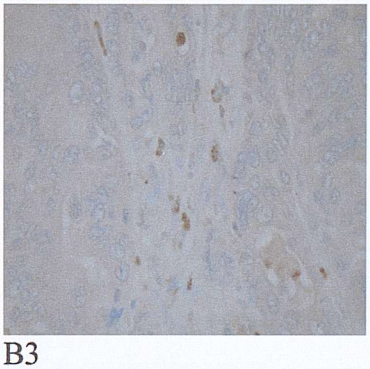


Distal liver x40

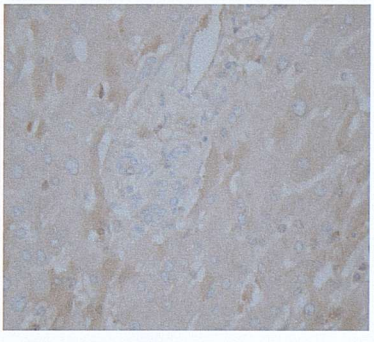


B2

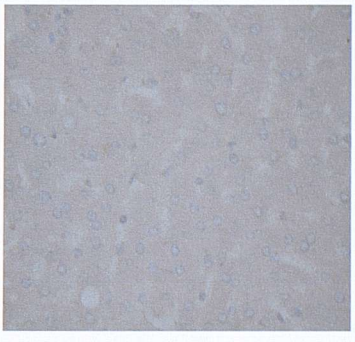
Met x40



Adj liver x40

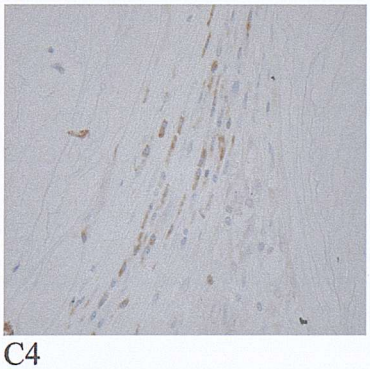


Distal liver x40

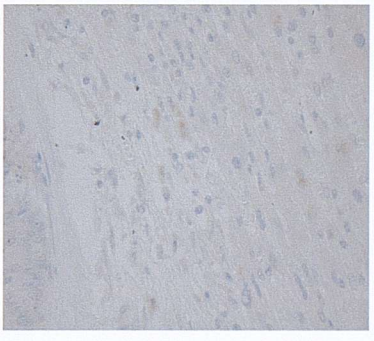


B3

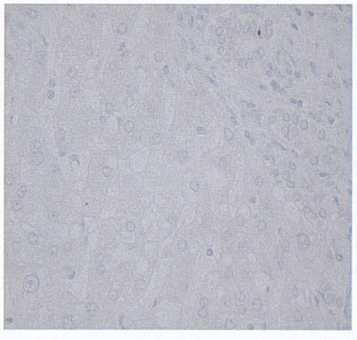
Met x40



Adj liver x40

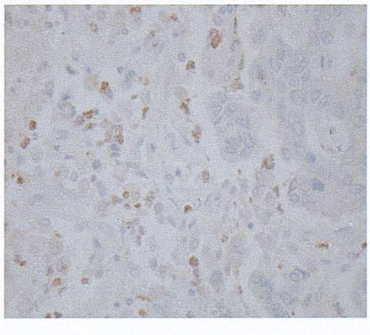


Distal liver x40

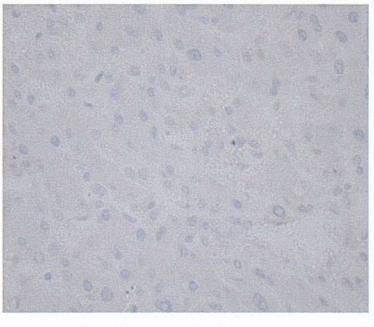


C4

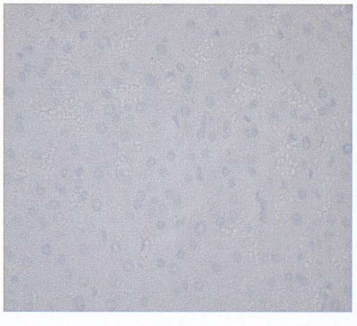
Met x40



Adj liver x40

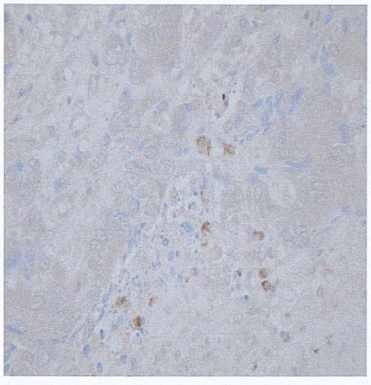


Distal liver x40

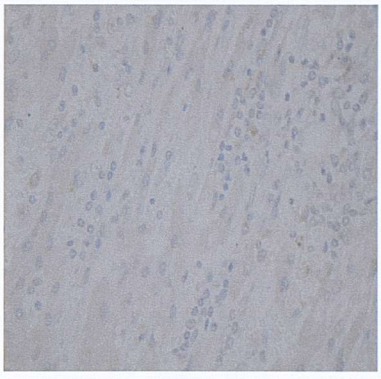


C5

Met x40



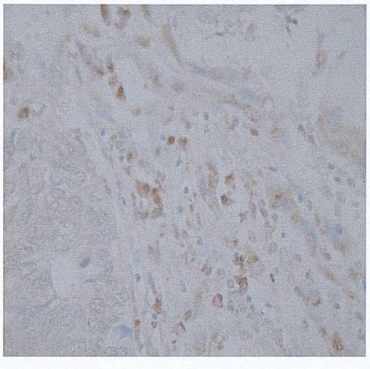
Adj liver x40



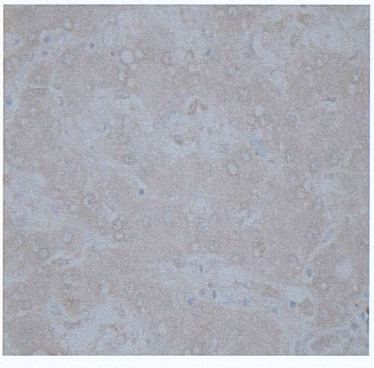
Distal liver x40

E2

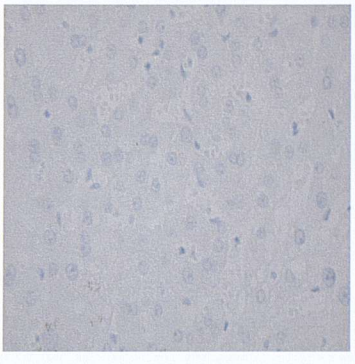
Met x40



Adj liver x40

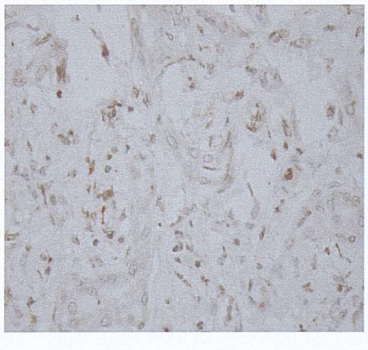


Distal liver x40

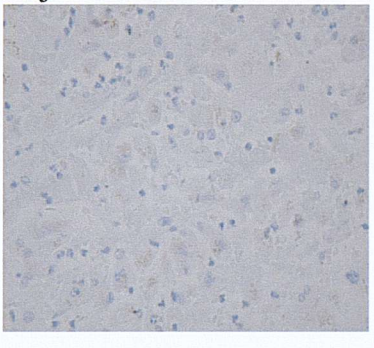


E3

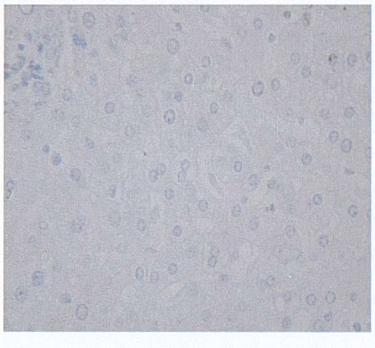
Met x40



Adj liver x40

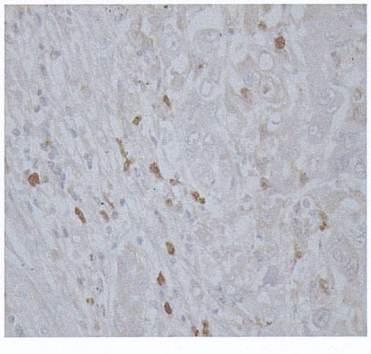


Distal liver x40

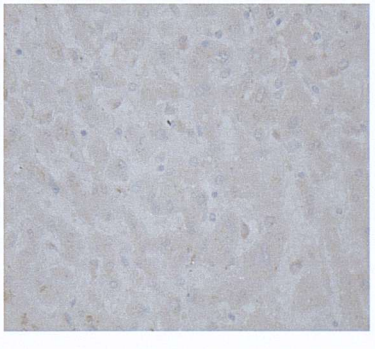


E4

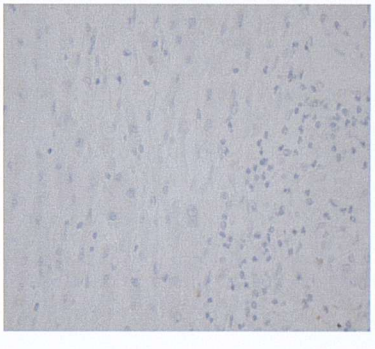
Met x40



Adj liver x40



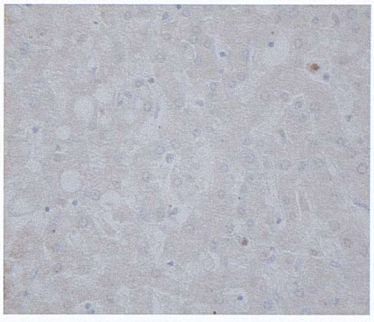
Distal liver x40



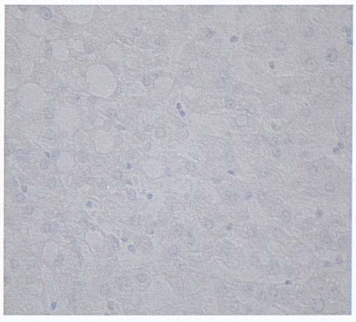
F2

Met x40

Adj liver x40

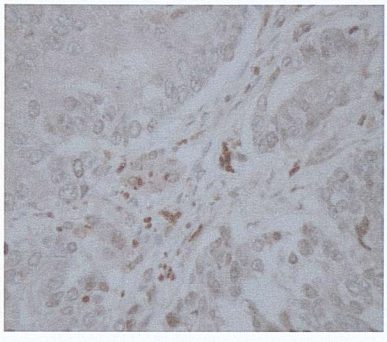


Distal liver x40



F5

Met x40



Adj liver x40

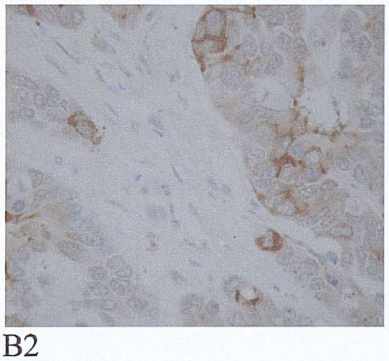
Distal liver x40

7.8e MMP 3 results

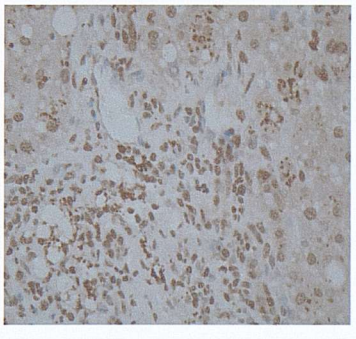
Figure 33: IHC results for MMP 3 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. MMP 3 expressed by both adenocarcinoma cells and tumour stromal macrophages as well as hepatocytes.

B1

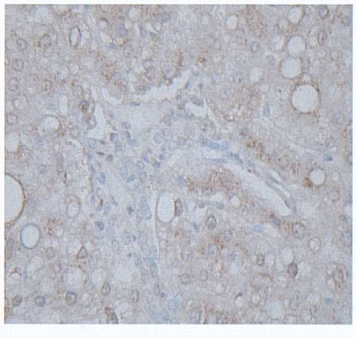
Met x40



Adj liver x40

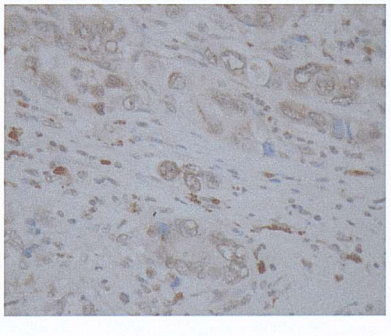


Distal liver x40

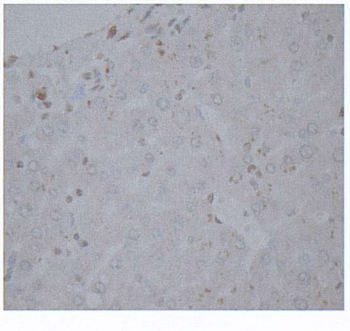


B2

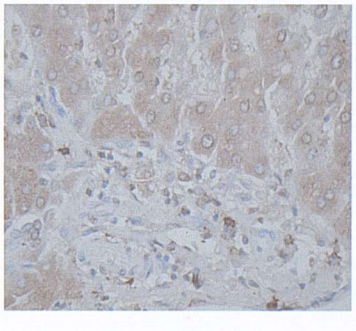
Met x40



Adj liver x40

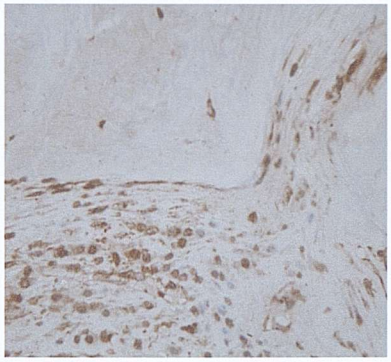


Distal liver x40

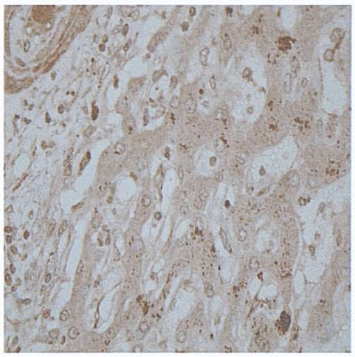


B3

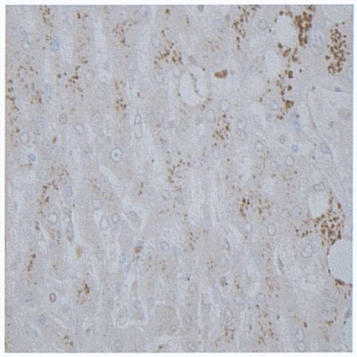
Met x40



Adj liver x40

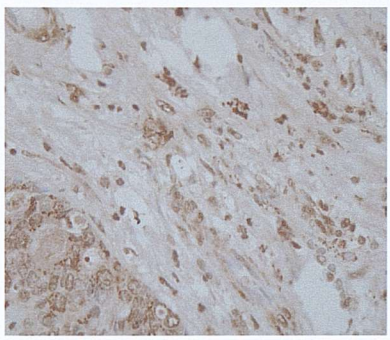


Distal liver x40

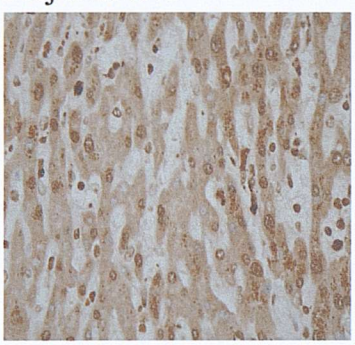


C4

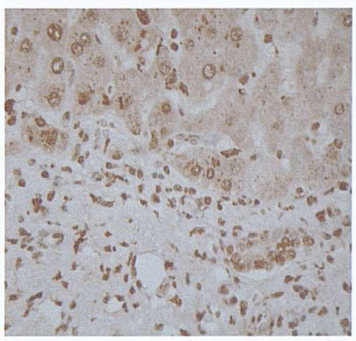
Met x40



Adj liver x40

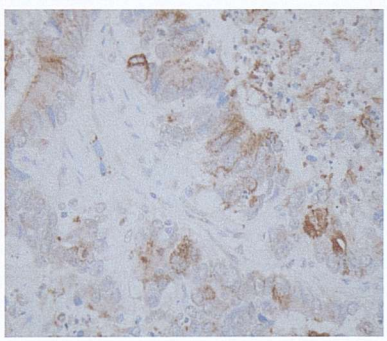


Distal liver x40

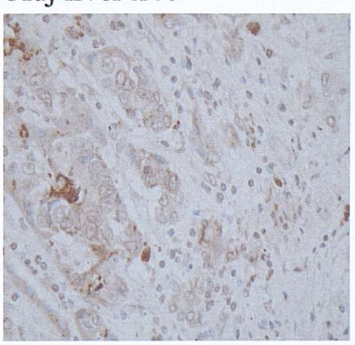


C5

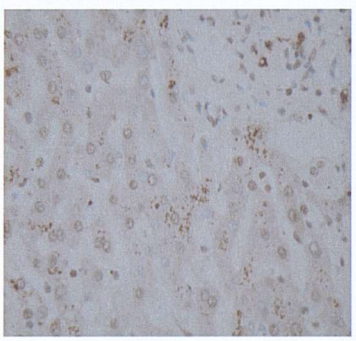
Met x40



Adj liver x40

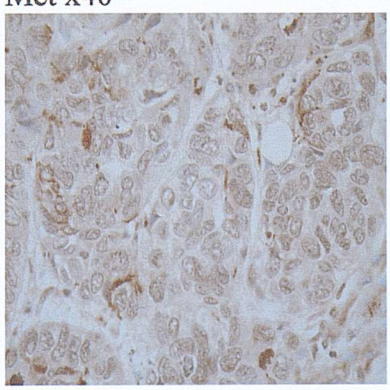


Distal liver x40

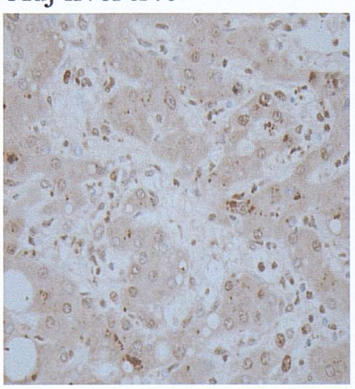


E2

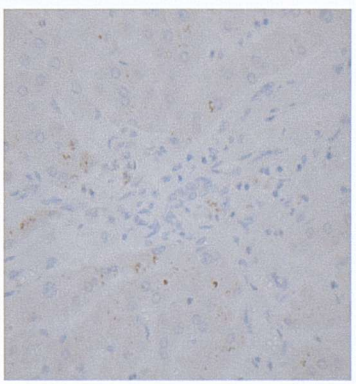
Met x40



Adj liver x40

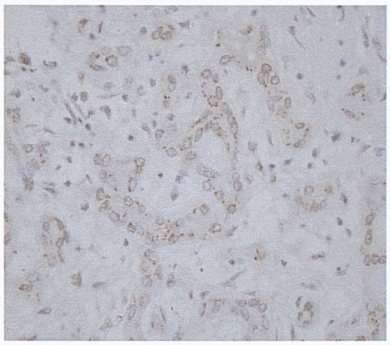


Distal liver x40

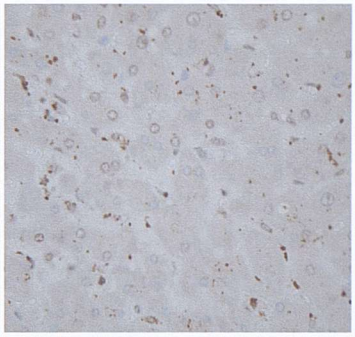


E3

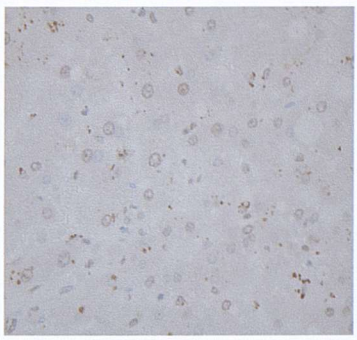
Met x40



Adj liver x40

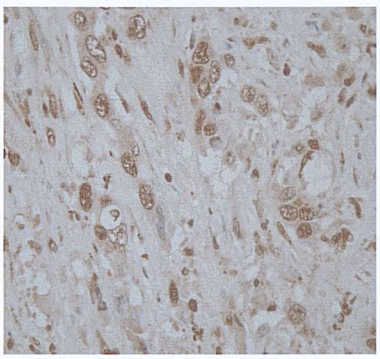


Distal liver x40

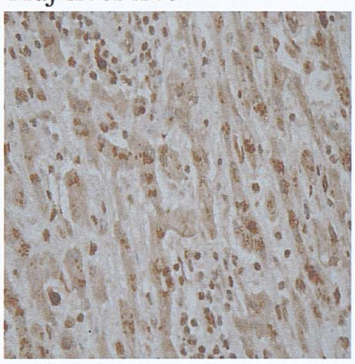


E4

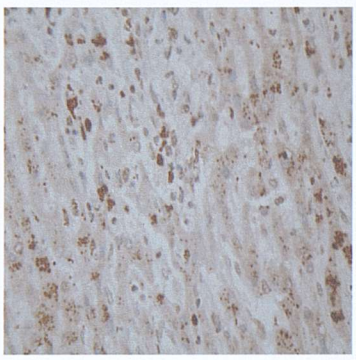
Met x40



Adj liver x40

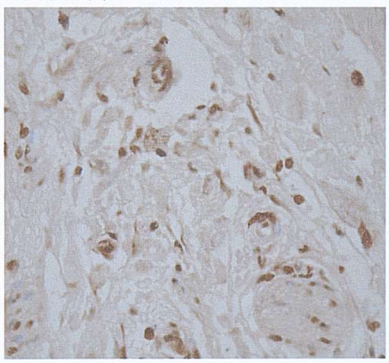


Distal liver x40

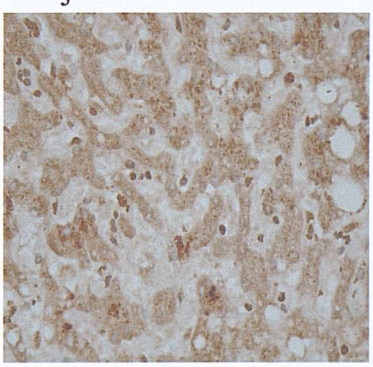


F2

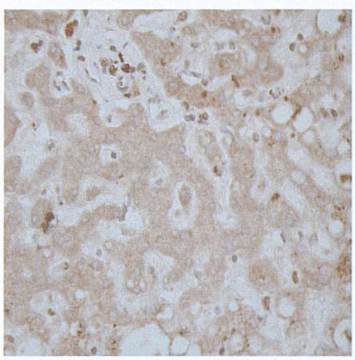
Met x40



Adj liver x40

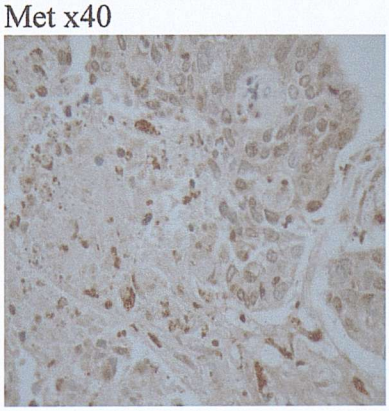


Distal liver x40

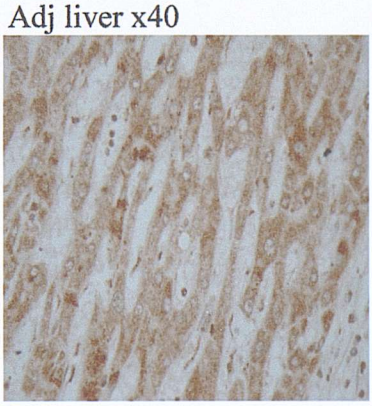


F5

Met x40



Adj liver x40



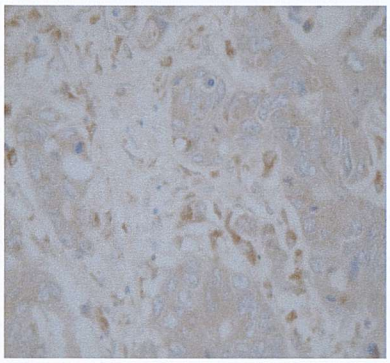
Distal liver x40

7.8f MMP 7 results

Figure 34: IHC results for MMP 7 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. MMP 7 was expressed by both adenocarcinoma cells and tumour stromal macrophages but only weakly in hepatocytes.

B1

Met x40

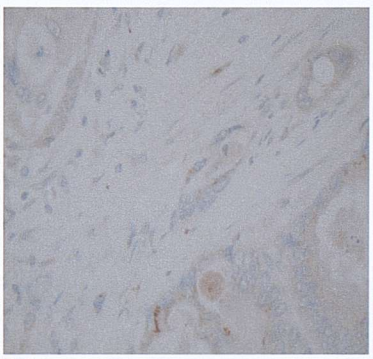


Adj liver x40

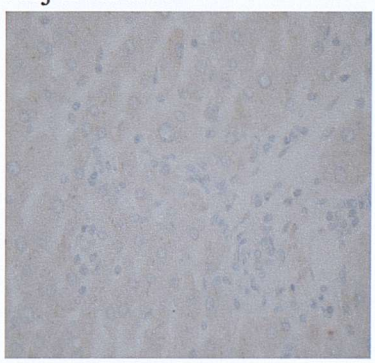
Distal liver x40

B2

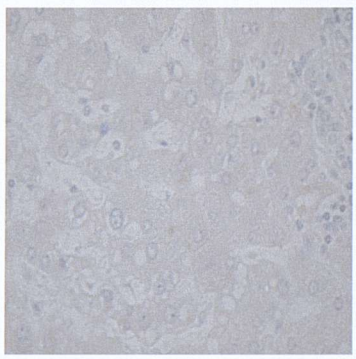
Met x40



Adj liver x40

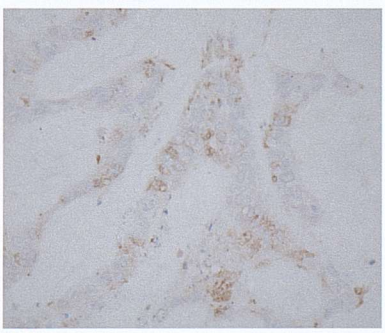


Distal liver x40

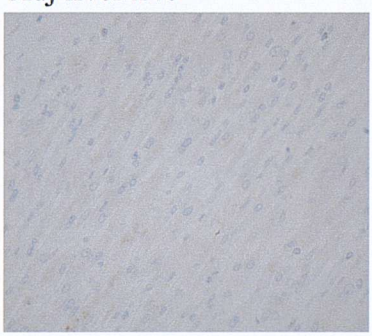


B3

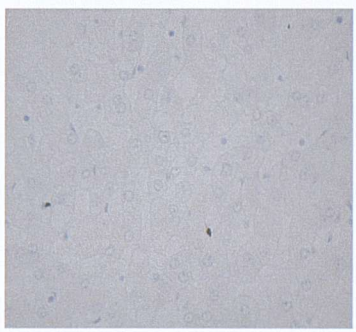
Met x40



Adj liver x40

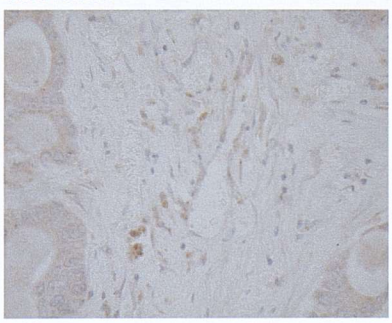


Distal liver x40

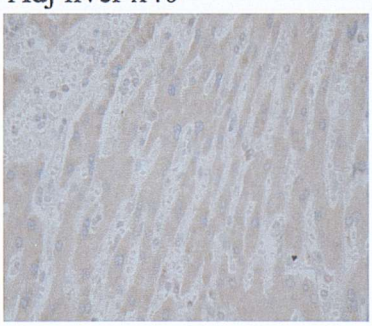


C4

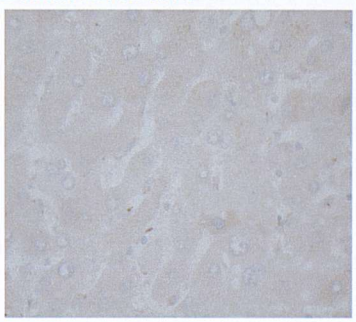
Met x40



Adj liver x40

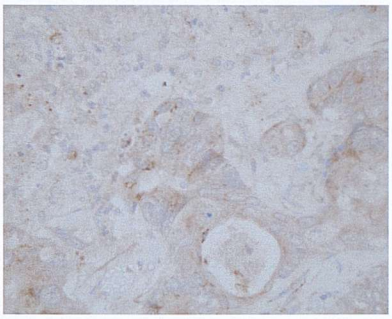


Distal liver x40



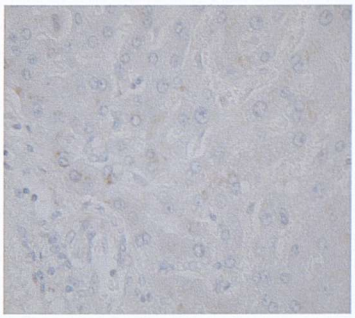
C5

Met x40



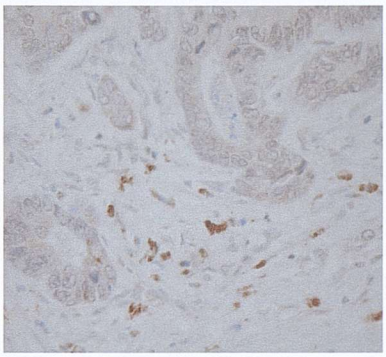
Adj liver x40

Distal liver x40

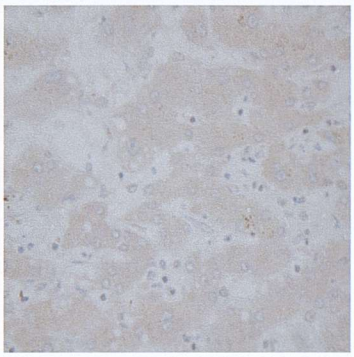


E2

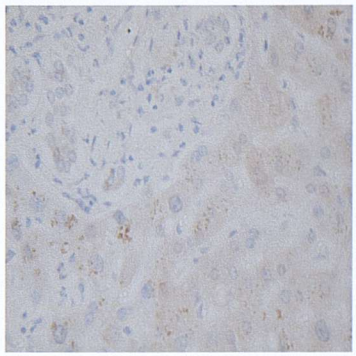
Met x40



Adj liver x40

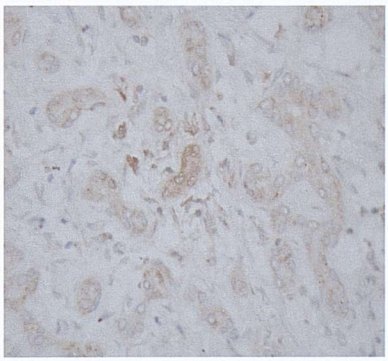


Distal liver x40

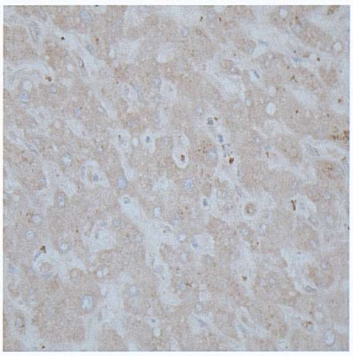


E3

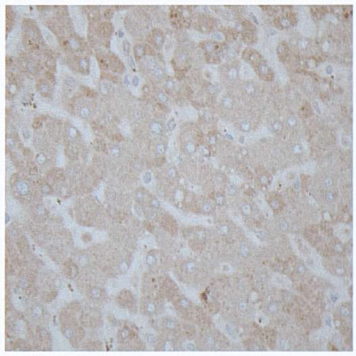
Met x40



Adj liver x40

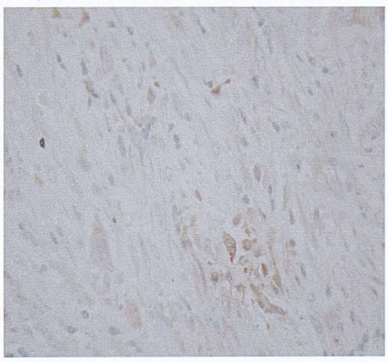


Distal liver x40

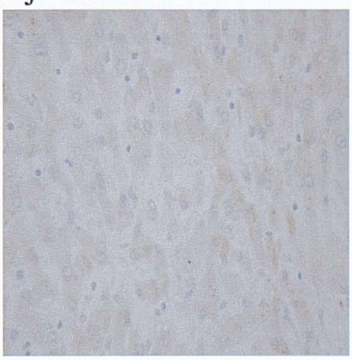


E4

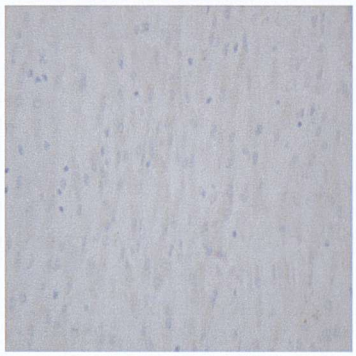
Met x40



Adj liver x40

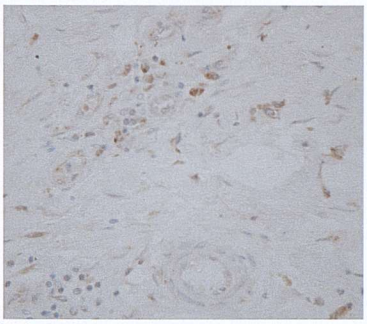


Distal liver x40

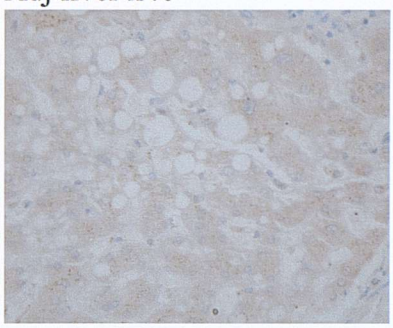


F2

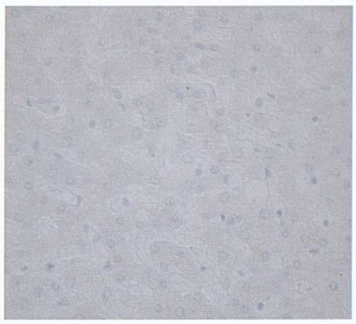
Met x40



Adj liver x40

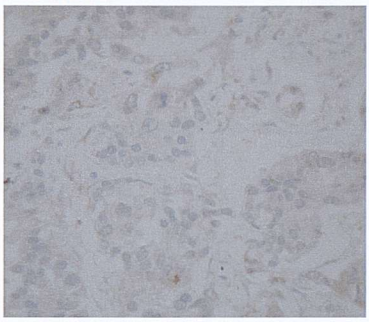


Distal liver x40

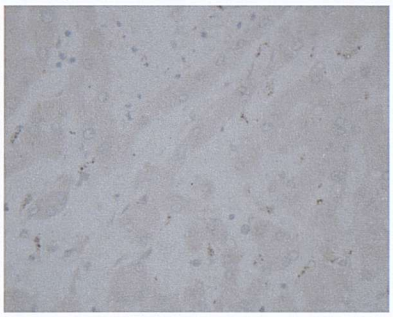


F5

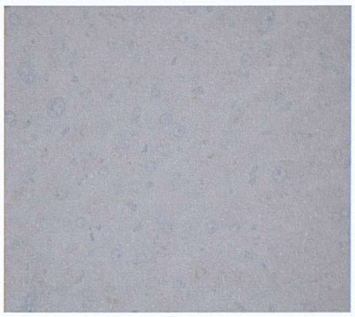
Met x40



Adj liver x40



Distal liver x40

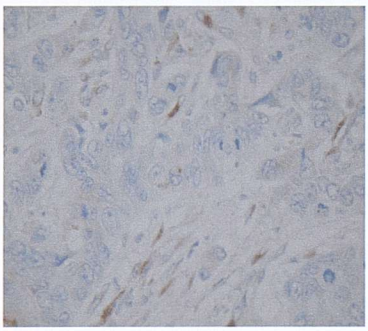


7.8g TIMP 1 results

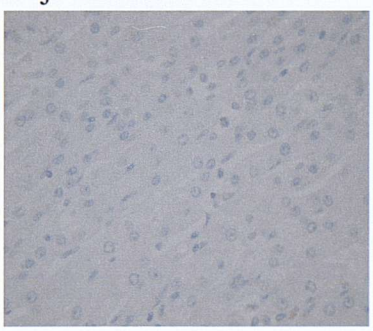
Figure 35: IHC results for TIMP 1 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. TIMP 1 expressed by macrophages in tumour stroma, absent in adjacent liver, expressed by hepatocytes in distal liver.

B1

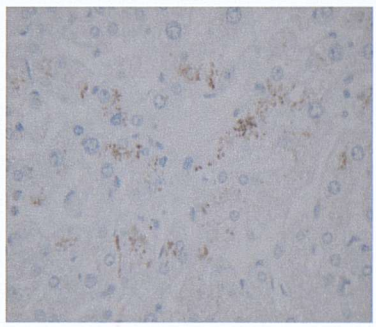
Met x40



Adj liver x40

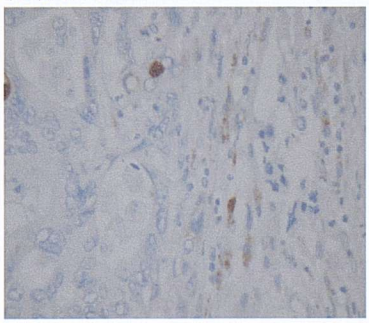


Distal liver x40

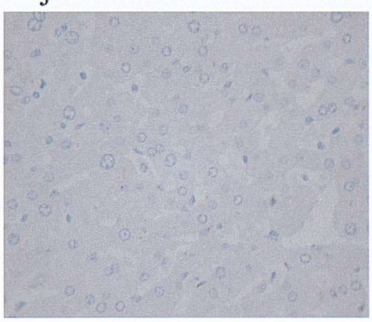


B2

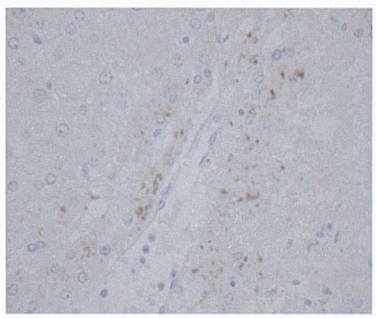
Met x40



Adj liver x40

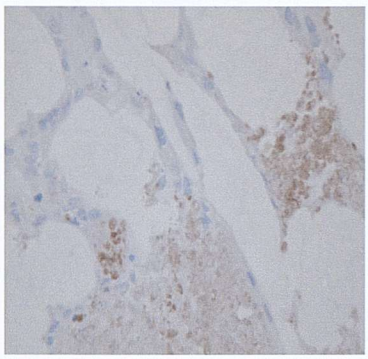


Distal liver x40

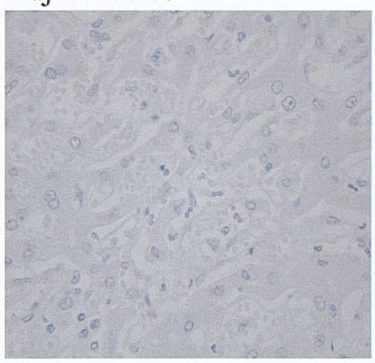


B3

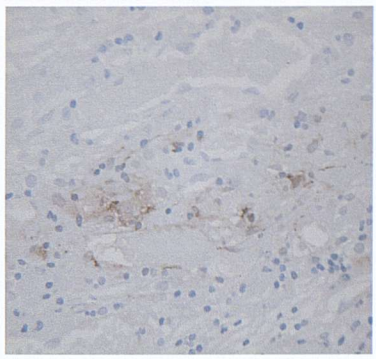
Met x40



Adj liver x40

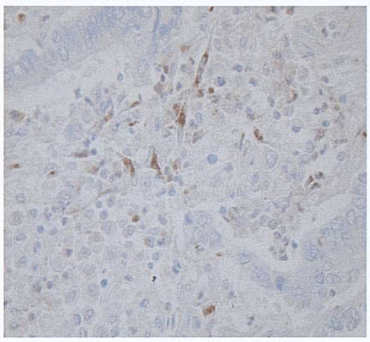


Distal liver x40

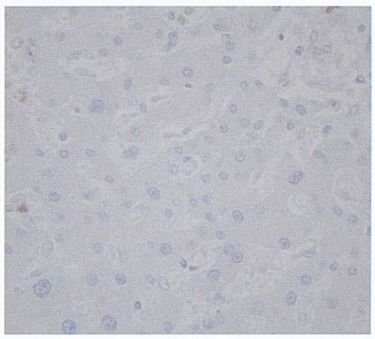


C4

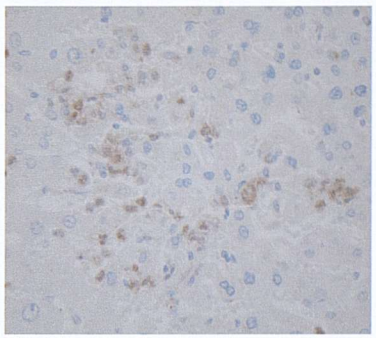
Met x40



Adj liver x40

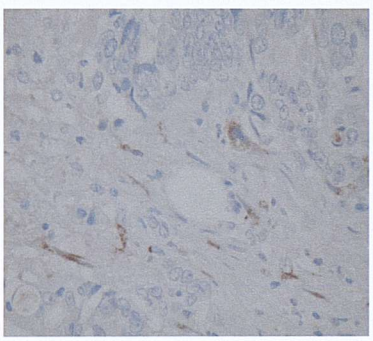


Distal liver x40

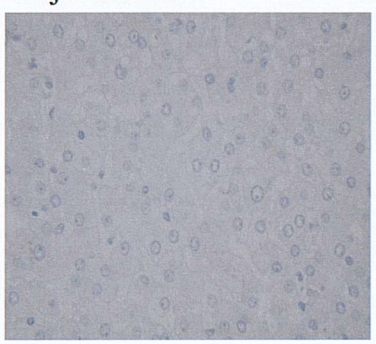


C5

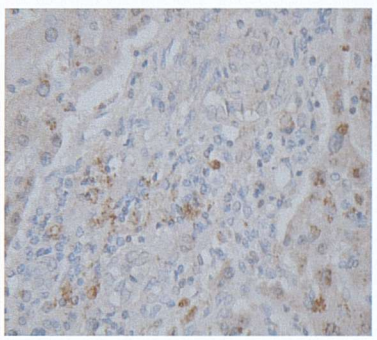
Met x40



Adj liver x40

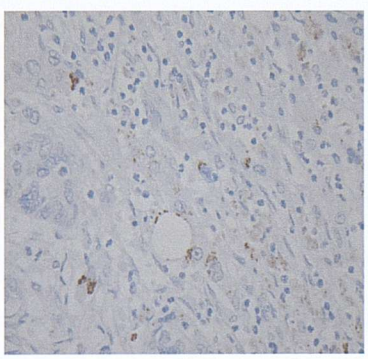


Distal liver x40

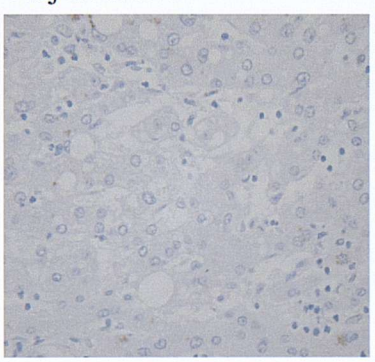


E2

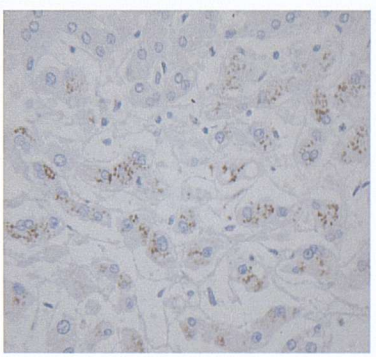
Met x40



Adj liver x40

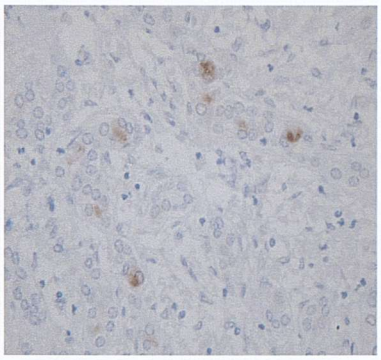


Distal liver x40

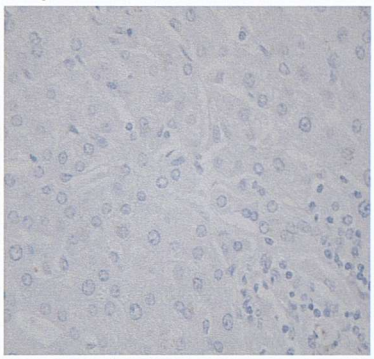


E3

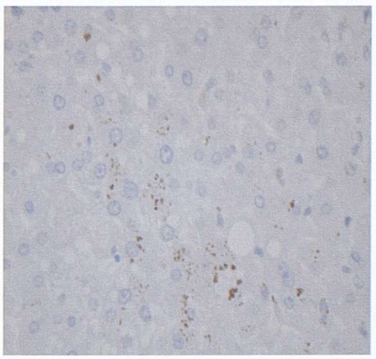
Met x40



Adj liver x40

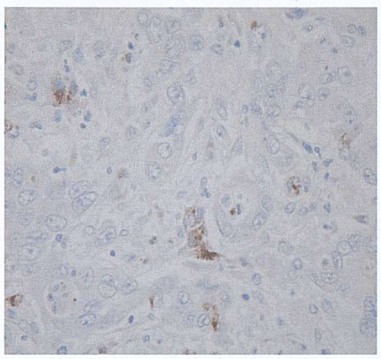


Distal liver x40

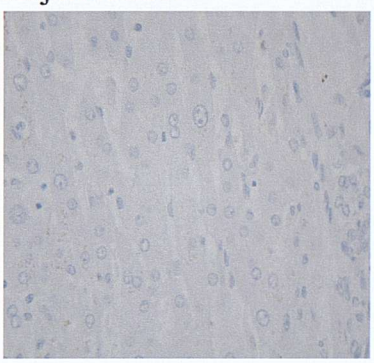


E4

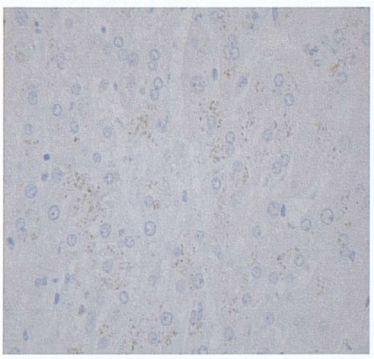
Met x40



Adj liver x40

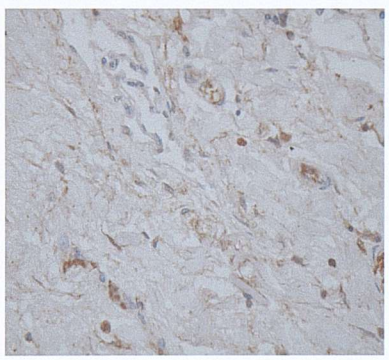


Distal liver x40

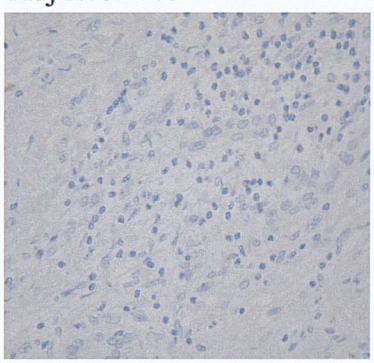


F2

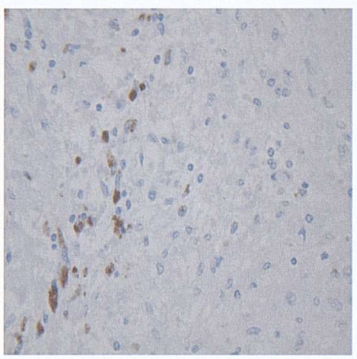
Met x40



Adj liver x40

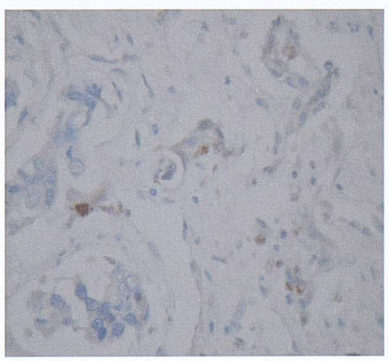


Distal liver x40

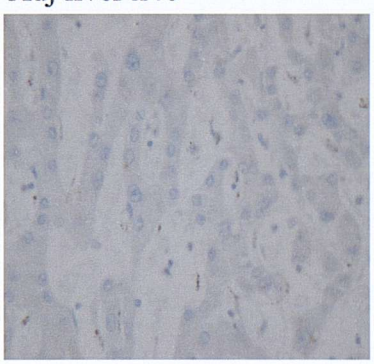


F5

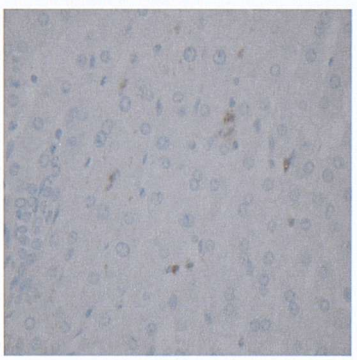
Met x40



Adj liver x40



Distal liver x40

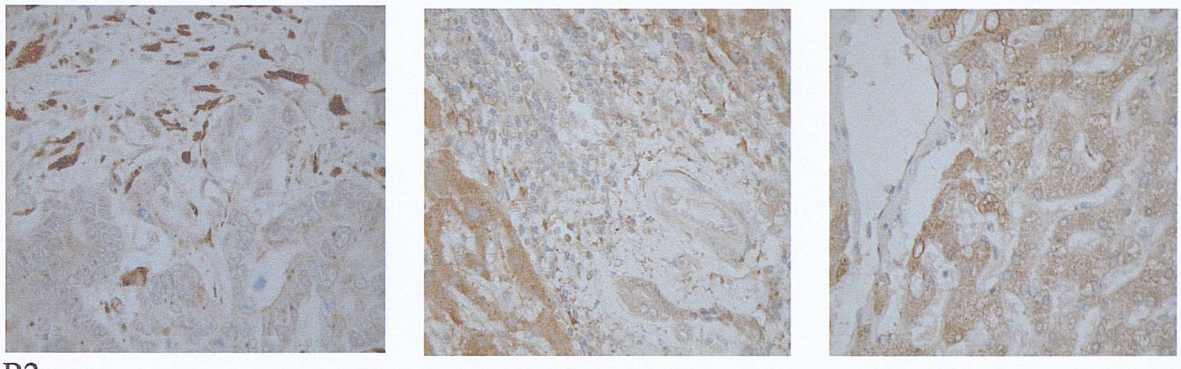


7.8h TIMP 2 results

Figure 36: IHC results for TIMP 2 on secondary tumour. The letter and number for each patient correspond to the batches of five in which the PCRs from these patients were analysed (see previous). Dark brown staining positive. TIMP 2 expressed by fibroblasts in tumour stroma, absent in adjacent liver, expressed by hepatocytes in distal liver.

B1

Met x40 | Adj liver x40 | Distal liver x40

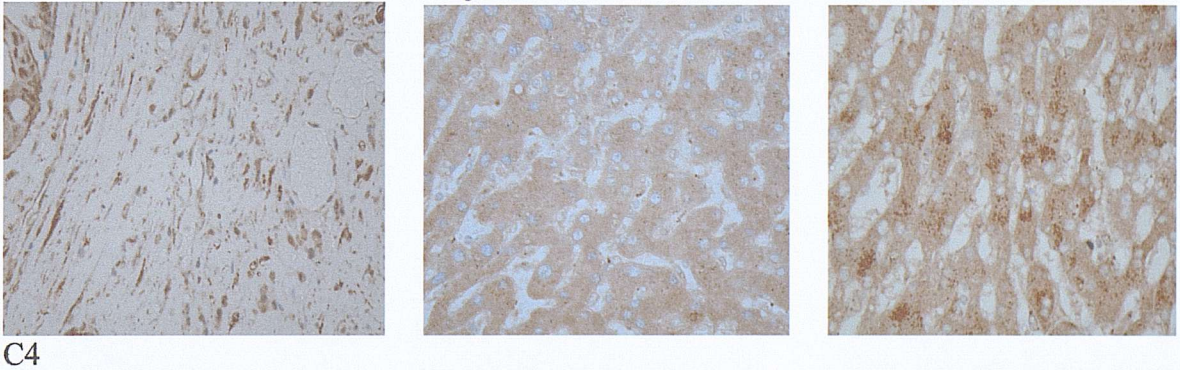


B2

Met x40 | Adj liver x40 | Distal liver x40

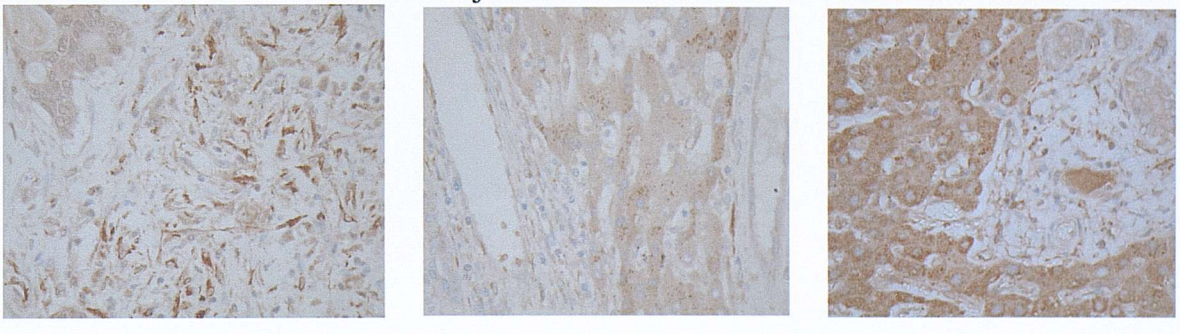
B3

Met x40 | Adj liver x40 | Distal liver x40



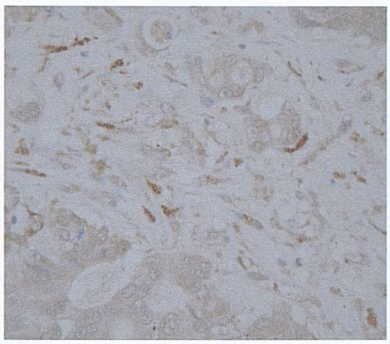
C4

Met x40 | Adj liver x40 | Distal liver x40

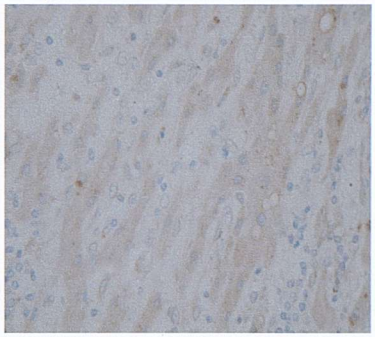


C5

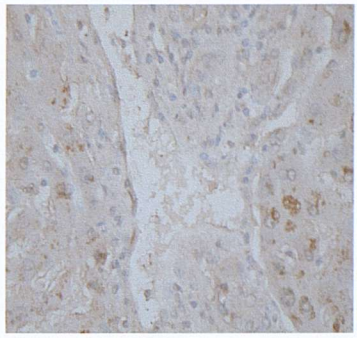
Met x40



Adj liver x40

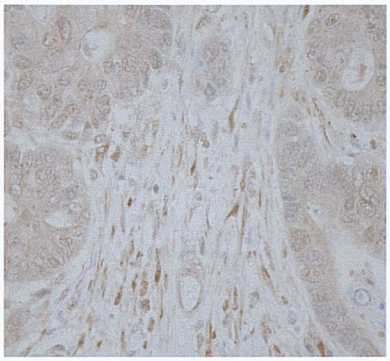


Distal liver x40

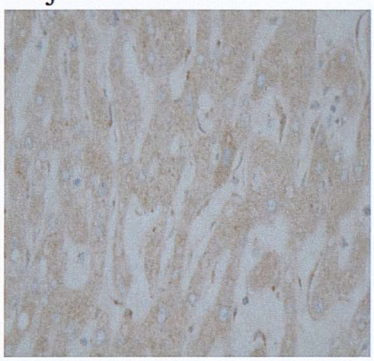


E2

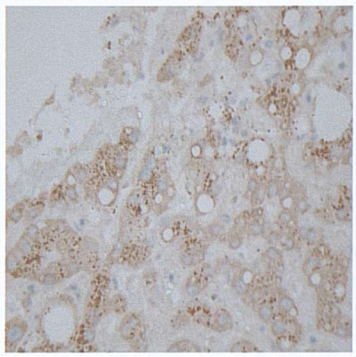
Met x40



Adj liver x40

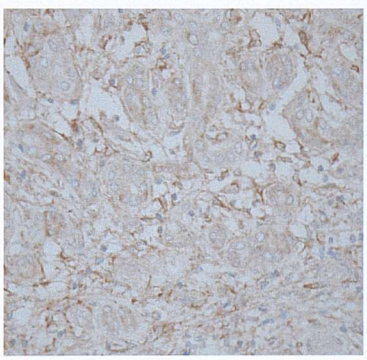


Distal liver x40

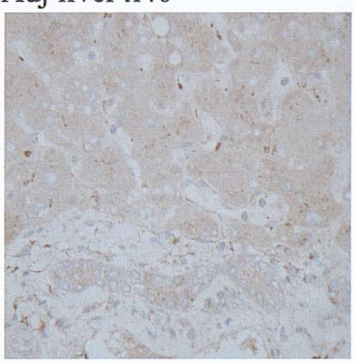


E3

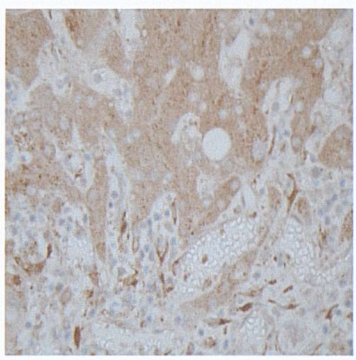
Met x40



Adj liver x40

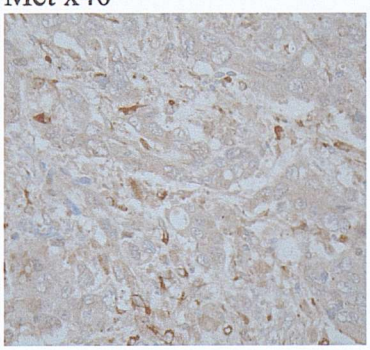


Distal liver x40

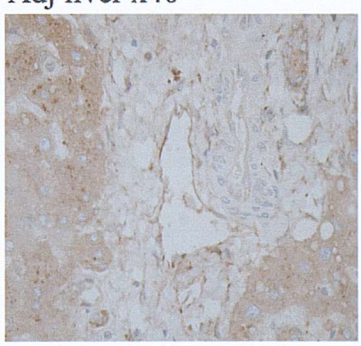


E4

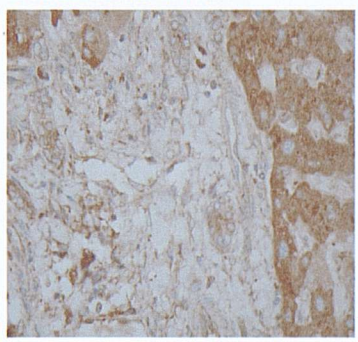
Met x40



Adj liver x40

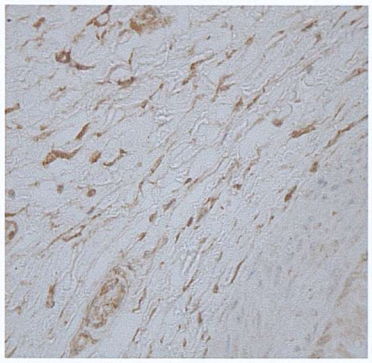


Distal liver x40

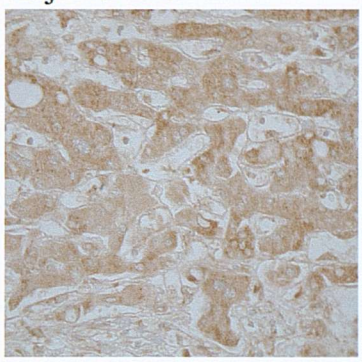


F2

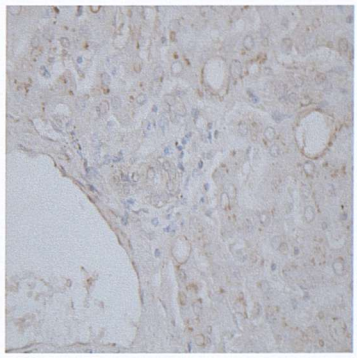
Met x40



Adj liver x40

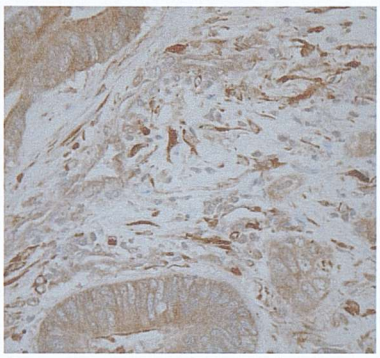


Distal liver x40

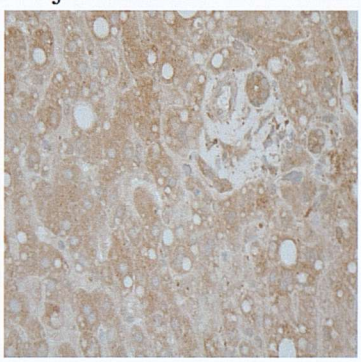


F5

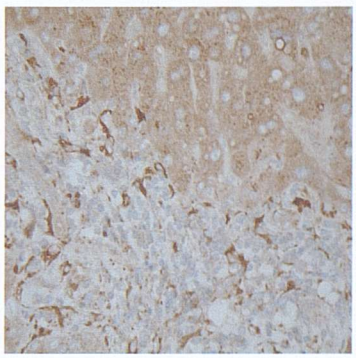
Met x40



Adj liver x40



Distal liver x40



7.9 Summary of IHC results for CRC liver metastases

MMPs 1, 8 and 2, 9 were expressed by the tumour stroma as opposed to adenocarcinoma cells. MMP 1 was predominately expressed by fibroblasts but was also present in macrophages and MMP 8 was expressed by fibroblasts. MMP2 was expressed by fibroblasts whereas MMP 9 was expressed by macrophages. MMPs 3 and 7 were expressed by both the adenocarcinoma cells and tumour stromal macrophages. TIMP-1 was expressed by macrophages in the tumour stroma, absent in the immediately adjacent liver tissue but expressed again in distal liver. TIMP-2 was expressed by fibroblasts in the tumour stroma, absent in the adjacent liver but expressed in the distal liver. The TIMP expression in liver appeared to be localised to the hepatocyte.

A comparison of the immunohistochemistry (protein) expression compared with

the PCR (mRNA) expression for the ten patients above is given in table 41 below (scoring 0 for no expression up to 3 for strong expression).

Table 41: Comparison of polymerase chain reaction (PCR) & Immunohistochemistry (IHC) results

	MMP 1		MMP 8		MMP 13		MMP 2		MMP 9		MMP 3		MMP 7		TIMP 1		TIMP 2	
	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot
B1 Met	3	3	1	1	0	-	1	1	1	1	1	2	1	2	2	2	1	2
Adj	1	1	1	1	0	-	1	1	1	1	1	1	1	1	1	0	1	1
Dist	0	0	1	1	0	-	1	1	0	0	1	1	1	1	2	2	1	2
B2 Met	3	3	1	1	0	-	1	1	2	2	2	1	1	1	1	1	2	2
Adj	1	2	1	1	0	-	1	1	1	1	0	1	1	1	0	0	0	0
Dist	2	2	1	1	0	-	1	1	1	1/2	1	1	2	1/2	2	1	2	1
B3 Met	2	2	1	1	0	-	1	1	2	2	2	1	2	1	2	2	2	2
Adj	1	1	1	1	0	-	1	1	1	1	1	1	1	1/2	1	1/2	0	0
Dist	1	1	1	1	0	-	1	1	1	1	1	1/2	1	0	1	1	2	2
C4 Met	3	2	1	1	0	-	1	1	2	2	1	1	1	1	2	2	2	2
Adj	0	1/2	1	1	0	-	1	0	1	0	1	1	1	1	1	1	1/2	1/2
Dist	1	1/2	1	1	0	-	1	0	1	0	1	1	1	0	1	2	2	2
C5 Met	2	2	1	1	0	-	2	1	3	2	1	2	2	1	2	2	2	2
Adj	1	1	1	1	0	-	2	1	1	1	1	2	0	1	0	0	1/2	1/2
Dist	2	1	1	1	0	-	2	1/2	1	1	1	2	2	1	2	2	2	2
E2 Met	3	3	1	2	0	-	1	1	0	2	1	1	1	2	1	2	2	2
Adj	1	1	1	1	0	-	1	1	1	0	1	1	0	0	1	1	0	0
Dist	1	1	1	1	0	-	1/2	1	0	0	1	1	1	1	1	2	1	2
E3 Met	2	2	0	1	0	-	1	1	1	1	1	1	1	1	1	1	2	2
Adj	0	1/2	1	1	0	-	1	1	0	0	1	1	1	1	1	0	1/2	1/2
Dist	0	1/2	0	1	0	-	1	1	0	0	1	1	1	1	1	1	2	2
E4 Met	2	2	1	1	0	-	1	1	1	1	1	1	0	1	2	2	2	2
Adj	1	1	1	1	0	-	1	1	0	0	1	1	1	1	1	0	1/2	1/2
Dist	0	0	0	1	0	-	1	1	0	0	1	1	1	1	2	1	2	2
F2 Met	1	2	1	2	0	-	2	1	1	1	1	1	1	2	1	1	2	2
Adj	2	2	2	1	0	-	2	1	0	0	1	1	2	1	0	0	0	0
Dist	1	1	1	1	0	-	2	1	0	0	1	1	0	0	1	1	2	1
F5 Met	3	2	2	1	0	-	1	1	3	2	2	2	2	1	3	1	1	2
Adj	0	0	1	1	0	-	1	1	1	1	1	1	0	0	1	0	1	1/2
Dist	0	0	1	1	0	-	1	1	1	0	1	1	1	0	1	1	1	2

Overall there is good correlation between the mRNA and the protein work.

7.9a Collagenases

For MMP 1 only 3 of the thirty specimens (10 patients with digitally captured imaging) compared showed disparity with no expression on the mRNA but weak (1/2)

expression on the immunohistochemistry and there was similar correlation with MMP 8. MMP 13 could not be compared due to the unavailability of a working antibody.

7.9b Gelatinases

MMP 2 showed good correlation with only 2 specimens showing mRNA expression but having no corresponding protein expression on immunohistochemistry. For MMP 9 1 specimen had no mRNA expression but subsequent protein expression, and 4 specimens with mRNA expression but no protein expression identified.

7.9c Stromelysins

For MMP 3 only 1 specimen showed no mRNA expression but weak protein expression and there were 2 such specimens for MMP 7. There were also 3 specimens that had MMP 7 mRNA expression but no protein expression demonstrated.

7.9d Tissue inhibitors of metalloproteinases

TIMP 1 showed good correlation between PCR and IHC. Interestingly 4 patients with weak expression of mRNA in the adjacent liver had no protein expression for this adjacent liver, further strengthening the observation of a down regulation of TIMP 1 in the adjacent liver surrounding the metastasis.

For TIMP 2 the correlation is again good with no protein expression if there was no mRNA expression. In five of the 30 specimens (10 patients) the protein expression is stronger than the mRNA, and in three weaker than the mRNA but even in these specimens the pattern of expression remained the same.

7.10 Benign liver lesions

7.10a PCR Results

The polyacrylamide gels are arranged in the order:

Hae III marker	
Negative control	
Benign 1	Adenoma
	Adj liver
	Normal liver
Benign 2	Adenoma
	Adj liver
	Normal liver
Benign 3	Cyst
	Normal liver
Positive control	

Except for MMP 2 which again has a β actin run with each specimen and has the orientation:

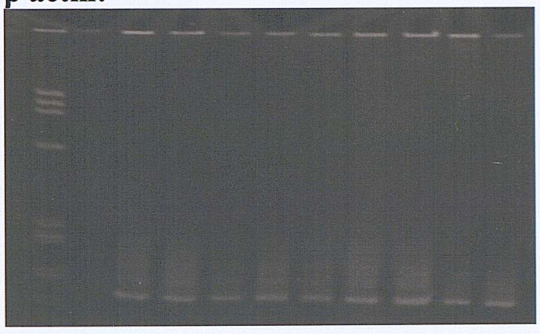
Hae III marker	
Benign 3	Cyst
	Adenoma
Benign 2	Adenoma
	Adj liver
	Normal liver
Empty lane	
Benign 1	Adenoma
	Adj liver
	Normal liver
Benign 1	Adenoma
	Adj liver
	Normal liver
Hae III marker	
2 negative lanes	
β actin positive	
MMP 2 positive	

Except for TIMP 2 which has the same arrangement of Hae III marker, negative control, benign 1, 2 and 3 three empty lanes and then the positive control.

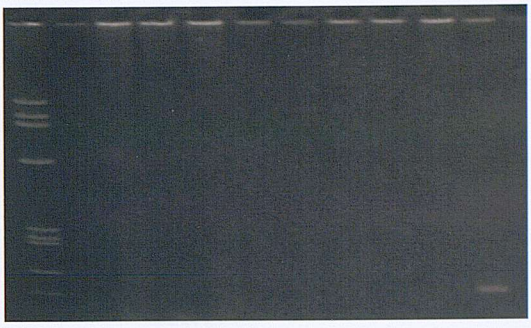
Figure 37: Polyacrylamide gels for benign liver lesions PCRs

Figure 37a: β actin and Collagenases. No expression of MMP 1 or 13. MMP 8 expressed by all 3 benign lesions.

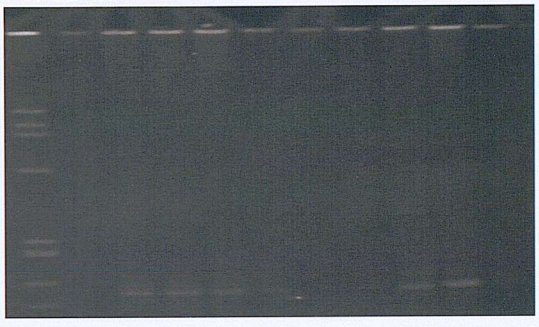
β actin:



MMP 1:



MMP 8:



MMP 13:

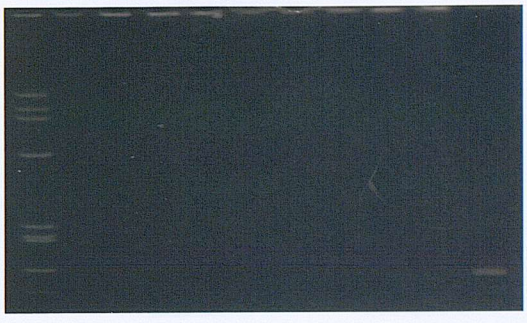
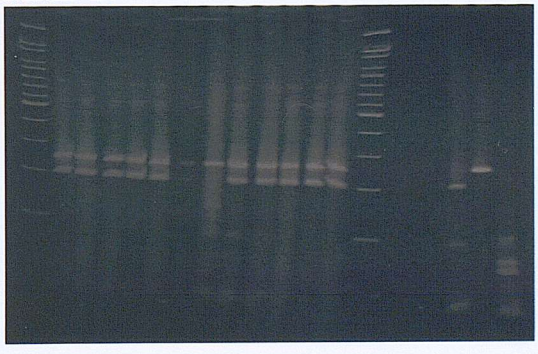


Figure 37b: Gelatinases. MMP 2 expressed by all 3 benign lesions, whereas MMP 9 only by one.

MMP 2:



MMP 9:

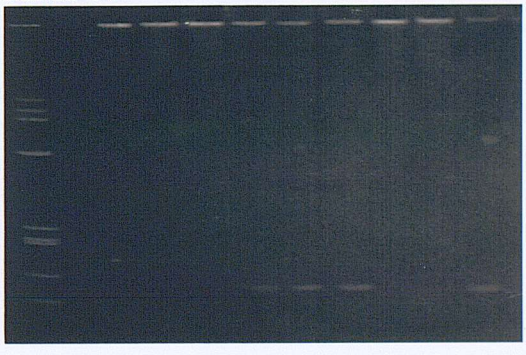
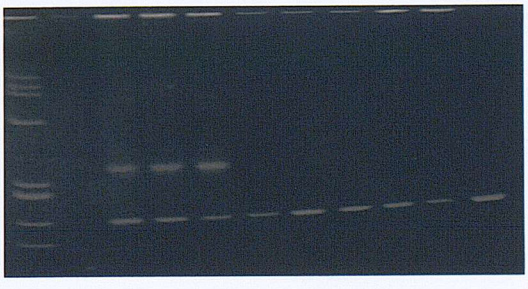


Figure 37c: Stromelysins. MMP 3 expressed by all 3 benign lesions. MMP 7 expressed at low levels by 2 of the benign lesions.

MMP 3:



MMP 7:

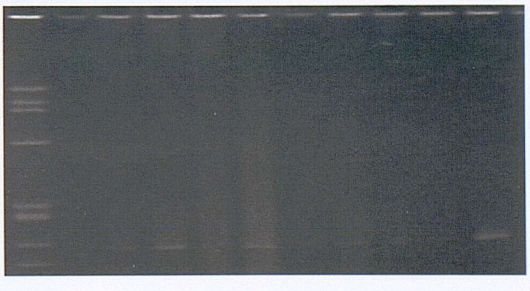
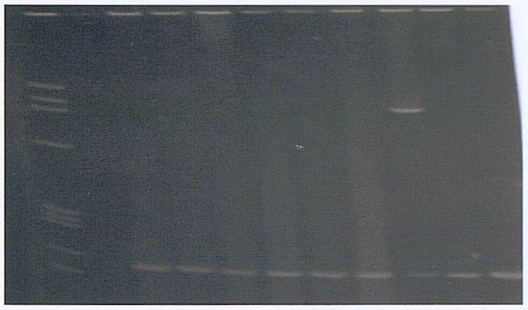


Figure 37d: Tissue inhibitors of metalloproteinase. Both TIMP 1and 2 were expressed at equal levels by metastasis, adjacent liver and distal liver of each benign lesion.

TIMP 1:



TIMP 2:

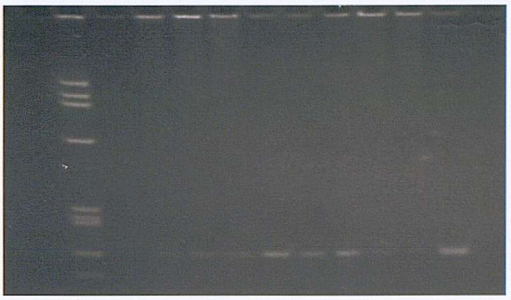


Table 42: Benign PCR Results. Median values (of three) with ranges

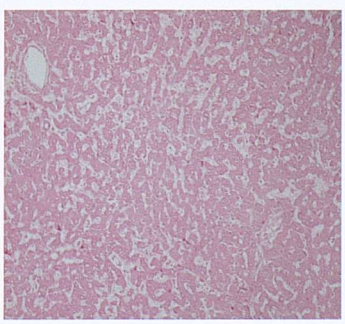
	Benign (B)	Adjacent liver (A)	Distal liver (D)
MMP 1	**0**	**0**	**0**
MMP 8	**0.39** (0.27-0.65)	**0.32** (0-0.63)	**0.71** (0.25-0.83)
MMP 13	**0**	**0**	**0**
MMP 2	**0.6** (0.5-0.7)	**0.5** (0.4-0.6)	**0.6** (0.45-0.75)
MMP 9	**0** (0-0.51)	**0.2** (0-0.39)	**0** (0-0.27)
MMP 3	**0.53** (0.11-0.62)	**0.44** (0.35-0.53)	**0.42** (0.22-0.7)
MMP 7	**0** (0-0.55)	**0.29** (0-0.58)	**0**
TIMP 1	**1.48** (0.45-1.85)	**1.57** (1.45-1.69)	**1.31** (0.84-1.4)
TIMP 2	**0.18** (0.08-0.49)	**0.42** (0.42-0.42)	**0.29** (0.17-0.32)

7.10b Immunohistochemistry results

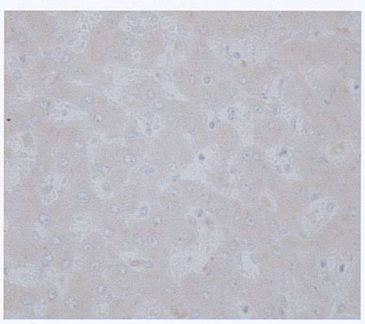
Figure 38: Immunohistochemistry for benign liver lesions

Figure 38a: Benign liver lesion 1 (adjacent liver) Dark brown staining positive

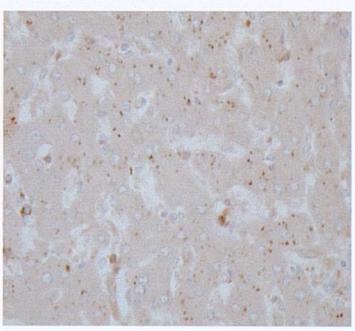
H&E x10



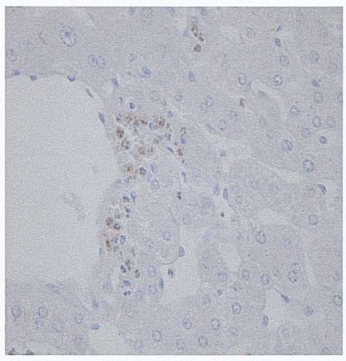
MMP 1 x40



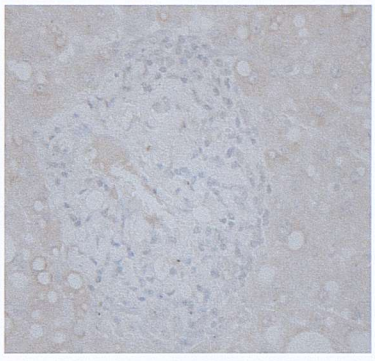
MMP 8 x40



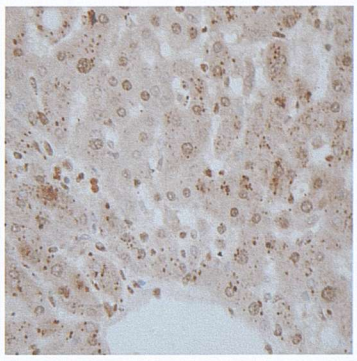
MMP 2 x40



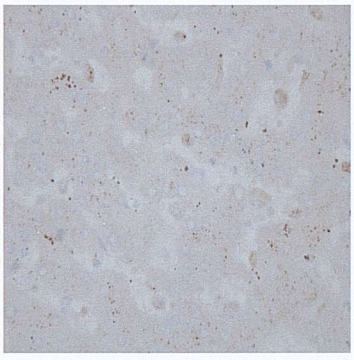
MMP 9 x40



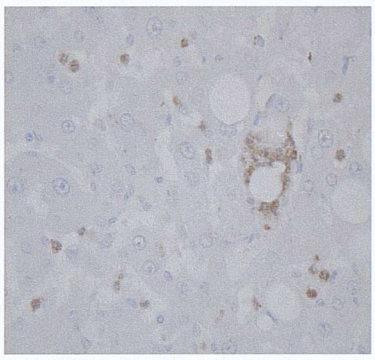
MMP 3 x40



MMP 7 x40



TIMP 1 x40



TIMP 2 x40

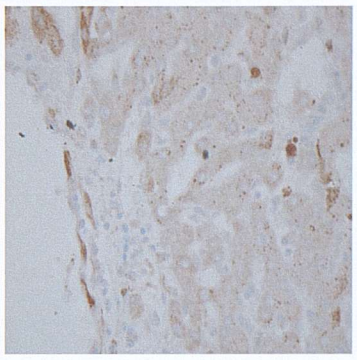
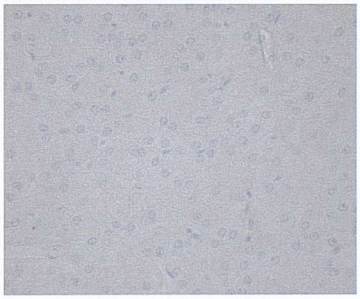


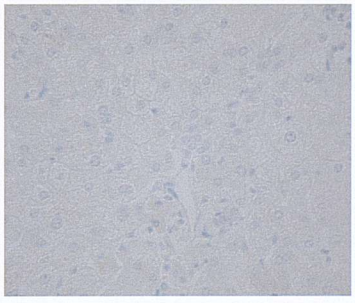
Figure 38b: Benign liver lesion 2 Dark brown staining positive

MMP 1

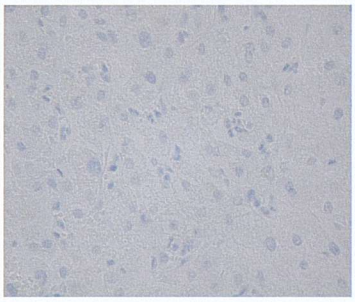
Benign lesion x40



Adjacent liver x40

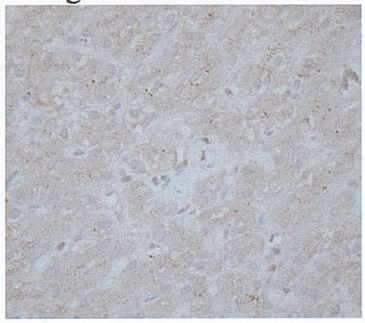


Distal liver x40

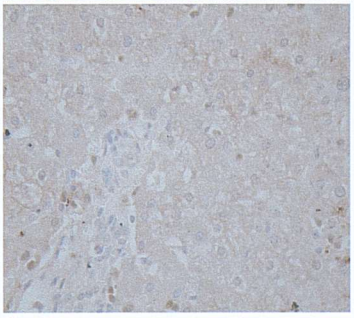


MMP 8

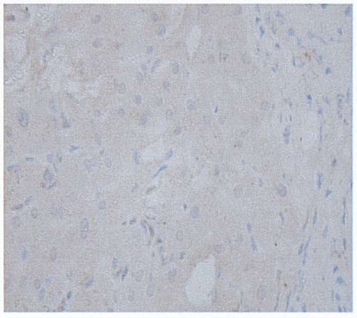
Benign lesion x40



Adjacent liver x40

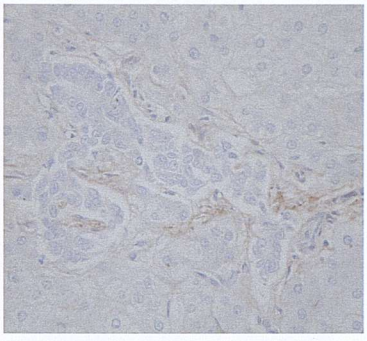


Distal liver x40

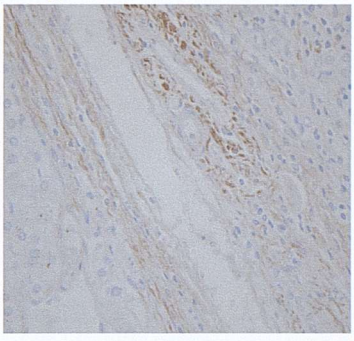


MMP 2

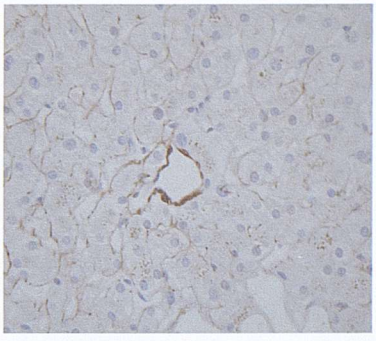
Benign lesion x40



Adjacent liver x40

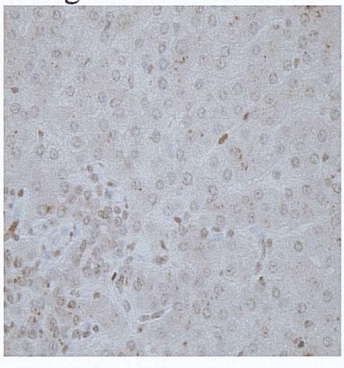


Distal liver x40

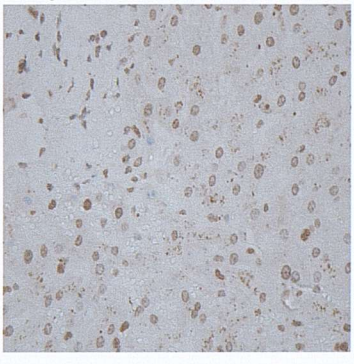


MMP 9

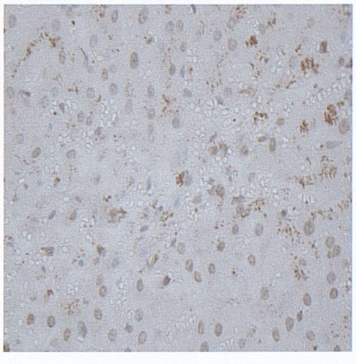
Benign lesion x40



Adjacent liver x40

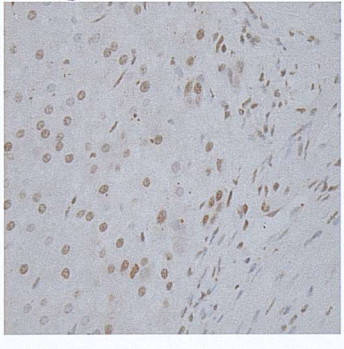


Distal liver x40

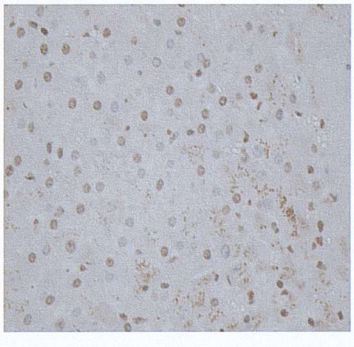


MMP 3

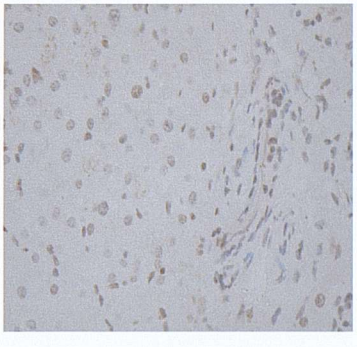
Benign lesion x40



Adjacent liver x40

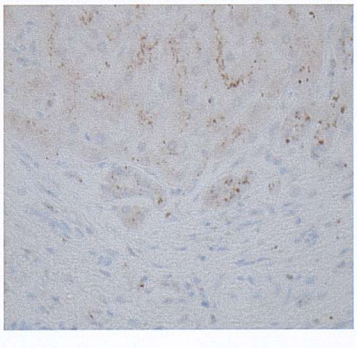


Distal liver x40

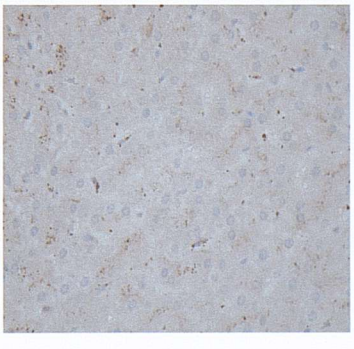


MMP 7

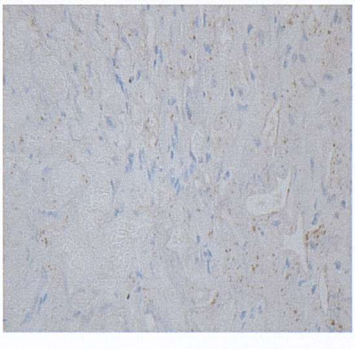
Benign lesion x40



Adjacent liver x40

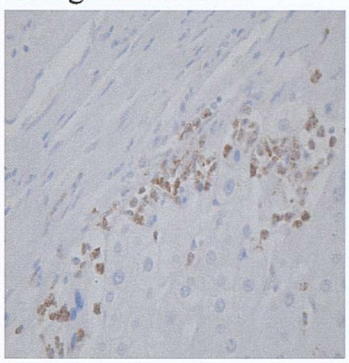


Distal liver x40

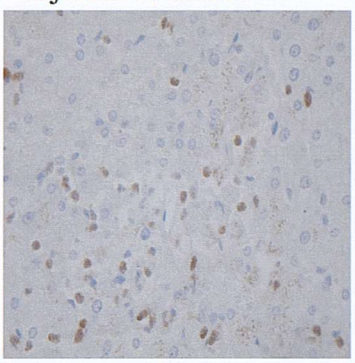


TIMP 1

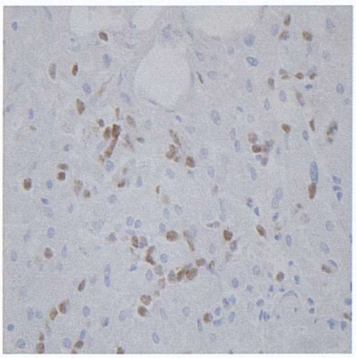
Benign lesion x40



Adjacent liver x40

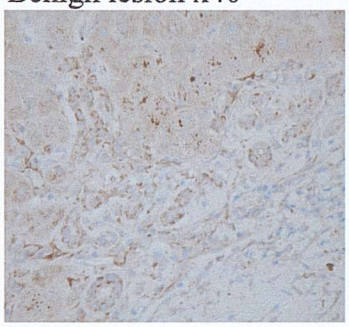


Distal liver x40

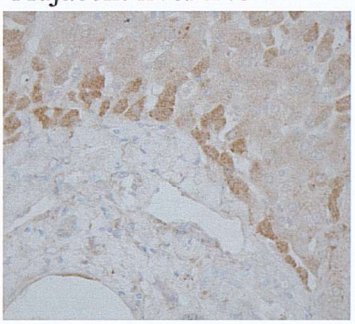


TIMP 2

Benign lesion x40



Adjacent liver x40



Distal liver x40

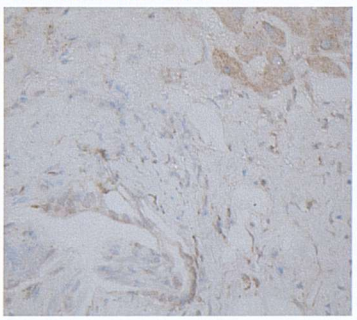
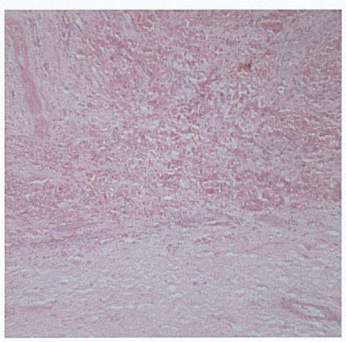
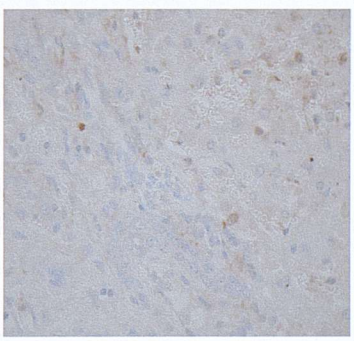


Figure 38c: Benign liver lesion 3 Dark brown staining positive

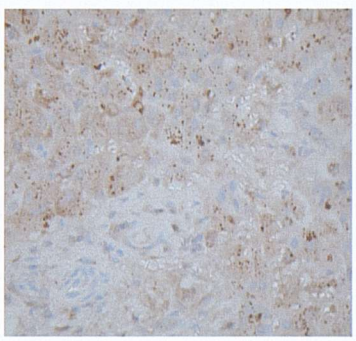
H&E x10



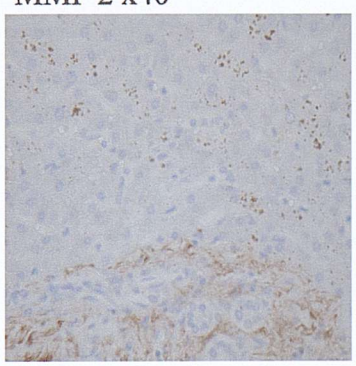
MMP 1 x40



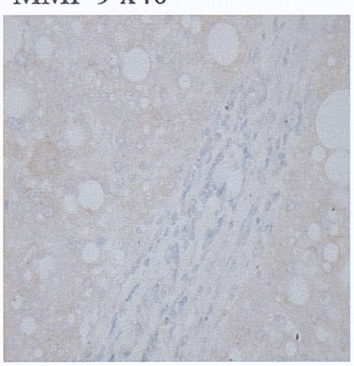
MMP 8 x40



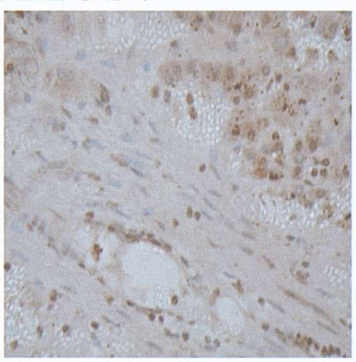
MMP 2 x40



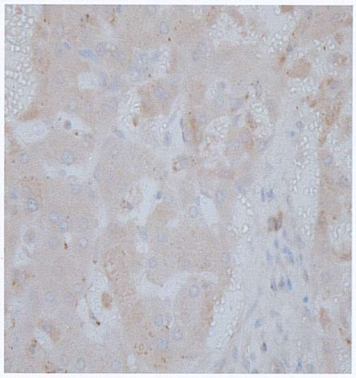
MMP 9 x40



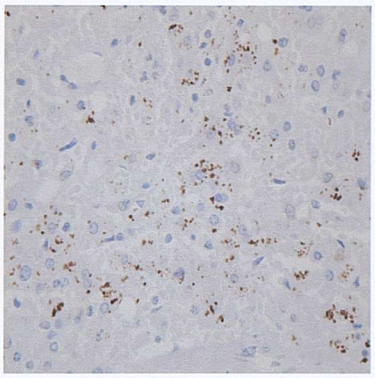
MMP 3 x40



MMP 7 x40



TIMP 1 x40



TIMP 2 x40

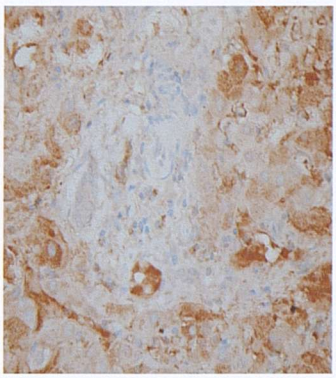


Table 43: Comparison between PCR and immunohistochemistry for benign lesions
(0 for no expression to 3 for strong expression; - no tissue/antibody available)

	MMP 1		MMP 8		MMP 13		MMP 2		MMP 9		MMP 3		MMP 7		TIMP 1		TIMP 2	
	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot	RNA	Prot
Benign 1																		
Adenoma	0	-	1	-	0	-	2	-	0	-	1	-	0	-	1	-	1	-
Adjacent	0	0	1	1	0	-	2	2	0	0	1	2	1	1	1	1	1	1
Normal	0	-	1	-	0	-	2	-	0	-	1	-	0	-	1	-	1	-
Benign 2																		
Adenoma	0	0	1/2	1/2	0	-	2	2	1	1	1	1	1	1	1	1	1	1
Adjacent	0	0	1/2	1/2	0	-	2	2	1	1	1	1	1/2	1	1	1	1	1
Normal	0	0	1/2	1/2	0	-	2	2	1	1	1	1	1/2	1	1	1	1	1
Benign 3																		
Cyst	0	-	1	-	0	-	2	-	0	-	1	-	0	-	1	-	1	-
Normal	0	1	1	1	0	-	2	2	0	0	1	1	0	0	1	1	1	1

7.10c Summary of comparison between PCR and IHC for benign liver lesions

Collagenases

There is good correlation between the mRNA work and the immunohistochemistry. There was no expression of MMP 1 mRNA and in only one of the three immunohistochemistry specimens was there some faint staining. For MMP 8 there was expression in both the mRNA and protein work. MMP 13 expression could not be compared as no antibody was available.

Gelatinases

MMP 2 was expressed in both RNA and protein work for all three benign lesions.

MMP 9 was expressed by benign 2 in both mRNA and protein work , whereas benign 1 and 3 had no expression either at mRNA or protein level.

Stromelysins

MMP 3 was expressed by all three benign lesions at the same intensity at both mRNA and protein level. MMP 7 was expressed by at low levels by both benign lesions 1 and 2 on RNA and protein work, but was not expressed by benign lesion 3.

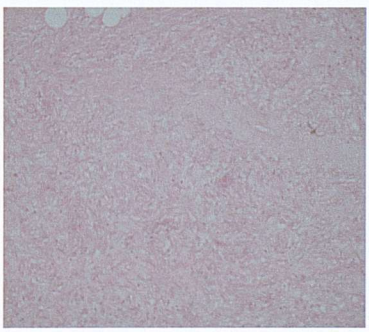
Tissue inhibitors of metalloproteinase

Both TIMP 1 and 2 were expressed at equal levels by the metastasis, adjacent liver and distal liver in all three benign specimens.

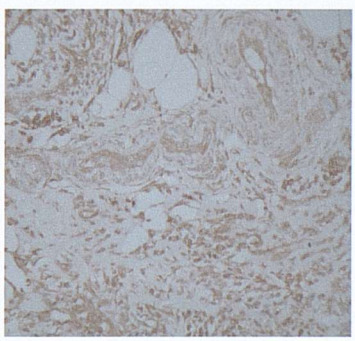
7.11 Immunohistochemistry (IHC) results for Primary tumours corresponding to previous secondary tumours

Figure 39: IHC on B1 primary tumour Dark brown staining positive. Dukes B

H&E x10

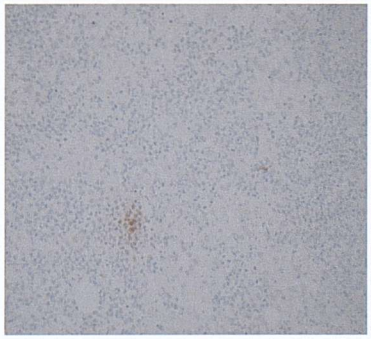


MMP 1 x20

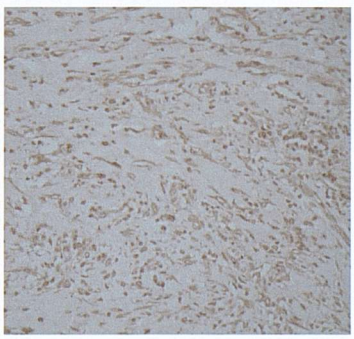


MMP 8 x20

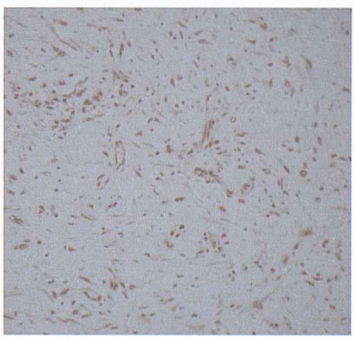
MMP 2 x20



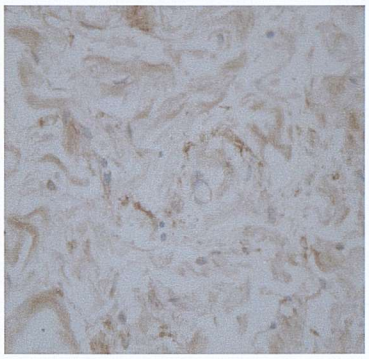
MMP 9 x20



MMP 3 x20



MMP 7 x40



TIMP 2 x20

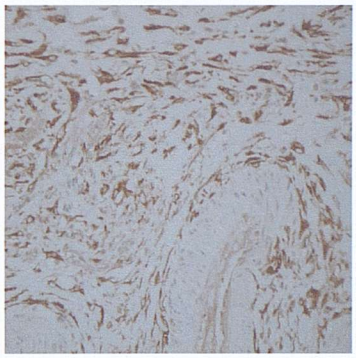
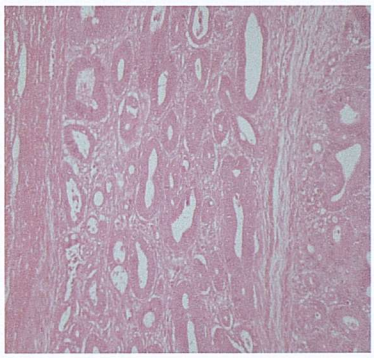
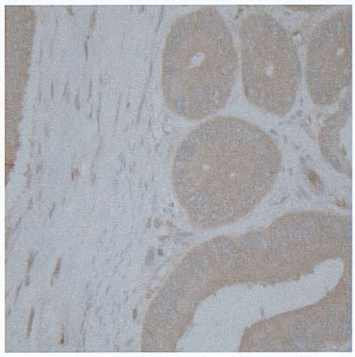


Figure 40: IHC on B2 primary tumour Dark brown staining positive. Dukes C

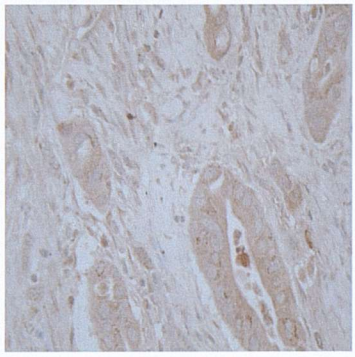
H&E x10



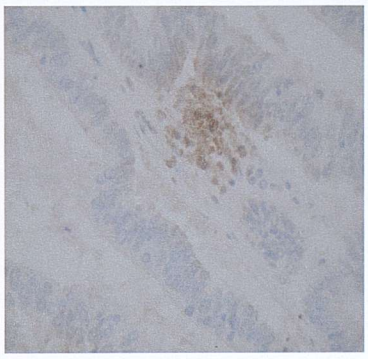
MMP 1 x40



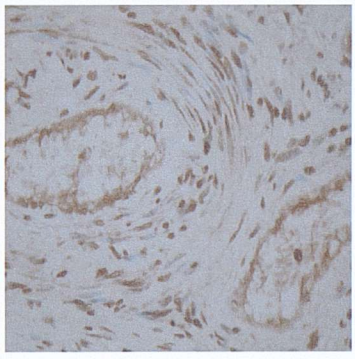
MMP 8 x40



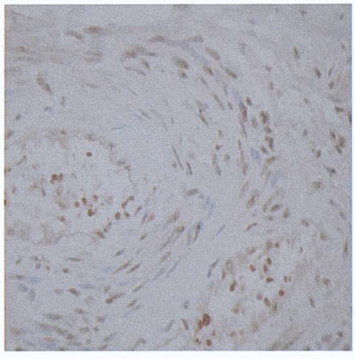
MMP 2 x40



MMP 9 x40



MMP 3 x40



MMP 7 x40

TIMP 2 x40

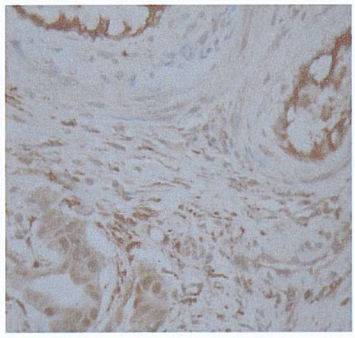
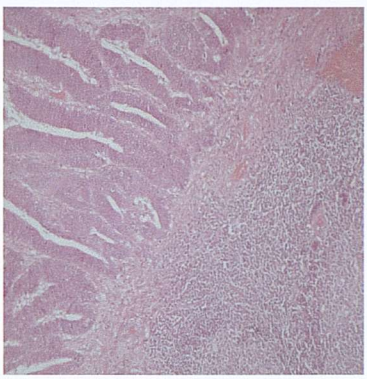
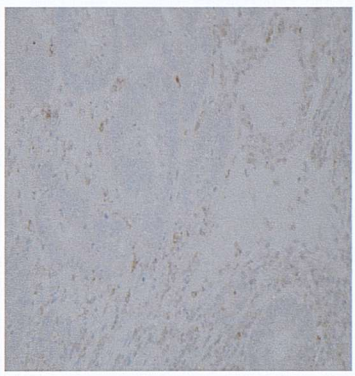


Figure 41: IHC on B3 primary tumour Dark brown staining positive. Dukes B

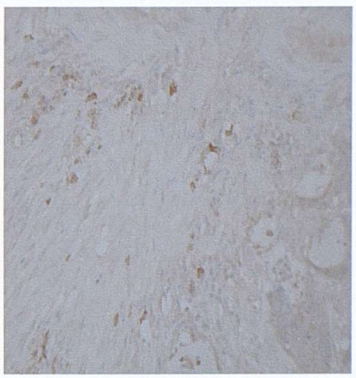
H&E x10



MMP 1 x20

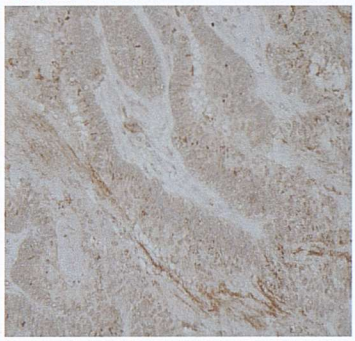


MMP 8 x20

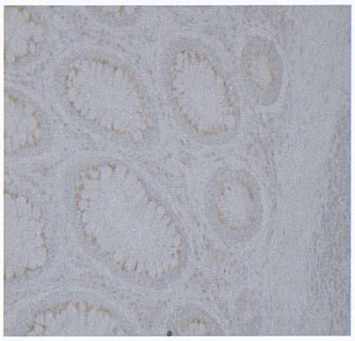


MMP 9 x20

MMP 3 x20



MMP 7 x20



TIMP 2 x20

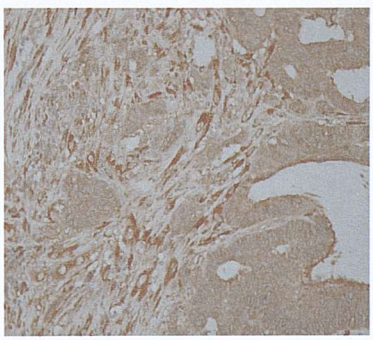
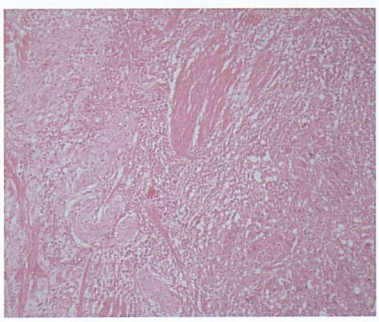
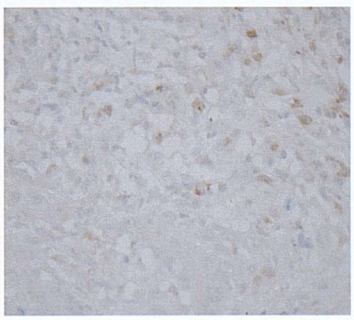


Figure 42: IHC on C4 primary tumour Dark brown staining positive. Dukes B

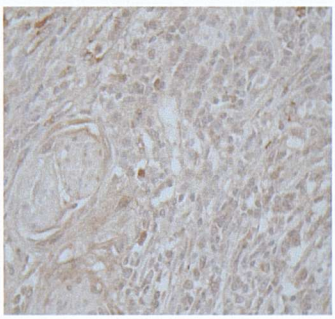
H&E x10



MMP 1 x40



MMP 8 x40



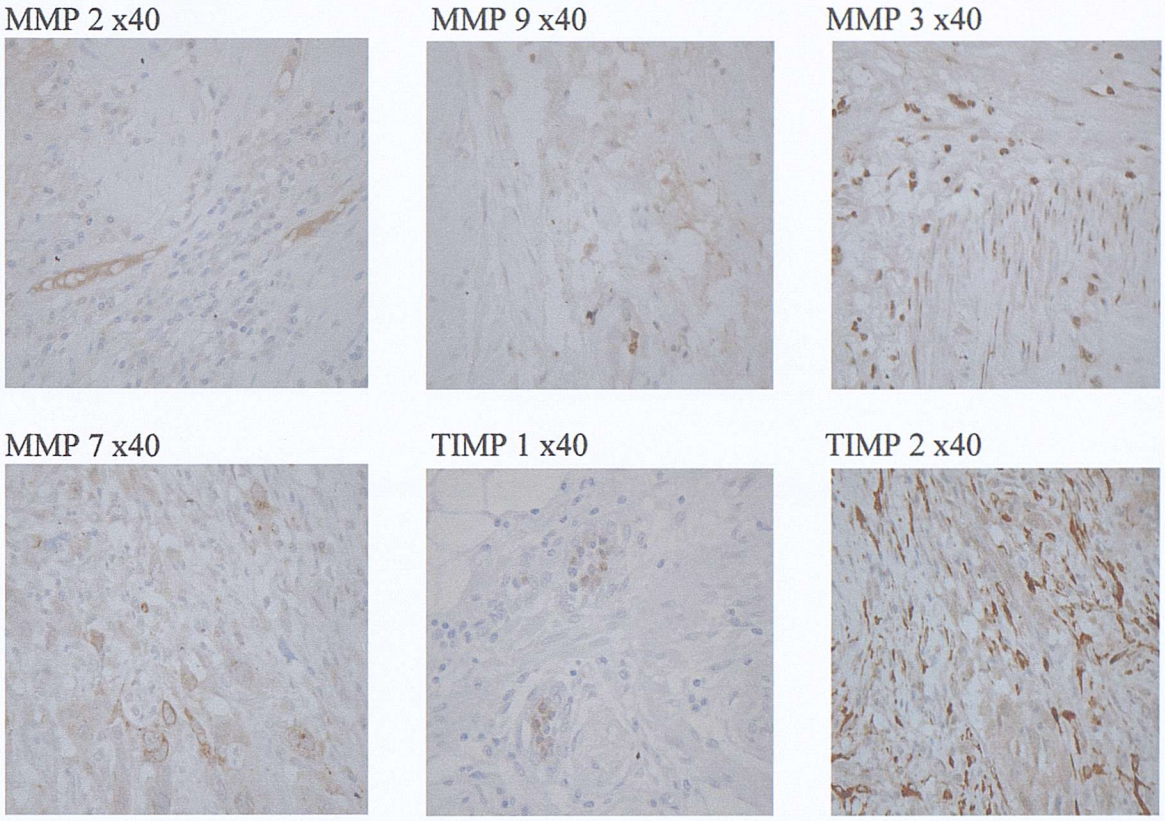
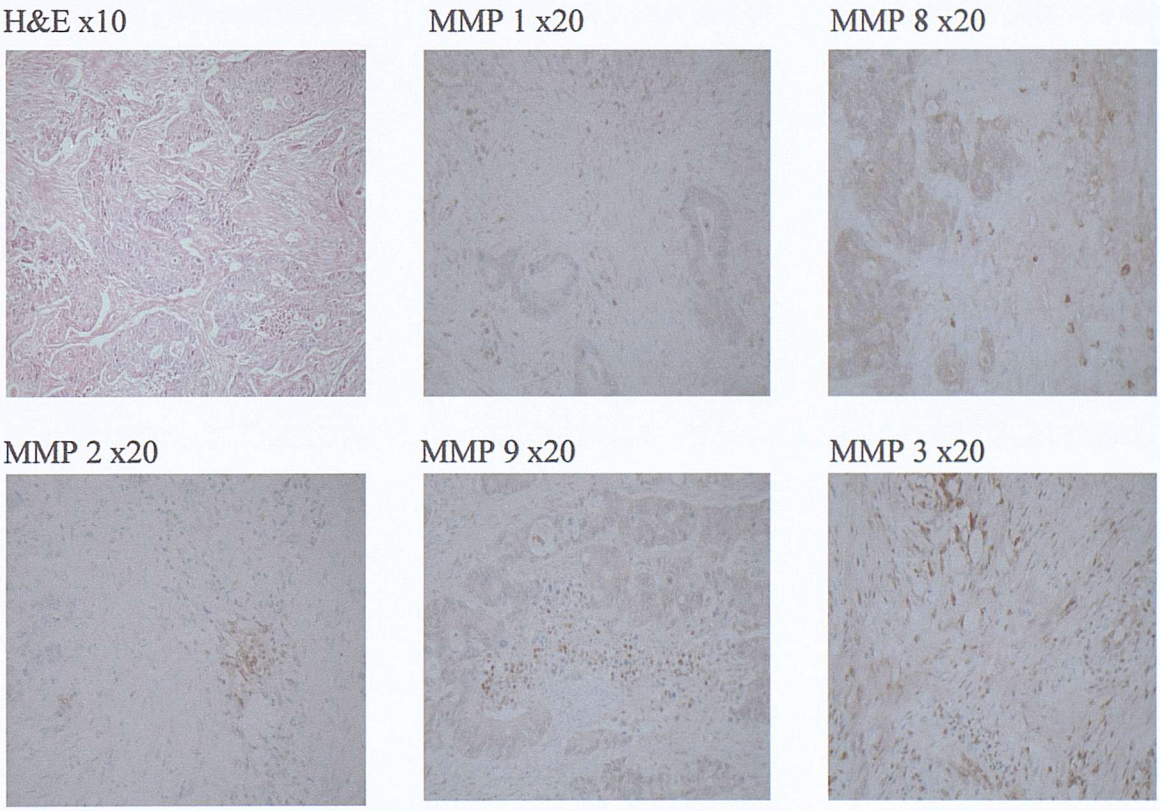
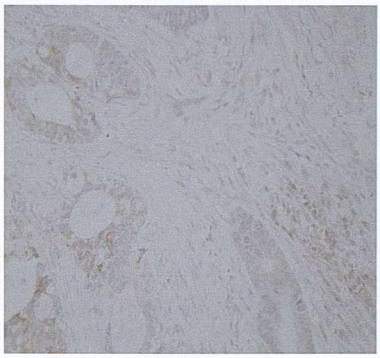


Figure 43: IHC on C5 primary tumour Dark brown staining positive. Dukes B



MMP 7 x20



TIMP 2 x20

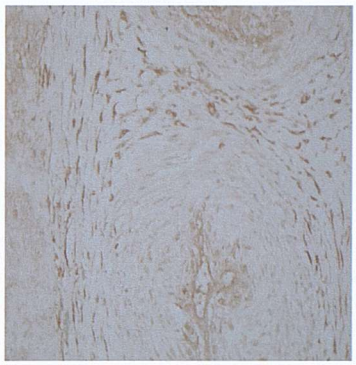
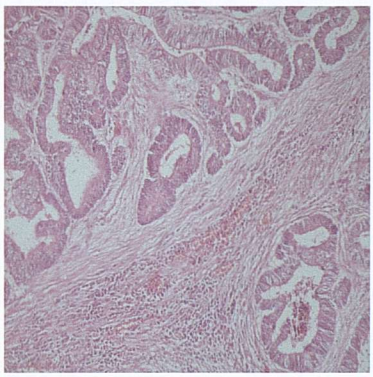
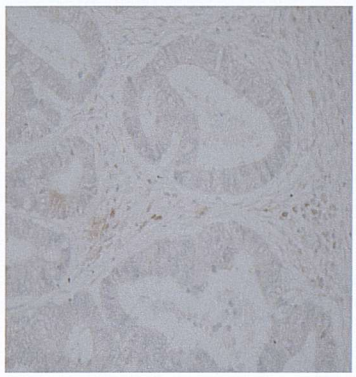


Figure 44: IHC on E2 primary tumour Dark brown staining positive. Dukes B

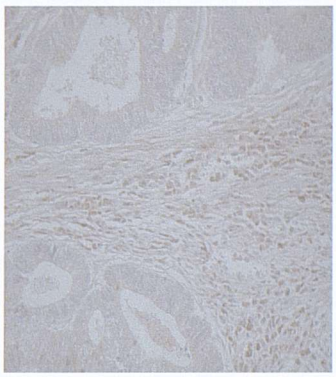
H&E x10



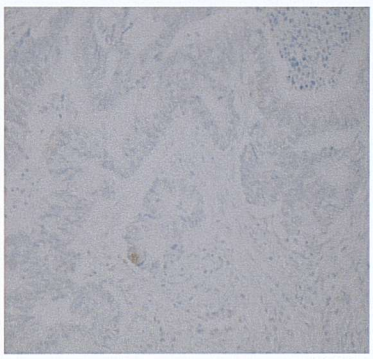
MMP 1 x20



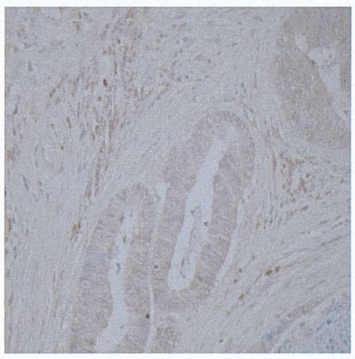
MMP 8 x20



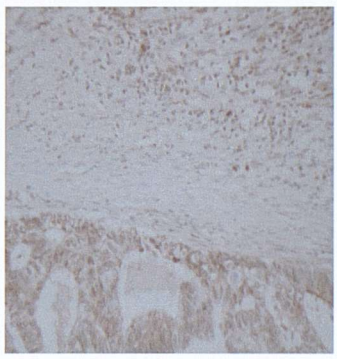
MMP 2 x20



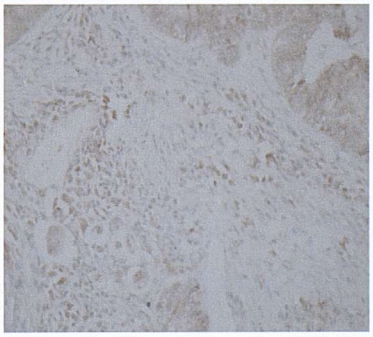
MMP 9 x20



MMP 3 x20



MMP 7 x20



TIMP 2 x20

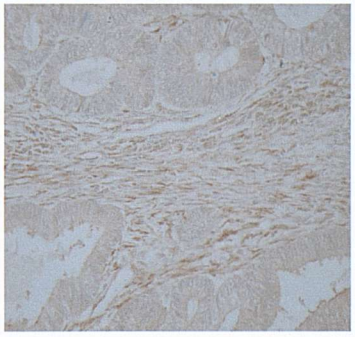


Figure 45: IHC on E3 primary tumour Dark brown staining positive. Dukes B

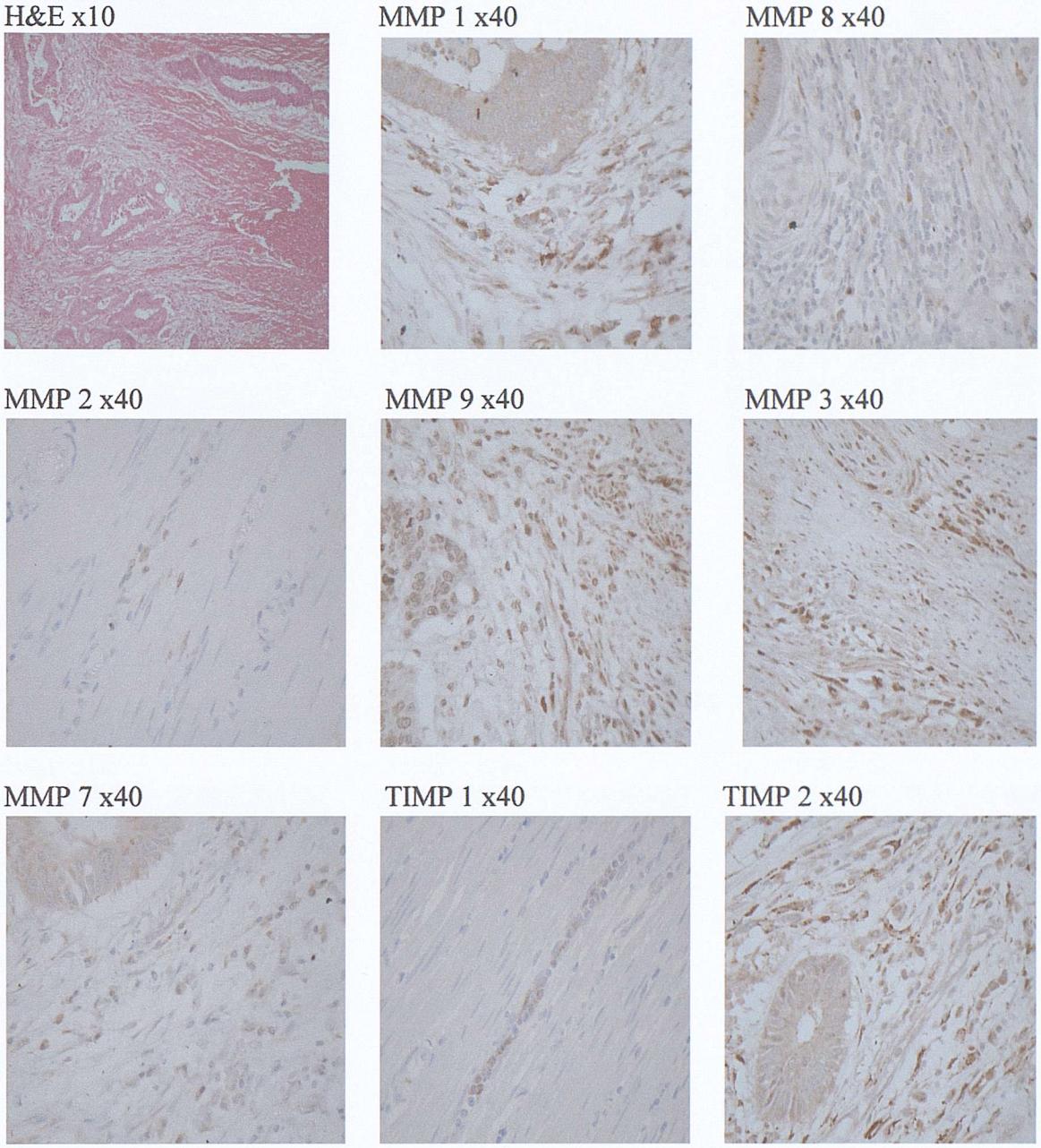
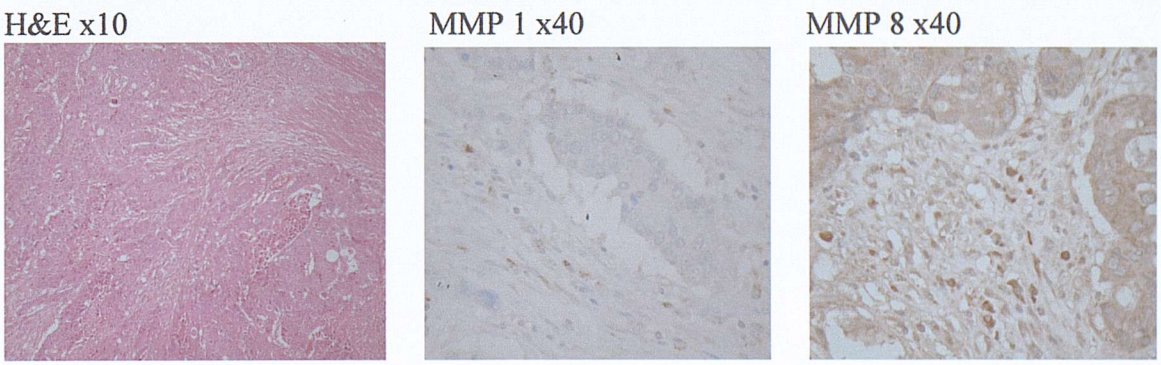
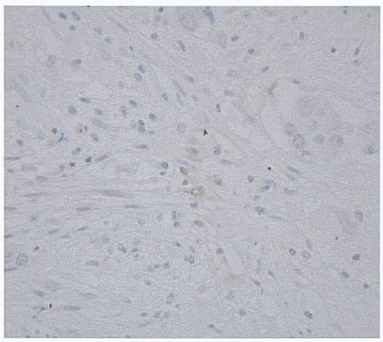


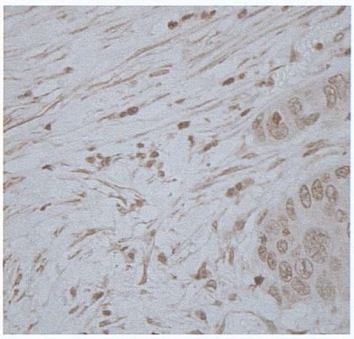
Figure 46: IHC on E4 primary tumour Dark brown staining positive. Dukes C



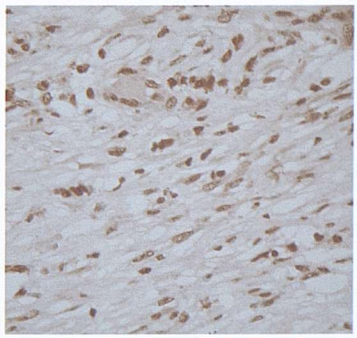
MMP 2 x40



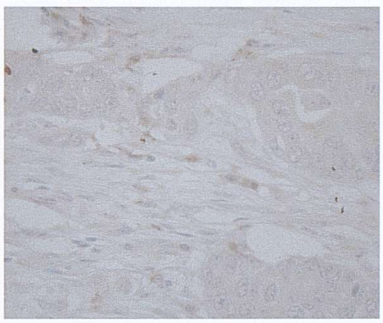
MMP 9 x40



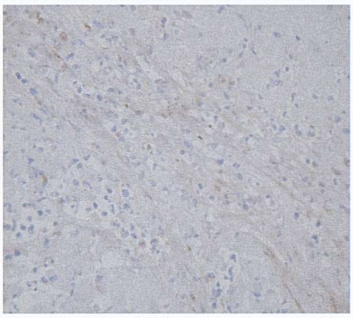
MMP 3 x40



MMP 7 x40



TIMP 1 x40



TIMP 2 x40

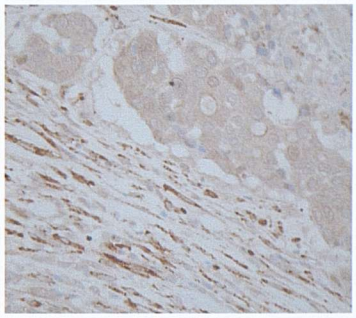
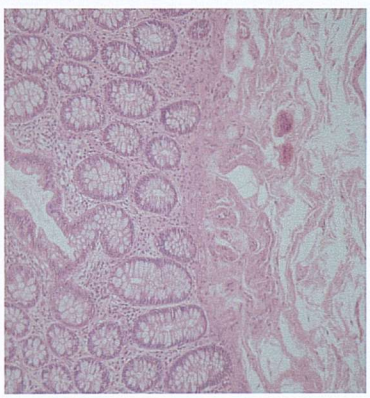
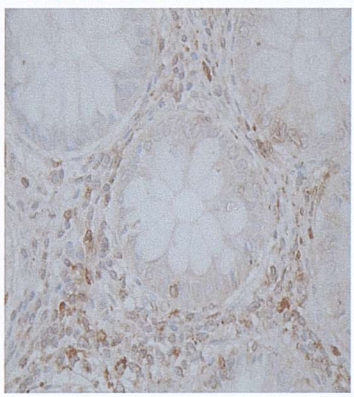


Figure 47: IHC on F2 primary tumour Dark brown staining positive. Dukes C

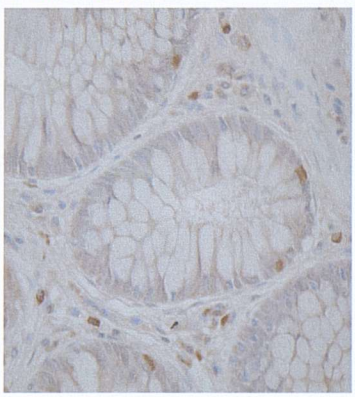
H&E x10



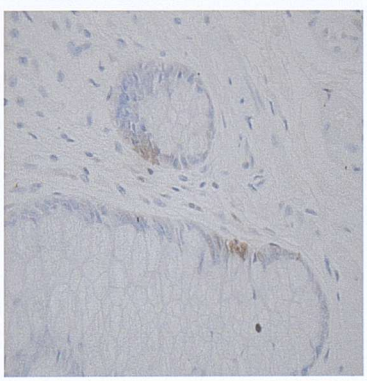
MMP 1 x40



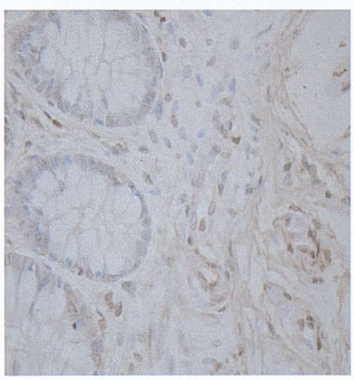
MMP 8 x40



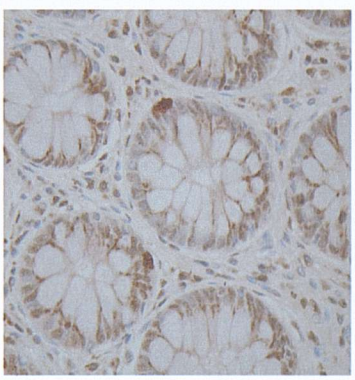
MMP 2 x40



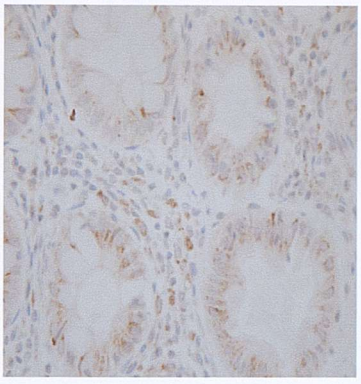
MMP 9 x40



MMP 3 x40



MMP 7 x40



TIMP 2 x40

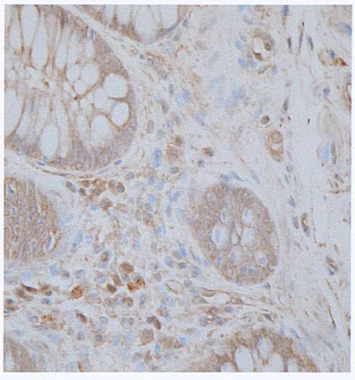
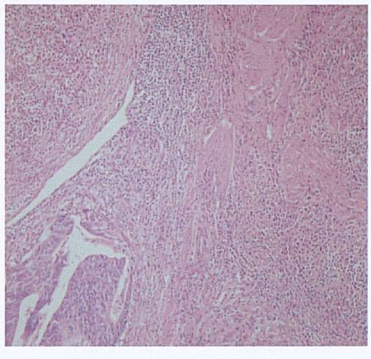
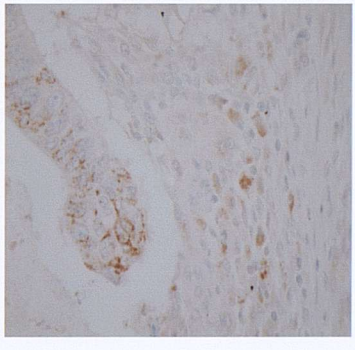


Figure 48: IHC on F5 primary tumour Dark brown staining positive. Dukes C

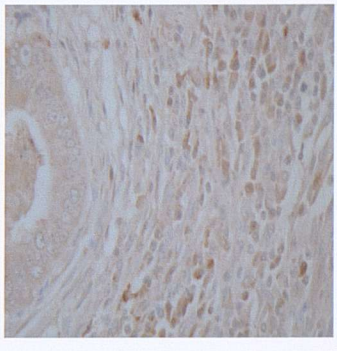
H&E x10



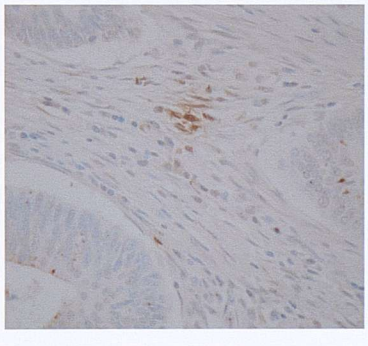
MMP 1 x40



MMP 8 x40



MMP 2 x40



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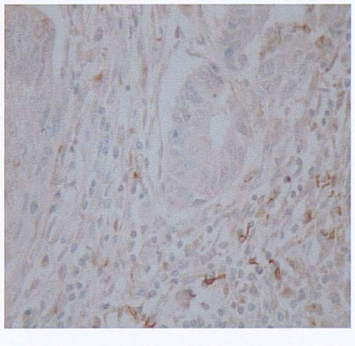
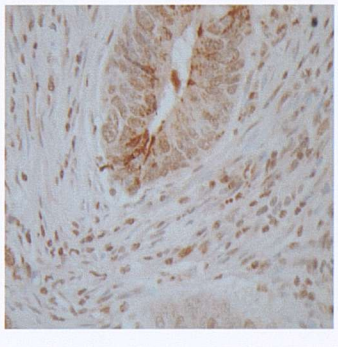
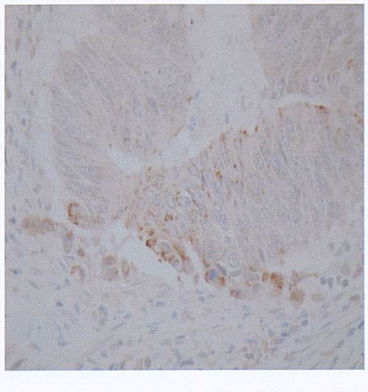


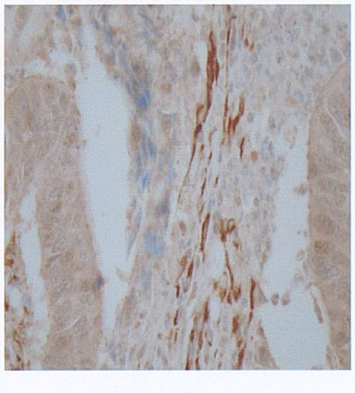
MMP 3 x40



MMP 7 x40



TIMP 2 x40



The primary tumour antibody work showed similar results to the secondary tumours. TIMP 1 was only run on three of the ten primary tumours whose antibody results were digitally imaged.

All the primary tumours expressed MMP 1,8,2,9,3,7 and TIMPs 1 and 2. MMPs 1, 8 and 2, 9 were expressed by the tumour stroma as opposed to adenocarcinoma cells. MMP 1 was predominately expressed by fibroblasts but was also present in macrophages and MMP 8 was expressed by fibroblasts. MMP2 was expressed by fibroblasts whereas MMP 9 was expressed by macrophages. MMPs 3 and 7 were expressed by both the adenocarcinoma cells and tumour stromal macrophages. TIMP-1 was expressed by macrophages in the tumour stroma. TIMP-2 was expressed by fibroblasts in the tumour stroma.

Chapter 8: DISCUSSION

The main findings of the research are outlined below. There is evidence that in metastatic colorectal carcinoma there is induced variation in the expression of MMP and TIMPs.

8.1 Collagenases

The mRNA work showed that MMP 1 is expressed at a significantly higher level in colorectal liver metastases than in the immediately adjacent liver and distal liver. This finding at the messenger level is confirmed at the protein level by the immunohistochemistry work, which shows expression by all thirty metastases with less expression by the adjacent liver and the distal liver. The MMP 1 was expressed predominantly by fibroblasts in the tumour stroma, but was also present in macrophages.

This high expression of MMP 1 in the colorectal liver metastases sits in line with the expression of MMP 1 by the stromal cells of primary colorectal carcinoma described by Hewitt, Gray, Urbanski and Otani (Hewitt RE 1991, Gray S 1993, Urbanski SJ 1993, Otani Y 1994). This high expression of MMP 1 by liver metastases also correlates well with the work by Murray et al that showed that although only 16% of the tumours they studied showed positive immunoreactivity for MMP 1, in those that did more than 90% of the cell were positive and it was associated with a poor prognosis that was independent of the Dukes' (A-C) staging. Patients with liver metastases would be expected to have more aggressive tumours and so MMP 1 would be expected to be expressed by these tumours and their liver metastases. The immunohistochemistry performed on the primary tumours giving rise to the liver metastases showed MMP 1 was indeed expressed by these tumours.

This concept is supported by the work of Sunami et al who showed that MMP 1 expression (identified by immunohistochemistry) by colorectal cancers was significantly correlated with haematogenous metastasis of colorectal cancer (Sunami E 2000).

Lunevicius et al have recently reported the presence of elevated levels of MMP 1 along the invading tumour margin in hepatic metastases (Lunevicius R 2001).

Further work by Baker et al using gelatin zymography, ELISA, and quenched fluorescent substrate hydrolysis has shown that the levels of MMP 1, 2 and 9, 3 and TIMP 1 were significantly greater in colorectal cancer tissue than normal colonic mucosa, and specifically showed that MMP 1 concentration correlated positively with Dukes staging, tumour differentiation and lymphatic invasion (Baker EA 2000). Conversely they found that TIMP 2 levels were decreased in colorectal cancer tissue compared to normal.

Shiozawa et al confirmed this role of MMP1 showing it to be present in 76% of colorectal adenocarcinomas and its expression to be significantly correlated with depth of tumour invasion, presence of lymphatic invasion, venous invasion, neural invasion, lymph node metastasis, hepatic metastasis and increasing Dukes staging (Shiozawa J 2000).

Work from Italy has shown that MMP 1 promoter polymorphism, producing 2G homozygotes which leads to more MMP 1 expression, favours growth and the metastatic process in colorectal cancer patients (Ghilardi G 2001), a conclusion confirmed by Hinoda et al (Hinoda Y 2002).

MMP 8 was expressed ubiquitously by the metastases, adjacent and distal liver at both the mRNA and protein levels, the immunohistochemistry showing that expression was in fibroblasts. Immunohistochemistry of the primary tumours again showed expression of MMP 8 by fibroblasts. No previous work with MMP 8 and colorectal carcinoma has been performed although its importance in breast carcinoma has been previously reported (Duffy MJ 1995).

No expression of MMP 13 at the mRNA level was seen in the liver metastases, adjacent liver or distal liver for any of the thirty patients examined. Unfortunately there was no antibody available in our laborotory to confirm this finding at the protein level,

either for the metastasis itself or the primary tumour. These findings are supported by work from California, using immunohistochemistry techniques, which examined MMP 13 expression by primary colorectal carcinomas and failed to show any expression (Bodey B 2000). However recent work by Leeman et al has disagreed with this and shown by immunohistochemistry that MMP 13 was expressed by 91% of cases (out of 249) of colorectal cancer, and a higher staining score showed a trend towards poorer survival (Leeman MF 2002).

8.2 Gelatinases

MMP 2 mRNA was expressed at a similar level in the metastasis, adjacent liver and distal liver of all 30 specimens and immunohistochemistry work confirmed this and showed the expression was by fibroblasts in the tumour stroma. The primary tumours also expressed MMP 2 in fibroblast stromal cells.

MMP 9 mRNA was expressed at significantly higher levels in the metastases than the immediately adjacent liver or the distal liver, and the levels in the adjacent liver were the same as those in the distal liver. Immunohistochemistry confirmed this pattern of expression and showed that the MMP 9 was expressed by macrophages in the tumour stroma. The primary tumours also expressed MMP 9 in tumour stromal macrophages. The gelatinases have for a long time been implicated in colorectal carcinogenesis, as discussed in Chapter 3. Recent work has confirmed this. Collins et al looked at MMP 2 and 9 expression in colorectal cancer cell lines and from human colorectal tumours. MMP 2 was not expressed by the colon cancer cell lines themselves but by the accompanying fibroblasts, which was confirmed by the human colorectal cancer tissue where MMP 2 mRNA was localised to the stromal component of the tumour tissue. The MMP 2/TIMP 2 ratio was increased in tumour tissue compared with normal colonic mucosa. MMP 9 was expressed by the metastatic cell line and was located to the inflammatory infiltrate in the

stroma (Collins HM 2001). Ornstein et al showed that the TIMP 2/MMP 2 ratio was two times lower in colorectal tumours compared to normal mucosa (Ornstein DL 2002).

Matsuyama et al used gelatin zymography and showed MMP 2 and 9 levels to be higher in colonic xenograft tumours in nude mice with liver metastases compared to those without (Matsuyama Y 2002). Roeb et al showed MMP 9 expression was greater in colon carcinoma compared with normal mucosa, but there was no difference between the expression in rectal cancer and its normal mucosa, both being high (Roeb E 2001).

The findings from this thesis are in agreement with the previous work done on gelatinases and liver metastases from colorectal cancer, MMP 2 and 9 being found in significantly higher levels in liver metastases compared with normal colorectal tissues (Murashige M 1996) and primary tumour (Karakiulakis G 1997). The importance of MMP 2 in the metastastic process in the liver was evaluated by both Brand and Nagatsuka. Brand et al used a highly metastatic cell line (LS174T) that was shown to secrete MMP 2 primarily. Transfection of TIMP 2 into the liver before innoculation of tumour or after tumour was established decreased tumour burden by 95% and 77% respectively. Reductions in proliferation and apoptosis were also seen (Brand K 2000). Nagatsuka et al working with a COX-2 inhibitor (JTE-522) in mice using a highly metastasizible colon carcinoma cell line, LM-H3 showed a reduction in the number of metastatic nodules on the surface of nude mouse livers. Using gelatin zymography, JTE-522 was shown to inhibit MMP 2 secretion by LM-H3 giving a potential mechanism of action for its reduction in metastatic nodules in the mouse model (Nagatsuka I 2002).

Zeng and Guillem studied MMP-9 and TIMP-1 mRNA expression in 10 liver metastases from colorectal cancer, using in situ hybridisation. Both MMP-9 and TIMP-1 mRNA were expressed in all 10 metastases, with MMP-9 localised within peritumour stroma or at the interface between the tumour stroma and normal liver. TIMP-1 mRNA on

the other hand was located throughout the malignant tumour stroma (Zeng ZS 1995). The same group then examined the cellular enzymatic expression of MMP-9 in 18 human colorectal cancer liver metastasis specimens using ELISA and zymography. ELISA revealed that the latent form of MMP-9 is present in both liver metastasis and paired adjacent normal liver tissue. Gelatin zymography showed that although the latent form of MMP-9 was present in both metastasis and normal liver, the active form of MMP-9 was seen only in liver metastasis (Zeng, Guillem 1998). The techniques we have used do not distinguish between latent and active forms of MMP-9 but these results are, in general, consistent with the findings of this research.

8.3 Stromelysins

With respect to the stromelysins, higher expression of MMP-3 mRNA has been shown in the metastases compared to the adjacent liver. There was no significant difference between MMP-3 expression in the distal liver compared with either the metastasis or the adjacent liver. The immunohistochemistry results showed MMP 3 was expressed by both the adenocarcinoma cells and tumour stromal macrophages in both the liver metastasis and the corresponding primary tumour.

MMP-7 mRNA is expressed at significantly higher levels in the metastases than the adjacent liver and distal liver. The immunohistochemistry showed MMP 7 being expressed by both adenocarcinoma cells and tumour stromal macrophages in both the metastases and the primary colorectal carcinoma.

MMP 3 has been shown to be expressed in the stroma of primary colorectal carcinomas (Newell KJ 1994, Gallegos NC 1995) compared with no expression in adenomas (Newell KJ 1994), especially in the extracellular matrix adjacent to blood vessels (Bodey B 2000). Moran et al looked at MMP 2 and 9 expression, and MMP 3 expression in sporadic colorectal cancer with varying microsatellite instability. The total

levels of MMP 9 were significantly higher in tumours with high microsatellite instability (MSI-H) (Moran A 2002). However the levels of active MMP 9 were significantly lower in these tumours. MMP 3 levels in these MSI-H tumours were much lower than the MSI-L tumours, and all of these MSI-H tumours showed nucleotide insertions and/or deletions in the MMP 3 promoter, whereas none of the MSI-L tumours did. The authors felt that the MMP 3 promoter constituted a novel target of the defective mismatch repair machinery in sporadic colorectal tumours. This leads to a dramatic decrease in the levels of active MMP 9 and might explain the reduced likelihood of metastases seen in MSI-H colorectal cancer (Gryfe R 2000). No work has been done on MMP 3 and colorectal liver metastases.

MMP-7 mRNA expression has been shown to increase moving from normal colorectal mucosa to adenomas (Heslin MJ 2001) and to carcinomas (Newell KJ 1994) and has been shown to be a significant determinant of malignant potential of early invasive colorectal carcinomas (Masaki T 2001). In liver metastases both Mori et al and Ishikawa et al have shown MMP-7 mRNA expression to be greater in liver metastases compared with the primary colorectal carcinoma (Mori M 1995, Ishikawa T 1996). This important role of MMP-7 in colorectal liver metastasis has been confirmed in vitro. An MMP-7-specific antisense oligonucleotide inhibited liver metastasis of human colon cancer cells in a nude mouse model (Hasegawa S 1998).

Adachi et al examined MMP 7 expression in 83 colorectal cancers and seven liver metastases and showed that 46% of primaries and all of the liver metastases expressed MMP 7 in the cancer cells (Adachi Y 1999). They also showed that matrilysin transfectants formed invasive tumours and multiple liver metastases in SCID mice, without producing any significant difference in subcutaneous tumour growth from mock transfectants. Zeng et al looked at MMP 7 expression in liver metastases and normal liver tissue in 44 patients at both the mRNA and protein levels. They showed that the

metastases overexpressed both latent and active forms of MMP 7 mRNA and protein compared with normal liver (Zeng ZS 2002).

8.4 Tissue inhibitors of metalloproteinases (TIMPs)

TIMP 1 mRNA was expressed by both colorectal carcinoma metastases and distal liver, but at significantly higher levels in the metastases. The expression of TIMP 1 mRNA by the immediately adjacent liver was markedly lower than both metastases and distal liver, being absent in 10 of the 30 patients. Immunohistochemistry backed up these findings at the protein level, TIMP 1 being expressed by macrophages in the tumour stroma, absent in the immediately adjacent liver tissue but expressed again in the hepatocytes of the distal liver. TIMP 1 was expressed by macrophages in the stroma of the primary colorectal carcinoma.

TIMP 2 mRNA was expressed by both colorectal carcinoma metastases and distal liver, but at significantly higher levels in the metastases. The expression of TIMP 2 mRNA in the immediately adjacent liver was significantly lower than both metastases and distal liver, being absent in 19 of the 30 patients. Immunohistochemistry backed up these findings at the protein level, TIMP 2 being expressed by fibroblasts in the tumour stroma, absent in the immediately adjacent liver tissue but expressed again in the hepatocytes of the distal liver. TIMP 2 was expressed by fibroblasts in the stroma of the primary colorectal carcinoma.

These results are the first to show a marked down regulation of TIMPs 1 and 2 in the liver adjacent to the colorectal metastases. They also confirm previous work showing TIMP 1 and 2 being expressed by stromal tissues in both primary colorectal carcinomas (Hewitt RE 1991, Poulsom R 1992, Newell KJ 1994, Tomita T 1996), and liver metastases (Zeng ZS 1995, Murashige M 1996, Zeng ZS 1998). The increase in TIMP 1 and 2 in colorectal carcinoma primaries and metastases probably reflects a growth

promoting function in the tumour (Gomez DE 1997, Chesler L 1995, Wingfield PT 1999, Jiang Y 2002).

In summary the results of this work show that there is marked down-regulation of TIMP-1 and 2 expression at the invading margin of colorectal liver metastases. This down regulation is seen at the mRNA and protein levels, as demonstrated by immunohistochemistry. This, together with the findings of high levels of expression of MMP-1, MMP-9, MMP-3 and MMP-7 provide evidence for the importance of MMPs and TIMPs in the process of malignant invasion.

One possible explanation for this down regulation of TIMP-1 and 2 expression at the invading margin of colorectal liver metastases is the effects of a space occupying lesion within the liver affecting TIMP 1 and 2 expression by the adjacent liver. In order to confirm that our findings were not related simply to the presence of a space occupying lesion within the liver we performed studies on the liver from 3 patients undergoing resection for benign disease. Benign liver lesions are seldom resected, hence the limited number of specimens.

These studies showed there was no decrease in TIMP-1 and 2 levels in the adjacent liver tissue. They also showed there was no expression of MMP1 and minimal expression of MMP 9 and 7 compared to the colorectal liver metastases. Thus, the changes observed with the colorectal liver metastases are not due to the presence of a space occupying lesion but may well be a consequence of cell-cell signalling between the cancer cells, stroma and hepatocyte.

As discussed previously, work using intravital videomicroscopy (Chambers AF 1997) first suggested that MMPs and TIMPs may play important roles regulating the growth environment of metastatic cancer and further work has confimed this (Egeblad M 2002). It is known that following liver resection for colorectal liver metastases long term

survival is more likely if a resection margin of >1 cm is achieved (Geoghegan 1999). This suggests there are factors within the liver immediately adjacent to the colorectal liver metastases that facilitate the spread of the metastases hence decreasing the survival of the patient if an adequate resection margin is not achieved.

The results suggest that as well as the high expression MMPs 1, 9, 3 and 7 in the colorectal carcinoma metastasis it is the marked down-regulation of TIMPs 1 and 2 in the liver adjacent to the metastases that facilitates the growth and potential spread of colorectal carcinoma liver metastases. This gives a theoretical basis for the use of synthetic MMP inhibitors in metastatic colorectal cancer. At present the results from trials using synthetic MMP inhibitors have been disappointing but these have mainly been in patients with advanced carcinoma unresponsive to more traditional oncological treatments (Coussens LM 2002). Alteration of the MMP/TIMP profile of these tumours is unlikely to have any major effect, apart from potentially slowing the progress of the disease, as they are all ready rapidly progressing tumours with the tumour/host microenvironment heavily weighted in favour of the tumour.

However despite some evidence of activity in colorectal liver metastases (Primrose JN 1999) the development of these agents has largely been abandoned (Zucker S 2000) as it is now apparent that MMP/TIMP interactions are more complex than originally thought (Egeblad M 2002). Thus the initial assumption that agents inhibiting MMPs be effective in treating metastases fails to appreciate their many, often opposing functions.

A more realistic use of synthetic MMP inhibitors would be as an adjuvant therapy. In the case of colorectal carcinoma liver metastases the results of this work would suggest their use after the resection of the primary tumour before any clinical or radiological liver metastases are present, to ensure that any metastatic cells in the liver have a hostile microenvironment in which to try and establish a secondary tumour. They could be used

along with conventional chemotherapy. Newer MMP inhibitors are being developed that are specific towards subtypes of MMPs, resulting in distinct efficacy against either the direct effects of MMPs on structural components of the extracellular matrix or on the release or processing of growth factors or inhibitors.

Ohta et al have examined the effects of MMI-166, a selective MMP inhibitor, on tumour growth, angiogenesis and metastasis in a liver metastatic model of human xenotransplanted colon cancer. They also investigated any synergistic effects of MMI-166 with mitomycin C, a conventional cytotoxic agent. They found that MMI-166 did not inhibit transplanted tumour growth but significantly inhibited liver metastasis compared with the control group and the mitomycin C group. They also showed that the combination of MMI-166 and mitomycin C had significant antitumour and antimetastatic effects (Ohta M 2001).

Miyazaki et al examined the use of an MMP 7 specific antisense phosphothioate oligodeoxyribonucleotide on in vitro invasion and liver metastasis in nude mice of two human colon carcinoma cell lines (CaR-1 and WiDr). In culture the antisense oligonucleotide effectively inhibited both the secretion of matrilysin by CaR-1 cells and their in vitro invasion through a reconstituted basement membrane. In the nude mouse model the antisense oligonucleotide suppressed the experimental liver metastasis of WiDr cells from the spleen. They concluded that MMP 7 has an important role in the liver metastasis of human colon cancer and that matrilysin antisense oligonucloetides have therapeutic potential for the prevention of metastasis (Miyazaki K 1999).

The Nottingham group have recently looked at the effect of preoperative radiotherapy on matrix metalloproteinase expression in resectable rectal cancer. As discussed in Chapter 1 the Dutch Colorectal Cancer Group have shown there is a decrease in local recurrence rates for rectal cancer if short course preoperative radiotherapy is given

(Kapiteijn E 2001) and this has become the standard of care. However Kumar et al have shown that the levels of MMP 2 and 9 (Kumar A 2000) and MMP 7 (Kumar A 2002) in resectable rectal cancer tissue are increased following short course radiotherapy. They suggest MMP inhibition may be a useful adjunct to radiotherapy in rectal cancer as, although radiotherapy has been shown to decrease local recurrence in rectal cancer it has no effect on overall survival. This increased MMP expression they have described may help to explain this, giving tumour cells more potential for metastatic spread and hence stopping any survival benefit for the radiotherapy.

Recent work looking at MMPs and TIMPs in hepatocellular carcinoma has shown similar, although not identical results to this work. Sakamoto et al showed MMP 1 and 9 expression to be significantly higher in tumour tissue than in non-tumour tissue, although MMP 2 and 7 were significantly lower (Sakamoto Y 2000). McKenna et al showed that there was an increase in the MMP 2:TIMP 2 mRNA expression ratio at the tumour margin in hepatocellular carcinomas but in this case due to an increase of MMP 2 as opposed to a decrease in TIMP 2. They too felt that this reflected host-tumour interaction regulating tumour metastasis (McKenna GJ 2002).

In this work a simple technique of semiquantitative PCR has been used rather than quantitative PCR. However the principal differences observed are not subtle and are qualitatively obvious using both RT-PCR and immunohistochemistry. This technique certainly lacks sensitivity when compared to real time PCR and it is likely that some additional small differences in expression may be revealed by a more sensitive approach. Similarly, with regard to MMP 2 and 9 zymography or an alternative technique may well reveal differences in active and latent forms of these enzymes. A quantitative PCR approach is, however, required if the prognostic significance of any of these enzymes is to be determined and is an avenue of future research.

Chapter 9: CONCLUSIONS

These results show that there is high expression of MMPs 1, 9, 3 and 7 in colorectal carcinoma liver metastasis, and down regulation of TIMP 1 and 2 in the immediately adjacent liver tissue.

Simplistically it can be concluded that this balance of MMPs and TIMPs at the interface between the metastases and the liver favours proteolysis and results in a cellular environment which facilitates local growth and further metastases of hepatic metastatic disease. This finding also provides a theoretical basis for the use of synthetic MMP inhibitors in patients with colorectal liver metastases.

However despite some evidence of activity in colorectal liver metastases the development of these agents has largely been abandoned as it is now apparent that MMP/TIMP interactions are more complex than originally thought. The nature of MMP/TIMP contribution to metastasis was assumed originally to be primarily facilitation of the breakdown of the physical barriers between a primary tumour and distant sites for metastasis. Recent evidence suggest that the action of MMPs at steps both before and after the breakdown of the apparent physical barriers to metastasis may in fact be of greater importance. MMPs and their inhibitors appear to be important regulators of the growth of tumours, both at the primary site and as metastases. Thus the initial assumption that agents inhibiting MMPs be effective in treating metastases fails to appreciate their many, often opposing functions.

The findings of this research require further studies to elucidate the mechanisms involved in these descriptive observations and to fully explain the complexities of the relationship between metastasis, liver, MMP and TIMP.

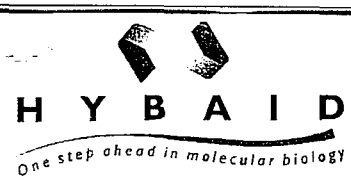
APPENDICES

Appendix i: Laboratory solutions used.

TAE:	50x	1000mls dH_2O 242g Tris Base 57.1ml glacial acetic acid 100ml 0.5M EDTA (pH 8.0)
	1x	0.04m Tris-acetate 0.001M EDTA
TBE:	5x	1000mls dH_2O 54g Tris Base 27.5g boric acid 20mls 0.5 EDTA (pH 8.0)
	1x	200mls 5xTBE + 800mls dH_2O
25% APS		20mls water for reactions 5g ammonium persulphate (invert until clear)
50/50		Bis-acrylamide Acrylamide (then vacuum for 10 mins)
Terrific Broth		To 900ml deionised water add: 12g bact-tryptone 24g bact-yeast extract 4ml glycerol

Shake until all solutes have dissolved and sterilize by autoclaving for 20 mins. Allow solution to cool to 60°C or less, and then add 100ml of a sterile solution of 0.17M KH_2PO_4, 0.72 M K_2HPO_4 (Made up by dissolving 2.31g of KH_2PO_4 and 12.54g K_2HPO_4 in 90mls deionised water. Add deionised water to make upto 100mls, then sterilise by autoclaving for 20 minutes).

Appendix ii: Hybaid Ribolyser ™ Kit-Green Protocol



HYBAID RIBOLYSER™ Kit-*GREEN* PROTOCOL

The Hybaid Ribolyser™ Kit GREEN is designed for the rapid isolation of total RNA from **plant and animal tissue** using the HYBAID RiboLyser™ Instrument.

Introduction

The unique aspects of the HYBAID RiboLyser™ system, which includes both the HYBAID RiboLyser™ instrument and kits, is the ability to lyse cells and stabilise RNA before RNA degradation occurs. The procedure eliminates the problematic steps in isolation of RNA from cells that are difficult to lyse without the use of enzymes or grinding and homogenising. More than anything it is these laborious and time-consuming lysis steps that allow time for nucleases to act. The HYBAID RiboLyser™ system, by use of high energy mechanical means and careful choice of reagents, disrupts whole tissues, lyses cells and stabilises RNA from any source in seconds. This eliminates the need for lysing enzymes or grinding and homogenising equipment.
The HYBAID RiboLyser™ instrument utilises a simultaneous shaking and twisting motion at very high speeds. The rotor holds 12 x 2ml tubes enabling 12 samples to be processed simultaneously. The HYBAID RiboLyser™ kits comprise 2ml tubes containing a lysing matrix optimised for maximum lysis of the sample being processed. The tubes also contain a chaotropic RNA stabilising reagent and phenol acid reagent which is a proprietary mixture of detergents, salts and acid phenol. The inactivate nucleases and they also provide lubrication during the lysing step to prevent shearing of the RNA. Different stabilising reagents are necessary to efficiently isolate intact RNA from different organisms.

Hybaid RiboLyser™ Kit GREEN, Cat.-No. RY62100

The HYBAID RiboLyser™ GREEN Kit uses a mixture of sizes of silica and ceramic particles that completely lyses cells in 20-120 seconds. The energy generated by friction during the lysis step is considerable causing a 20-40°C temperature increase in the tube. This rise in temperature facilitates the inactivation of nucleases and does no harm to the RNA. The temperature rise most likely contributes in a positive way as the use of hot phenol is a well-known method for RNA isolation[1].
The kit included chaotropic RNA stabilising reagent is a modification of the reagents used by Cheung and Eberhardt[2] that has been optimised for stability. The mixture is excellent for many plant species and tissues and results in pure RNA with no contaminating DNA, as evidenced by gel electrophoresis analysis. Experiments using a guanidine thiocyanate/acid phenol mixture[3] with the HYBAID RiboLyser™ instrument produced RNA but with measurable DNA content.

Hybaid Limited
Office: 111-113 Waldegrave Road, Teddington, Middlesex TW11 8LL
Tel: 0181-614 1000 Fax: 0181-977 0170

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Reg. Office: Crown House, 51 Aldwych, London WC2B 4LS. Reg. No. 2015834

A LIFE SCIENCES INTERNATIONAL COMPANY

Kit components

One kit contains most of the materials and reagents for 100 preps using 50-250mg of tissue or 10^7 cells with yields of 20-100µg RNA.
Reagents supplied by the user are high grade water saturated phenol, chloroform, ethanol and isopropanol.

100 x 2.0ml	HYBAID RiboLyser™ tubes with GREEN CAPS containing acid-washed, RNase-free, silica/ceramic matrix for optimum animal or plant cell lysis.
55ml	Reagent A (Chaotropic RNA Stabilising Reagent GREEN)
16.5ml	Buffered salt solution for the preparation of Reagent B (Phenol Acid Reagent GREEN)
2.5ml	Isoamyl alcohol for the preparation of Reagent C (Chloroform Isoamylalcohol GREEN)
1ml	DEPC-treated salt solution for the preparation of Reagent D (DEPC-treated Isopropanol Precipitation Solution GREEN)
13ml	DEPC-treated salt solution for the preparation of Reagent E (Salt/Ethanol Wash Solution GREEN)
12ml	Reagent F (DEPC-treated Water for Elution)
12ml	Reagent G (DEPC-treated Water with 0.5mM EDTA)
12ml	Reagent H (DEPC-treated Water with 0.5% SDS)
200µl	Reagent I (Gel Loading Dye)
5ml	Reagent J (LiCl 12M Solution in DEPC-treated Water)

1. Preparation of Reagent B (Phenol Acid Reagent GREEN)

The amber glass bottle labelled Reagent B contains a buffered salt solution, pH4, that is ready for the addition of high grade phenol. The phenol should be from a fresh unopened bottle of water saturated phenol or from a non-saturated source that is solid at room temperature. Solid phenol must be heated to 50°C until liquefied. Add an equal volume of water (phenol will usually dissolve 15-20ml of water per 100ml of phenol) to saturate it before adding to 16.5ml of the buffered salt solution premeasured in the Reagent B bottle. You will need to add **55ml** of water-saturated phenol. Mix the phenol and the salt solution and let it stand overnight or until there is an obvious interface. Reagent B is stable for 1-2 months at 4°C or until it develops colour in which case it should not be used.

2. Preparation of Reagent C (Chloroform Isoamyl Alcohol GREEN)

The amber glass bottle labelled Reagent C contains 2.5ml of isoamyl alcohol. Add 60ml of chloroform and mix to make 62.5ml of a 24:1 mixture. Once the chloroform has been added, the solution is ready to be used. Store tightly capped at room temperature.

1. Wallace, D.M., *Methods in Enzymology* (Breger, Kimmel, Eds.) 152, 38.
2. Cheung, A.L., Eberhardt, K.J., and Fischetti, V.A. (1994) *Anal. Biochem.* 222, 511.
3. Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156.

3. Preparation of Reagent D (Isopropanol Precipitation Solution GREEN)

Add 54ml isopropanol to the bottle labelled Reagent D which already contains 1ml of DEPC-treated salt solution, to make a total of 55ml. Shake and store tightly capped at room temperature.

4. Preparation of Reagent E (Salt/Ethanol Wash Solution GREEN)

Add 42ml ethanol to the bottle labelled Reagent E which already contains 13ml of RNase-free salt solution, to make a total of 55ml. Shake and store tightly capped at room temperature.

EXPERIMENTAL PROCEDURE

Notes: Wear gloves during the entire procedure to avoid introducing RNases
Wear safety glasses when working with phenol.

1. Add 500µl of Reagent A (Chaotropic RNA Stabilising Reagent GREEN)
   Add 500µl of Reagent B (Phenol Acid Reagent GREEN) (bottom phase)
   Add 100µl of Reagent C (Chloroform Isoamyl Alcohol GREEN)
   to the HYBAID RiboLyser™ matrix tube and chill on ice for 10 mins.

2. Add 50-250mg of tissue to HYBAID RiboLyser™ GREEN tube (or pellet 10^7 cells and suspend in 100µl of PBS and add resuspended cells to the HYBAID RiboLyser™ GREEN tube and reagents.
   Note: The volumes are calculated to leave an air space of approximately 0.5cc. If less air space is present there is a likelihood of sample loss due to tube failure or deformation around the cap allowing sample to bubble out. Sample loss is caused by an increase in pressure due to temperature rise during HYBAID RiboLyser™ runs, especially during high speed settings or long runs where the temperature inside the tube can exceed the boiling point of chloroform (61°C). The presence of 0.5cc of air space in the tube is sufficient to prevent sample loss during routine HYBAID RiboLyser™ runs.

3. Place tube in HYBAID RiboLyser™ instrument and process tissue culture cells, whole tissues, flowers, fruits or seedlings for 20 seconds at a speed rating of 5. Additional time (up to 2 minutes) is usually required for stems, leaves, seeds or roots.

4. Remove tube from the instrument and place on ice for 10 minutes.
   Note: If tube is opened without cooling the positive pressure will cause some of the sample to squirt out and be lost. It is not necessary to cool below room temperature because the RNA is stabilised by the reagents added.

5. Spin in a microcentrifuge for >5 minutes to separate phases. (Extending spin to 15 minutes can enhance elimination of DNA and excessive debris from large samples or from cells with complex cell walls).

Note: Check that tubes are balanced by weight and that the bottom or side of the tubes will not scrape the wall of your microcentrifuge as this will cause rapid loss of sample. If clearance is questionable, transfer the solution mixture (not the lysing matrix) with a pipette to a 1.5ml microcentrifuge tube before spinning.

6. Remove the top phase to a microcentrifuge tube avoiding the interphase. (Leave 10-15% of the solution behind to minimise disturbance of the interphase and contamination of final sample with protein and DNA).
Note: An additional extraction with 0.5 volumes Phenol Acid Reagent GREEN can be performed at this stage for improved quality RNA.

7. Add 300µl of Reagent C (Chloroform Isoamyl Alcohol). Vortex for 10 seconds. Place on ice for 5 minutes. Centrifuge at high speed for 2 minutes to separate phases. Remove top phase to a microcentrifuge tube avoiding interphase material.

8. Add 500µl of Reagent D (DEPC-treated Isopropanol Precipitation Solution), mix and incubate at room temperature for 1-2 minutes. Centrifuge for 5 minutes to pellet precipitated RNA. (Pellet should be quite large.)

9. Wash pellet once with 500µl of Reagent E (Salt/Ethanol Wash) or twice with 250µl for maximum efficiency: Add Reagent E and swirl tube (it is not necessary to resuspend pellet), centrifuge briefly and remove liquid with small bore pipette tip. Air dry pellet for 5-10 minutes.

10. Dissolve pellet in 50-100µl of Reagent F (DEPC-treated water). Alternatively, depending on subsequent needs, use DEPC-treated water and EDTA (Reagent G) or SDS (Reagent H) or a combination to dissolve pellet. (Solutions containing EDTA and SDS will inhibit nucleases to some extent if introduced accidentally. Store purified RNA at -70°C.

Optional: For added purity an optional lithium chloride precipitation can be performed to minimise carbohydrate contamination of the final RNA sample. However, this is not recommended if cDNA libraries are being constructed. Add 20µl of Reagent J (LiCl solution) to each 100µl of the dissolved RNA in step 10. Incubate 5-15 mins. on ice (or in refrigerator overnight, or indefinitely as a storage method). Centrifuge for 5 mins. Wash pellet with Reagent E (Salt/ethanol wash) as in step 9. Carefully remove solution with a small bore pipette tip. Air dry for 5 mins. Resuspend as in step 10.

Anticipated results: 20-100µg total RNA from 10^7 cells or 100mg of tissue.

In the event of any queries or for further information, please contact HYBAID Ltd., 111-113 Waldegrave Road, Teddington, Middlesex TW11 8LL. Tel: 0181 614 1000 Fax: 0181 977 0170

Protocol version 5/96
This kit is for research use only.

Appendix iii: Cloning of PCR products: The pGEM® -T Easy Vector System (Promega)

I. Introduction

The pGEM®†-T and pGEM®-T Easy Vector Systems are convenient systems for the cloning of PCR products. The vectors are prepared by cutting Promega's pGEM®-5Zf(+) and pGEM®-T Easy Vectors with *Eco*R V and adding a 3′ terminal thymidine to both ends. These single 3′-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (1,2). As summarized in Table 1, these polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3′-ends of the amplified fragments (3,4).

The high copy number pGEM®-T and pGEM®-T Easy Vectors contain T7 and SP6 RNA Polymerase promoters flanking a multiple cloning site (MCS) within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates. The multiple cloning region of the two vectors includes restriction sites conveniently arranged for use with Promega's Erase-a-Base® System (Cat.# E5750) for generating nested sets of deletions.

Both the pGEM®-T and pGEM®-T Easy Vector contain multiple restriction sites within the MCS. These restriction sites allow for the release of the insert by digestion with a single restriction enzyme. The pGEM®-T Easy Vector MCS is flanked by recognition sites for the restriction enzymes *Eco*R I, *BstZ* I and *Not* I, thus providing three single-enzyme digestions for release of the insert, while the pGEM®-T Vector cloning site is flanked by recognition sites for the enzyme *BstZ* I. Alternatively, a double-digestion may be used to release the insert from either vector.

The pGEM®-T and pGEM®-T Easy Vectors also contain the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA (ssDNA; see Section VII). The ssDNA molecule exported corresponds to the bottom strand shown in Figure 1A and 1B for the pGEM®-T and pGEM®-T Easy Vectors (nonrecombinant), respectively.

Table 1. Comparison of Thermostable DNA Polymerases.

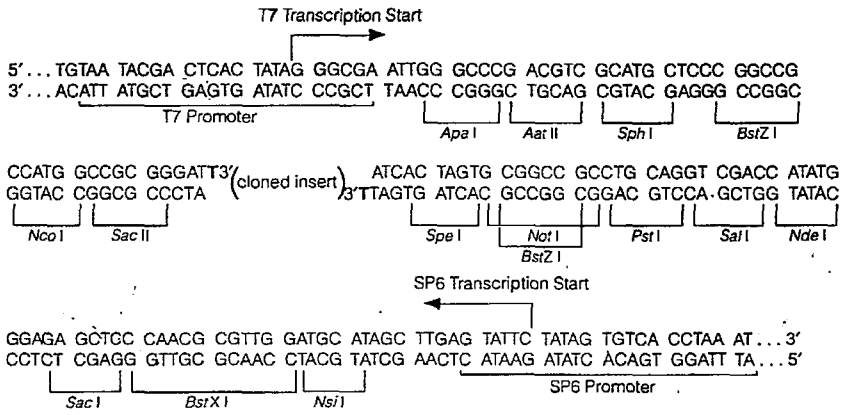
Characteristic	*Taq*‡**	*Tfl***	*Tth***	*Tli*** (Vent$_R$®)	Deep Vent$_R$®**	*Pfu***	*Pwo***
Resulting DNA ends	3′ A	3′ A	3′ A	>95% Blunt	>95% Blunt	Blunt	N.A.
5′→3′ exonuclease activity	Yes	Yes	Yes	No	No	No	No
3′→5′ exonuclease activity	No	No	No	Yes	Yes	Yes	Yes

‡AmpliTaq®
N.A.: not available

†U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

**Some applications in which this product may be used are covered by patents issued and applicable in certain countries. Because purchase of this product does not include a license to perform any patented application, users of the product may be required to obtain a patent license depending upon the particular application and country in which the product is used. For more specific information, please contact Promega.

Panel A



Panel B

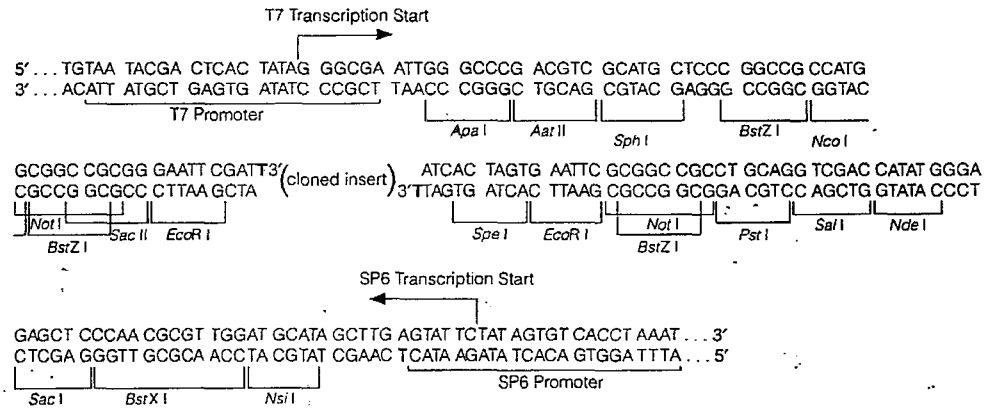
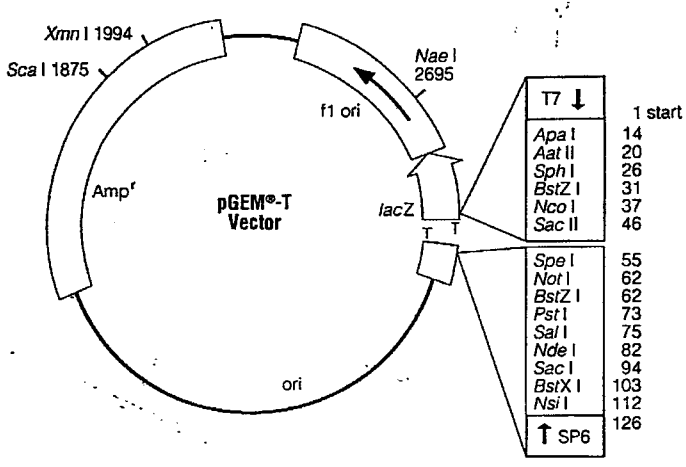


Figure 1. The promoter and multiple cloning sequence of the pGEM®-T (Panel A) and pGEM®-T Easy (Panel B) Vectors. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA Polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA Polymerase.



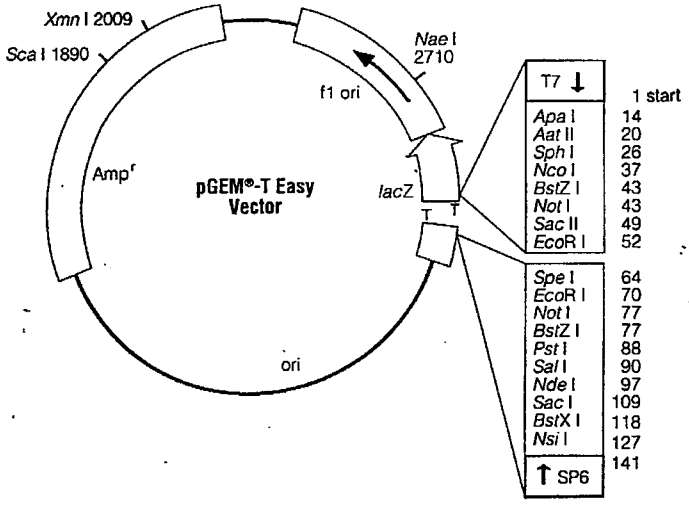


Figure 2. pGEM®-T and pGEM®-T Easy Vector circle maps.

Figure 2 Circle Map Notes:

pGEM®-T Vector Sequence reference points:	
T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	126
T7 RNA Polymerase promoter	2987-6
SP6 RNA Polymerase promoter	121-143
multiple cloning site	10-113
lacZ start codon	165
lac operon sequences	2824-2984, 151-380
lac operator	185-201
β-lactamase coding region	1322-2182
phage f1 region	2368-2823
binding site of pUC/M13 Forward Sequencing Primer	2944-2960
binding site of pUC/M13 Reverse Sequencing Primer	161-177

pGEM®-T Easy Vector Sequence reference points:	
T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter	3002-6
SP6 RNA Polymerase promoter	136-158
multiple cloning site	10-128
lacZ start codon	180
lac operon sequences	2839-2999, 166-395
lac operator	100-216
β-lactamase coding region	1337-2197
phage f1 region	2383-2838
binding site of pUC/M13 Forward Sequencing Primer	2959-2975
binding site of pUC/M13 Reverse Sequencing Primer	176-192

Specialized applications of the pGEM®-T and pGEM®-T Easy Vectors:

1. cloning PCR products
2. used with the Erase-a-Base® System for construction of unidirectional nested deletions
3. ssDNA production
4. blue/white screening for recombinants
5. transcription *in vitro* from dual opposed promoters (For protocol information, please request Promega's *Riboprobe®* in vitro *Transcription Systems*†† *Technical Manual,* #TM016.)

Use the T7 Promoter Primer or the pUC/M13 Forward Primer to sequence ssDNA produced by the pGEM®-T and pGEM®-T Easy Vectors.

††U.S. Pat. No. 5,552,302 has been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI). Inhibitors of Angiogenin, which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows of Harvard College and exclusively licensed to Promega Corporation.

II. Ordering Information

Product	Size	Cat.#
pGEM®-T Vector System I	20 reactions	A3600

Includes:

- 1.2µg pGEM®-T Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl T4 DNA Ligase 10X Buffer

Storage Conditions: Store all components at –20°C or –70°C. pGEM®-T Vector System I is guaranteed for at least 6 months from date of purchase when stored and handled properly.

Product	Size	Cat.#
pGEM®-T Vector System II	20 reactions	A3610

Includes:

- 1.2µg pGEM®-T Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl T4 DNA Ligase 10X Buffer
- 1.2ml JM109 Competent Cells, High Efficiency (6 x 200µl)

Storage Conditions: Store the Competent Cells at –70°C. All other components can be stored at –20°C or –70°C. pGEM®-T Vector System II is guaranteed for at least 6 months from date of purchase when stored and handled properly.

Product	Size	Cat.#
pGEM®-T Easy Vector System I	20 reactions	A1360

Includes:

- 1.2µg pGEM®-T Easy Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl T4 DNA Ligase 10X Buffer

Storage Conditions: Store all components at –20°C or –70°C. pGEM®-T Easy Vector System I is guaranteed for at least 6 months from date of purchase when stored and handled properly.

Product	Size	Cat.#
pGEM®-T Easy Vector System II	20 reactions	A1380

Includes:

- 1.2µg pGEM®-T Easy Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl T4 DNA Ligase 10X Buffer
- 1.2ml JM109 Competent Cells, High Efficiency (6 x 200µl)

Storage Conditions: Store the Competent Cells at –70°C. All other components can be stored at –20°C or –70°C. pGEM®-T Easy Vector System II is guaranteed for at least 6 months from date of purchase when stored and handled properly.

III. General Considerations

A. PCR Product Purity

An aliquot of the PCR reaction should be analyzed on an agarose gel before using it in the ligation reaction. The PCR product to be ligated can be gel-purified or directly purified using the Wizard® PCR Preps DNA Purification System (Cat.# A7170) or used directly from the reaction. Exposure to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers. If smearing of the PCR product or inappropriate banding is observed on the gel, excise the bands to be cloned from a low-melt agarose gel and purify the DNA with Wizard® PCR Preps or Agar*ACE*™ Agarose-Digesting Enzyme (Cat.# M1741). Even if distinct bands of expected size are observed, primer-dimers should be removed. Wizard® PCR Preps can be used to purify the bands of interest directly from the reaction mix. Use of crude PCR product may also produce successful ligations in some cases; however, the number of white colonies containing the relevant insert may be reduced due to preferential incorporation of primer-dimers or other extraneous reaction products. It may, therefore, be necessary to screen numerous colonies in order to identify clones which contain the PCR product of interest.

B. Optimizing Insert:Vector Molar Ratios

The pGEM®-T and pGEM®-T Easy Vector Systems have been optimized using a 1:1 molar ratio of the Control Insert DNA to the Vectors. However, ratios of 8:1 to 1:8 have been successfully used. If initial experiments with your PCR product are suboptimal, ratio optimization may be necessary. Ratios from 3:1 to 1:3 provide good initial parameters. The concentration of PCR product should be estimated by comparison to DNA mass standards on a gel or by using a fluorescent assay (5). The pGEM®-T and pGEM®-T Easy Vectors are approximately 3kb and are supplied at 50ng/µl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Sufficient pGEM®-T or pGEM®-T Easy Vector is provided to vary insert:vector ratios as recommended and to perform control reactions.

Example of insert:vector ratio calculation:

How much 0.5kb PCR product should be added to a ligation in which 50ng of 3.0kb vector will be used if a 3:1 insert:vector molar ratio is desired?

$$\frac{50\text{ng vector} \times 0.5\text{kb insert}}{3.0\text{kb vector}} \times \frac{3}{1} = 25\text{ng insert}$$

Note: Using the same parameters for a 1:1 insert:vector molar ratio, 8.3ng of a 0.5kb insert would be required.

C. Screening Transformants for Inserts

Successful cloning of an insert in the pGEM®-T and pGEM®-T Easy Vectors interrupts the coding sequence of β-galactosidase; recombinant clones can usually be identified by color screening on indicator plates. However, the characteristics of PCR products cloned into the pGEM®-T and pGEM®-T Easy Vectors can significantly affect the ratio of blue:white colonies obtained following transformation of competent cells. Clones which contain PCR products, in most cases, produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lacZ* gene. Such fragments are usually a multiple of 3 base pairs long (including the 3′-A overhangs) which do not contain in-frame stop codons. It has been reported in the literature that DNA fragments up to 2kb have been cloned in-frame and produced blue colonies.

Even if your PCR product is not a multiple of 3 bases long, the amplification process can introduce mutations (e.g., deletions or point mutations) that may result in blue colonies when competent cells are transformed with the fragment inserted into the pGEM®-T or pGEM®-T Easy Vectors.

The Control Insert DNA supplied with the pGEM®-T and pGEM®-T Easy Systems is a 542bp fragment from Promega's pGEM®-*luc* DNA. This sequence has been mutated to contain multiple stop codons in all six reading frames, which ensures a low background of blue colonies for the control reaction. Results obtained with the Control Insert DNA may not be representative of those achieved with your PCR product.

D. Experimental Controls

Promega strongly recommends performing the controls detailed below. These are necessary to accurately assess the performance of the pGEM®-T and pGEM®-T Easy Vector Systems.

Positive Control

Set up a ligation reaction with the Control Insert DNA as described in the protocol (Section IV.A) and use it for transformations as described in Section V. This control will allow you to determine whether the ligation is proceeding efficiently. Typically, approximately 100 colonies should be observed, 10-30% of which are blue, when competent cells that have a transformation efficiency of 1 x 10^8 cfu/µg DNA are transformed. Greater than 60% of the colonies should be white, and therefore recombinant since the Control Insert DNA is designed to reduce the number of background blue colonies (discussed in Section III.C). Background blue colonies arise from non-T-tailed or undigested pGEM®-T or pGEM®-T Easy Vector. These blue colonies are a useful internal transformation control; if no colonies are obtained, the transformation has failed. If blue colonies are obtained, but no whites, the result suggests that the ligation reaction failed. If <50% white colonies are seen in this positive control reaction, then the ligation conditions were probably suboptimal.

The concentration of the Control Insert DNA has been optimized such that 2µl (4ng/µl) can be used in a 10µl ligation reaction to achieve a 1:1 molar ratio with 50ng of the pGEM®-T or pGEM®-T Easy Vectors.

Background Control

Set up a ligation reaction with 50ng of pGEM®-T or pGEM®-T Easy Vector and no insert as described in the protocol (Section IV.A) and use it for transformations as described in Section V. This ligation will allow determination of the number of background blue colonies resulting from non-T-tailed or undigested pGEM®-T or pGEM®-T Easy Vector alone. If the recommendations in Section V are followed closely, 10-30 blue colonies will, typically, be observed if the transformation efficiency of the competent cells is 1 x 10^8 cfu/µg DNA. (Under these conditions, cells that are 1 x 10^7 cfu/µg DNA would yield 1-3 blue colonies and cells with a transformation efficiency of 1 x 10^9 cfu/µg DNA would yield 100-300 blue colonies). Compare the number of blue colonies obtained with this background control to the number of blue colonies obtained in the standard reaction using the PCR product. If ligation of the PCR product yields dramatically more blue colonies than the background control reaction, then recombinants are probably among these blue colonies (see Section III.C).

Transformation Control

Check the transformation efficiency of the competent cells by transforming them with an uncut plasmid and calculating cfu/µg DNA. If the transformation efficiency is lower than 1 x 10^8 cfu/µg DNA, prepare fresh cells (Competent cells are available from Promega. See Section X.B). If you are not using JM109 High Efficiency Competent Cells (provided with pGEM®-T and pGEM®-T Easy Vector Systems II; Cat.# A3610 and A1380, respectively), be sure the cells are compatible with blue/white screening and standard ampicillin selection, and are at least 1 x 10^8 cfu/µg DNA.

Example of transformation efficiency calculation:

After 100µl competent cells are transformed with 0.1ng uncut plasmid DNA, the transformation reaction is added to 900µl of SOC medium (0.1ng DNA/ml). From that volume, a 1:10 dilution with SOC medium (0.01ng DNA/ml) is made and 100µl plated on two plates (0.001ng DNA/100µl). If 200 colonies are obtained (average of two plates), what is the transformation efficiency?

$$\frac{200\text{cfu}}{0.001\text{ng}} = 2 \times 10^5 \text{ cfu / ng} = 2 \times 10^8 \text{ cfu / µg DNA}$$

IV. Ligations Using the pGEM®-T and pGEM®-T Easy Vectors

A. Protocol

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tube.

2. Set up ligation reactions as described below. **Note:** Use 0.5ml tubes known to have low DNA-binding capacity (e.g., Sarstedt Cat.# 72687005).

	Standard Reaction	Positive Control	Background Control
T4 DNA Ligase 10X Buffer	1µl	1µl	1µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl*	—	—
Control Insert DNA	—	2µl	—
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
deionized water to a final volume of	10µl	10µl	10µl

*Molar ratio of PCR product:vector may require optimization (see Section III.B).

3. Mix the reactions by pipetting. Incubate the reactions overnight at 4°C.

Notes:

1. Use only Promega T4 DNA Ligase supplied with this system in performing pGEM®-T and pGEM®-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities which may remove the terminal thymidines from the vector.

2. T4 DNA Ligase 10X Buffer contains ATP which degrades during temperature fluctuations. Avoid multiple freeze-thaws by making single-use aliquots of the buffer.

3. If a precipitate is present in the thawed T4 DNA Ligase 10X Buffer, vortex the buffer until the precipitate is back in solution. (It may help to warm the solution by rolling the tube between your fingers before vortexing.)

4. Low temperature ligations are necessary for annealing of single-base overhangs. Ligation temperatures higher than 15°C may significantly reduce the number of recombinants. In our experience, ligations incubated at temperatures higher than 15°C give rise to a high background of blue colonies resulting from optimization of blunt-end ligations of non-T-tailed vector.

5. Shorter incubation times (**minimum** of 3 hours at room temperature) may be used, but may result in lower colony numbers.

V. Transformations Using the pGEM®-T and pGEM®-T Easy Vector Ligation Reactions

Use high efficiency competent cells (1 x 10^8 cfu/µg DNA) for transformations. The ligation of fragments with a single-base overhang can be inefficient, so it is essential to use cells that are 1 x 10^8 cfu/µg DNA (or higher) in order to obtain a reasonable number of colonies (see Section III.D).

We recommend using JM109 High Efficiency Competent Cells (Cat.# L2001); these are provided with the pGEM®-T and pGEM®-T Easy Vector Systems II. Other host strains may be used, but they should be compatible with blue/white color screening and standard ampicillin selection. JM109 cells should be maintained on M9 minimal medium plates supplemented with thiamine hydrochloride prior to the preparation of competent cells. This selects for the presence of the F′ episome which carries both the *pro*AB genes, which complement proline auxotrophy in a host with a (*pro*AB) deletion, and *lacI*^{q}ZΔM15, which is required in the blue/white color screening process. **If you are using competent cells other than JM109 High Efficiency Competent Cells purchased from Promega, it is important that the appropriate transformation protocol be followed.** Selection for transformants should be on LB/ampicillin/IPTG/X-Gal plates (see Section X.A). For best results, do not use plates more than 30 days old.

The genotype of JM109 is *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_{K-},m_{K+}), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lacI*^{q}ZΔM15] (6).

A. Protocol

Reagents to Be Supplied by the User
(Solution compositions are provided in Section X.A.)
- LB plates with ampicillin/IPTG/X-Gal
- SOC medium

1. Prepare 2 LB/ampicillin/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency (see Section III.D). Equilibrate the plates to room temperature prior to plating (Step 10).

2. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 2µl of each ligation reaction to (a) sterile 1.5ml microcentrifuge tube(s) on ice (see Note 1). Set up another tube on ice with 0.1ng uncut plasmid for determination of the transformation efficiency of the competent cells (see Section III.D).

3. Remove tube(s) of frozen JM109 High Efficiency Competent Cells from –70°C storage and place them in an ice bath until just thawed (about 5 minutes). Mix the cells by **gently** flicking the tube.

4. **Carefully** transfer 50µl of cells into each tube prepared in Step 2 (100µl cells for determination of transformation efficiency). **Avoid excessive pipetting as the cells are very fragile.**

5. **Gently** flick the tubes to mix and place them on ice for 20 minutes.

6. Heat shock the cells for 45-50 seconds in a water bath at exactly 42°C (DO NOT SHAKE).

7. Immediately return the tubes to ice for 2 minutes.

8. Add 950µl room temperature SOC medium to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid (LB broth may be substituted, but colony number may be lower).

9. Incubate for 1.5 hours at 37°C with shaking (~150rpm).

10. Plate 100µl of each transformation culture onto duplicate antibiotic plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 x g for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of 2 plates.

11. Incubate the plates overnight (16-24 hours) at 37°C. In our experience approximately 100 colonies per plate are routinely seen when using competent cells that are 1 x 10^8 cfu/µg DNA, if 100µl is plated. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to facilitate blue/white screening. White colonies generally contain inserts; however, inserts may also be present in blue colonies. Please see Section III.C for more information.

Notes:

1. In our experience the use of larger (17 x 100mm) polypropylene tubes (e.g., Falcon Cat.# 2059) has been observed to increase transformation efficiency. Tubes from some manufacturers bind DNA, thereby decreasing the colony number, and should be avoided.

2. Colonies containing β-galactosidase activity may grow poorly relative to cells lacking this activity. After overnight growth, the blue colonies may be smaller than the white colonies which are approximately one millimeter in diameter.

VI. Isolation of Recombinant Plasmid DNA

A standard plasmid miniprep procedure, which takes 30-60 minutes to perform, is described in Promega's *Protocols and Applications Guide* (7). Various other miniprep protocols are available, but few have proven to be consistently reliable. The miniprep process can be both laborious and time-consuming, particularly when large numbers of minipreps are required. A convenient and reliable method is the Wizard® *Plus* Minipreps DNA Purification System.

The Wizard® line of DNA purification products offers five alternatives for plasmid DNA preparation which are distinguished by the scale of the isolation desired (see Table 2). Wizard® *Plus* Miniprep DNA isolations can be completed in 15 minutes and the DNA is ready for other molecular biology applications without prior ethanol precipitations. For best results, a vacuum manifold, such as Promega's Vac-Man® (Cat.# A7231) or Vac-Man® Jr. (Cat.# A7660) Laboratory Vacuum Manifold, should be used to process the minipreps. If a vacuum source is not available, the minipreps may be

processed individually using a disposable 3ml Luer-Lok® syringe and a microcentrifuge. A vacuum source and manifold is required for the Wizard® *Plus* Midipreps, Maxipreps, Megapreps and Series 9600™ DNA Purification Systems. Refer to Section X.B for ordering information.

Table 2. Wizard® *Plus* Plasmid DNA Purification Systems Selection Guide.

Wizard® *Plus* DNA Purification System	Number of Isolations	Culture Volume	Typical DNA Yields*
Minipreps	50-250	1-3ml	3-10µg
Midipreps	25	10-100ml	10-200µg
Maxipreps	10	100-500ml	300-1,000µg
Megapreps	5	500-1,000ml	700-3,000µg
Series 9600™	8-192	1-5ml	5-20µg

*Typical DNA yields are based on results obtained with high copy number plasmids.

VII. Generation of Single-Stranded DNA from the pGEM®-T and pGEM®-T Easy Vectors

For induction of ssDNA production, bacterial cells containing either the pGEM®-T or pGEM®-T Easy Vector are infected with an appropriate helper phage. The plasmid then enters the f1 replication mode and the resulting ssDNA is exported as an encapsulated virus-like particle. The ssDNA is purified from the supernatant by simple precipitation and extraction procedures which are described in detail in Technical Bulletin #TB187, *pGEM® Vector Cloning and Single-Stranded DNA Generation.* For further information, please contact your local Promega Branch Office or Distributor. In the U.S., contact Technical Services at 1-800-356-9526.

VIII. Troubleshooting

Symptoms	Possible Causes	Comments
No colonies	A problem has occurred with the transformation reaction or the cells have lost competence	Background undigested vector and religated non-T-tailed vector should yield 10-30 blue colonies, independent of the presence of insert DNA. Check the background control (Section III.D).
		Use high efficiency competent cells (1 x 10^8 cfu/µg DNA). Test the efficiency of the cells by transforming them with an uncut plasmid that allows for antibiotic selection, such as the pGEM®-5Zf(+) Vector. If the recommendations in Section V.A are followed, cells that are 1 x 10^8 cfu/µg DNA typically yield 100 colonies. Therefore, you would not see any colonies from cells that are 1 x 10^6 cfu/µg DNA (Section III.D).

Appendix iv: Sequencing of PCR products (ThermoSequenase Amersham Life Science)

INTRODUCTION

This sequencing kit combines two revolutionary innovations for sequencing DNA using radioactive labels. First, the label is incorporated into the DNA sequencing reaction products by the use of four [α-^{33}P]dideoxynucleotide (ddNTP) terminators (G,A,T,&C). The labeled ddNTP's are more efficient for labeling sequencing experiments than other labeled nucleotides because they specifically label only the properly terminated DNA chains. Also, since prematurely terminated chains are not labeled, 'stop' artifacts and most background bands are eliminated. As an additional benefit, the absence of artifact bands allows the routine use of dITP, which can eliminate even very strong compression artifacts.

The second innovation is the use of Thermo Sequenase DNA polymerase. This enzyme has been engineered to efficiently incorporate dideoxynucleotides, allowing the use of very low amounts of isotope ([α-^{33}P]ddNTP) for the termination reactions. Thermo Sequenase DNA polymerase is also thermostable and performs very well in convenient and sensitive cycle or non-cycle sequencing protocols. This polymerase produces very uniform band intensities (with dGTP), so mixed sequences (such as those of heterozygotes) can be easily identified.

Thus, the kit offers:

- Clean, background free sequences
- Complete elimination of compressions
- Efficient use of labeled nucleotides, less than 1μCi per sequence
- Convenient single-step protocol
- Uniform band intensities for identification of mixed sequences (*e.g.* heterozygotes)
- Sensitive cycle-sequencing protocols for sequencing 20fmol or less of template
- Overnight exposures with ordinary autoradiography film—same day possible with fast films
- Exceptionally easy-to-read sequences
- ^{33}P for sharp autoradiogram resolution
- Can hold samples for 1-2 days prior to gel running

Chain termination sequencing

This kit is designed to eliminate sequencing artifacts such as stops (or BAFLs—bands across four lanes) and background bands. BAFLs can result from the enzyme pausing at regions of secondary structures in GC-rich templates, producing prematurely aborted primer extension products of the same length. Background bands can be caused by primer extensions aborting prematurely at random positions, such as when a template is rich in a certain base and the complementary nucleotide in the reaction becomes depleted.

Traditional chain termination sequencing methods (1) involve the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at the site where a primer anneals to the template. Elongation of the 3' end of the annealed primer is catalyzed by a DNA polymerase in the presence of 2'-deoxynucleoside-5'-triphosphates (dNTPs), and is terminated by the incorporation of a 2',3'-dideoxynucleoside-5'-triphosphate nucleotide analog (ddNTP) that will not support continued DNA elongation (hence the name 'chain termination'). Four separate reactions, each with a different ddNTP, (ddG, ddA, ddT, or ddC) give complete sequence information. A radiolabeled dNTP (2,3) or primer is normally included in the synthesis, so the labeled chains of various lengths can be visualized after separation by high-resolution gel electrophoresis (4,5). In this kit, a radioactive label is incorporated into the sequencing reaction products at the 3' end by the use of an [α-^{33}P]ddNTP, thus ensuring that only properly terminated DNA strands are labeled and are visible in the sequence. This results in a cleaner, more reliable, and easier to read sequence with fewer background bands and virtually no BAFLs.

The accuracy and readability of the sequence obtained depends strongly on the properties of the polymerase used for chain termination. Some polymerases, such as T7 Sequenase™ version 2.0 DNA polymerase, generate much more uniform, readable bands than others like Klenow and *Taq* DNA polymerase (6,7,8). Thermostable polymerases, such as *Taq* polymerase, can be used for multiple rounds (cycles) of DNA synthesis, generating stronger signals. Tabor and Richardson (9) have discovered that DNA polymerases can be modified to accept dideoxynucleotides as readily as the normal deoxynucleotide substrates. Using this technology, Amersham has developed a new DNA polymerase for DNA sequencing. This enzyme, called Thermo Sequenase DNA polymerase, is thermostable and possesses many of the excellent DNA sequencing qualities of T7 Sequenase DNA polymerase. The properties of this DNA polymerase include activity at high temperature and absence of associated exonuclease activity. Like T7 Sequenase DNA polymerase, it readily uses dideoxynucleoside triphosphates, generating uniform band intensities in sequencing experiments (with dGTP). These properties make the enzyme ideal for generating high-quality DNA sequences using cycle-sequencing methods. It is stable at 90°C for at least 1 hour and retains 50% of its activity when incubated at 95°C for 60 minutes. The Thermo Sequenase polymerase in this kit combines the advantages of both T7 Sequenase DNA polymerase and *Taq* DNA polymerase. It produces bands (with Mg^{2+}) that are nearly as uniform as those produced with T7 Sequenase DNA polymerase with Mn^{2+} (10), yet is thermostable like *Taq* DNA polymerase.

Cycle sequencing is the name given to the process of using repeated cycles of thermal denaturation, primer annealing, and polymerization to produce greater amounts of product in a DNA sequencing reaction. This amplification process employs a single primer so the amount of product DNA increases linearly with the number of cycles. (This distinguishes it from PCR* which uses 2 primers so that the amount of product can increase exponentially with the number of cycles.)

The earliest examples of cycle sequencing used ^{32}P-labeled primers and a non-thermostable polymerase which was added after each denaturation cycle (11,12). Later improvements included the use of thermostable *Taq* polymerase (13,14) and the use of alpha-labeled dNTPs in place of the labeled primer using mixtures of nucleotides similar to those used originally by Sanger (15,16). The labeled-primer methods make efficient use of ^{32}P giving a sequence with as little as 4µCi of [γ-^{32}P]ATP (14). The methods using internally-labeled products were less efficient, requiring 20µCi of [α-^{35}S]dATP for a sequence. This is a consequence of the relatively low specific radioactivity and the small number of labeled bases in short product molecules. This kit makes very efficient use of [α-^{33}P]ddNTP, requiring less than 1µCi of ^{33}P per sequence. Cycle sequencing is necessary with this kit when using less than 0.2-0.5pmol of template DNA. Non-cycle (or very few cycle) protocols may be used with more than ~0.5pmol of template.

MATERIALS NOT SUPPLIED

Necessary reagents:

Water—Only deionized, distilled water should be used for the sequencing reactions.

Specialized sequencing primers—Some sequencing projects will require the use of primers which are specific to the project. For most sequencing applications, 0.5-2.5pmol of primer should be used for each set of sequencing reactions. Always determine the concentration of the primer by reading the optical density at 260nm (OD_{260}). If the primer has N bases, the approximate concentration (pmol/µl) is given by the following formula:

Concentration (pmol/µl)=OD_{260}/(0.01 x N) where N is the number of bases.

Gel reagents—Sequencing gels should be made only from fresh solutions of acrylamide and bis-acrylamide. Other reagents should be electrophoresis grade materials.

Necessary equipment:

Liquid handling supplies such as vials, pipettes and a microcentrifuge—All sequencing reactions are run in plastic microcentrifuge tubes (typically 0.5ml) suitable for thermal cycling.

Electrophoresis equipment—While standard, non-gradient sequencing gel apparatus is sufficient for much sequencing work, the use of field-gradient

*See license information on back cover.

('wedge') or salt-gradient gels will allow much greater reading capacity on the gel (4,5,17). A power supply offering constant voltage operation at 2000V or greater is essential.

Gel handling—For ^{33}P sequencing, a large tray for washing the gel (to remove urea) and a gel drying apparatus are highly recommended. For best results, gels containing ^{33}P must be exposed dry in direct contract with the film at room temperature.

Autoradiography—Any large format autoradiography film such as Amersham's Hyperfilm™ MP (RPN 30) or Hyperfilm βmax (RPN 11), and a large film cassette.

Thermal cycler—Sequencing will require thermally cycled incubations between 50°C and 95°C (1-100 cycles).

BRIEF PROTOCOL FOR SEQUENCING CLONES IN PLASMID OR M13 VECTORS

1. **Termination mixes**— Mix 2µl of termination master mix (either dGTP or dITP—see note below) and 0.5µl of [α-^{33}P]ddNTP to produce a termination mix for each ddNTP. Label, fill and cap four tubes ('G', 'A', 'T', 'C') with 2.5µl of each termination mix. It is more accurate and convenient to prepare batches of termination mixes sufficient for all sequences to be performed, then dispense 2.5µl from this batch to each vial for the termination reactions.
   Note: For determination of new sequences, or of sequences with high G-C content, the dITP termination master mix is recommended. This will eliminate all compression artifacts but will result in somewhat uneven band intensities, especially in the 'G' lane. When perfectly uniform band intensities are desired, such as when examining sequences from potentially heterozygous individuals, the dGTP mixture should be used.
2. **Reaction mixture:**

Reaction buffer	2µl	
DNA	3µl	(50-500ng or 25-250fmol)
Primer	3µl	(0.5-2.5pmol)
H_2O	__µl	(To adjust total volume to 20µl)
Thermo Sequenase polymerase (4U/µl)	2µl	(8 units polymerase-add LAST)
Total	20µl	

3. **Cycling termination reactions**
   Transfer 4.5µl of reaction mixture (prepared in step 2) to each termination tube ('G', 'A', 'T' and 'C') from step 1. Mix well and overlay with 10-20µl of mineral oil (if needed). Cap and place the tube in the thermal cycling instrument. Cycle 30-60 times as follows (conditions are appropriate for the kit control primer—fewer (1-10) cycles may produce better results when using 200-500fmol DNA):

dGTP	dITP
95°C, 30s	95°C, 30s
55°C, 30s	50°C, 30s
72°C, 1min	60°C, 5-10min

4. Add 4µl of stop solution to each termination reaction (see alternative below). Heat samples at 70°C for 2-10 minutes immediately before loading onto glycerol tolerant sequencing gel. Load 3-5µl in each lane using a standard pipette tip, avoiding oil. Do not load oil.
   Alternative: Remove 6µl from each termination reaction avoiding oil and transfer to a fresh vial. Add 3.5µl of stop solution, heat at 70°C for 2-10 minutes and load 3-5µl in each gel lane.

DETAILED SEQUENCING PROTOCOL

General guidelines

- Since the popular multiple cloning sites all derive from similar sequences, one primer can serve for the sequencing of insert DNA in most of the common vectors. Among the vectors compatible with the primer supplied in the Thermo Sequenase radiolabeled terminator cycle sequencing kit are M13mp8, M13mp9, M13mp12, M13mp13, M13mp18, M13mp19, mWB2348, mWB3295, mWB3225, pUC18, pUC19, and virtually any vector featuring blue/white screening with β-galactosidase activity.
- Good sequences can be obtained using as little as 0.05µg of M13 DNA, 0.1µg of plasmid DNA, or 50fmol of PCR product. Mix reagents by gently 'pumping' the pipettor. The total volume of the reaction mix should be 20µl—the volumes of DNA and primer added will depend on their concentration. Adjust the amount of distilled water so that the total volume of DNA, primer and water is 16µl.
- The specific cycling parameters used will depend on the primer sequence and the amount and purity of the template DNA. See cycle conditions below.
- The dGTP termination master mix should be used if the sequence is already known to be free of compression artifacts and the benefits of uniform band intensities are desired. The uniform band intensities can aid in finding heterozygotes or in other cases where mixed sequence may be present. If compressions are a problem when using dGTP, gels containing formamide can be used as described in the 'Denaturing gel electrophoresis' section of this booklet.
- For running sequences where compressions are a problem, the dITP termination master mix included in this kit can be substituted for the dGTP termination master mix. See 'Elimination of compressions' section for details. Note: When using dITP, use an 'extension' temperature of 60°C with a duration of at least 4 minutes.
- Whenever possible, tubes should be kept capped and on ice to minimize evaporation of the small volumes employed. Additions should be made with disposable-tip micropipettes and care should be taken not to contaminate stock solutions. The solutions must be thoroughly mixed after each addition, typically by 'pumping' the solution two or three times with a micropipette, avoiding the creation of air bubbles. At any stage where the possibility exists for some solution to cling to the walls of the tubes, the tubes should be centrifuged. With care and experience these reactions can be completed in 15-20 minutes.

Reaction mixture

Preliminary notes: Prepare the reaction mix on ice. For multiple (n) reactions with different primers and/or templates, prepare a n+1 batch and aliquot; then add the unique primer and/or template in the appropriate concentration and volume to the aliquots. For each termination reaction described below, add 4.5µl of the 20µl reaction mix to the termination tube (with 2.5µl termination mix).

Reaction buffer	2µl	
DNA	__µl*	(50-500ng or 25-250fmol)
Primer	__µl*	(0.5-2.5pmol)
H_2O	__µl	(To adjust total volume to 20µl)
Thermo Sequenase polymerase (4U/µl)	2µl	(8 units polymerase-add LAST)
Total	20µl	

*For the control reaction, use 10µl of control DNA and 1µl of control primer.

Cycling termination reactions

Preliminary notes: Prepare the termination mixes on ice. Prepare termination mixes by combining 2µl of termination master mix (either dGTP or dITP) and 0.5µl of [α-^{33}P]ddNTP (G, A, T, or C—one of each per sequence). Label tubes, place on ice, and add termination reagents. It is more accurate and convenient to prepare batches of termination mixes sufficient for all sequences to be performed, then aliquotting 2.5µl to each vial. Cap tubes. Note: The termination tubes can be left uncapped until all reagents have been added if the tubes are kept on ice and the reaction mixture is added within a few minutes.

1. Have on ice 4 tubes per sequence, labeled 'G', 'A', 'T' and 'C'.
2. Place 2.5µl of the ddGTP termination mix (from batch as described above) in the tube labeled 'G'. Similarly, add 2.5µl of the ddATP, ddTTP and ddCTP termination mixes to the 'A', 'T' and 'C' tubes, respectively. Cap the tubes to prevent evaporation (see option above).

3. Add 4.5µl of the reaction mix from above. Top with 10-20µl mineral oil (or one drop from a 200µl micropipette tip) unless using a thermal cycling system that does not require oil (such as a cycler with a heated lid). Cap the tubes and place them in the thermal cycler. Note: When sequencing single-stranded DNA, the primer may anneal to the template with reduced specificity while the tubes are on ice, and extension of these primers can occur as the thermal cycler heats up during the first cycle. To minimize nonspecific extension products, the cycler can be pre-heated to 85-95°C or pre-cooled to 4°C.
4. Start the cycling program. Note: The specific cycling parameters used will depend on the primer sequence and the amount and purity of the template DNA. For the primers included in the kit and the suggested amount of purified DNA (25-250fmol), cycle 30-60 times as follows:

dGTP	dITP
95°C, 30s	95°C, 30s
60°C, 30s	55°C, 30s
72°C, 60-120s	60°C, 5-10min
(typically 30 cycles taking 2-3h)	(typically 30 cycles taking 3-5h)

Fewer (1-10) cycles may produce better results when using 250-500fmol DNA.

5. Add 4µl of stop solution to each of the termination reactions, mix thoroughly and centrifuge briefly to separate the oil from the aqueous phase. Alternatively, remove 6µl from each termination reaction and transfer to a fresh tube containing 3-4µl of stop solution. Samples may be stored frozen until ready to load the sequencing gel.
6. When the gel is ready for loading, heat the samples to 70°C for 2-10 minutes and load immediately on the gel —3-5µl in each gel lane. NOTE: Heating in open vials will promote evaporation of water from the formamide-reaction mixture. This is not normally necessary, but will increase the signal by concentrating the isotope and will promote more complete denaturation of the DNA. This may improve results when using older ^{35}P ddNTPs. Avoid complete evaporation to dryness by prolonged heating.

Cycle conditions and template quantity

The temperatures used for cycling the termination reactions should be determined from the characteristics of the sequencing primer, the template, and the length of the termination product desired. The number of cycles required will depend on the quantity and quality of the template DNA used. The following guidelines should assist in choosing cycling parameters.

Cycling temperatures

The melting temperature of the primer should be kept in mind when choosing cycle temperatures. The control primer included in the kit is moderately long (23 bases) with 50% G/C content. The melting temperature of this primer is ~73°C under sequencing reaction conditions, and best results are achieved by cycling between 60°C and 95°C. The duration of the steps does not seem to be critical, and even brief pauses at these temperatures seem to be effective (except with dITP as described above).

As another example, when using the universal -40 17-mer, which has a melting temperature of about 50-55°C, cycling between 45°C and 95°C is effective. If in doubt, choose a wide temperature range with brief pauses at the extremes of temperature.

The termination reaction cycles should always have a denaturation temperature of 95-98°C (however, avoid extended steps at 98°C since at this temperature the enzyme has a half-life of less than one hour). Since the optimum temperature for polymerization is about 70-75°C, 72°C is a good choice for the termination step (except when using dITP, which requires a maximum temperature of 55-60°C). An annealing step (*e.g.* <60°C) is required only with primers less than ~24 bases.

Number of cycles and quantity of template

The number of cycles required will primarily depend on the amount of template DNA (in fmols) used for sequencing. It will also depend on the purity of the DNA, and the sensitivity of autoradiographic detection. The minimum quantities of highly-purified DNA which we have been able to sequence using these methods are about 5fmol of M13mp18 DNA and about 15fmol of pUC18 DNA. (For routine sequencing, we recommend 25fmol of M13 and 75fmol of plasmid DNA). When sequencing very small amounts of template, it has been observed that the number of cycles has a strong influence on sequence intensity. Increasing the number of cycles from 30 to 60 will increase the signal significantly when using less than ~50fmol of template DNA, whereas increasing the number of cycles with more than ~100fmol is of little benefit, and may even produce background sequence. So in general, use more cycles when template amounts are limited. Also, a modest improvement can sometimes be achieved by increasing the amount of primer 2-5 fold.

Designing a new sequencing primer

The length of the primer (and its sequence) will determine the melting temperature and specificity. For the cycling temperatures normally used, the primer should be about 18-25 nucleotides long. It is also a good idea to check the sequence of the primer for possible self-annealing (dimer formation could result) and for potential 'hairpin' formation, especially those involving the 3' end of the primer. Finally, check for possible sites of false priming in the vector or other known sequence if possible, again stressing matches which include the 3' end of the primer.

Appendix v: Streptavidin-Biotin Peroxidase Complex (StABCPx) immunostaining technique for fixed, paraffin embedded sections using monoclonal or polyclonal antibodies

Laboratory: LE73 No of Pages: 2 Prepared by: JHD Williams (SOPJ1) Date: 13.12.96

PRINCIPLE: Antigen binds to either a monoclonal or polyclonal antibody which is then detected using a three stage Streptavidin-Biotin peroxidase complex technique. The enzyme label peroxidase is then demonstrated using the chromogen diaminobenzidine (DAB) or 3-amino-9-ethyl-carbazole (AEC).

RISK ASSESSMENT: Observe relevant COSHH requirement for all chemicals and reagents.

REQUIREMENTS :

Equipment: Immunostaining trays, pH meter, balance.

Reagents:

1. Inhibitor for endogenous peroxidase (0.5% H2O2 in methanol):-
   Hydrogen peroxide (30%) 0.2m1
   Methanol 11.8ml

2. TRIS buffered saline pH 7.6 (TBS):- Sodium chloride 80g
   TRIS hydroxymethyl methylamine (TRIS) 6.05g 1M hydrochloric acid 38m1
   RO water to 10L

3. Bovine serum albumin (BSA):-
   To one aliquot (1m1) of 10% BSA (in freezer), add 9m1 TBS.

Related Protocols:
SOP 73/la or b Demonstration of the Enzyme Peroxidase using DAB or AEC.
SOP 73/3a or b Use of Microwaves or Pressure Cooker.
SOP 73/5a or b Use of Trypsin or Pronase.

Quality Control: All new batches of antibodies must be titrated before use (see

SOP73/2).

METHOD/PROCEDURE:

NOTE:

a. For each case (profile of antibodies) include a section in which the primary antibody is replaced with TBS.
b. Where necessary use control sections in which there is likely to be positive and negative staining .

1. De-paraffinise section in xylene (2 x 5 minutes) and take through 100% alcohol to 70% (1 minute each) .

2. Inhibit endogenous peroxidase by treating with freshly prepared inhibitor (Reagent 1) for 10 minutes (30 minutes for tissues containing large amounts of endogenous peroxidase, eg, bone marrow and spleen).

3. Wash well in RO water.

4. Consult antibody register before proceeding:
    a. If antigen retrieval is not necessary , apply 1 % BSA in TBS for 30 minutes (Reagent 3). Drain and apply primary antibody, Step 5.
    b. Antigen retrieval by microwave use SOP 73/3a.
    c. Antigen retrieval by pronase, use SOP 73/5b.
    d. Antigen retrieval by pressure cooker, use SOP 73/3b.

5. Apply primary antibody correctly diluted in TBS and incubate either at room temperature for 30 minutes or at 4°C for 18-24 hours (overnight) .Consult antibody register for recommended incubation time for each antibody.

6. If incubated overnight at +4 °C allow to warm up to room temperature before

   washing (approx 15 minutes).

   Wash in TBS 3 x 5 minutes.

7. Apply either biotinylated sheep anti-mouse Ig (BAM) for monoclonal antibodies or

   biotinylated swine anti-rabbit Ig (BAR) for polyclonal antibodies, at current

   dilution in TBS (1 in 200), for 30 minutes at room temperature.

8. Prepare peroxidase labelled complexes for Step 10 now by adding the equivalent of *2µl* of solution A, *2µl* of solution B to *396µl* of TBS making a 1:200 dilution. Leave on bench to complex (for at least 30minutes).

9. Wash in TBS for 3 x 5 minutes.

10. Apply pre-prepared Streptavidin/Biotin peroxidase complexes (Step 8) for 30 minutes at room temperature .

11. Wash in TBS 3 x 5 minutes.

12. Apply DAB substrate (see SOP 73/la) for 10 minutes, or AEC (see SOP 73/1b).

13. Rinse in TBS, followed by a wash in running tap water for 2 minutes.

14. Rinse in 70% alcohol.

15. Counterstain with Harris' haematoxylin, for 2 minutes.
Rinse in tap water for one minute.
Differentiate in 1% acid alcohol for 5 seconds.
Blue in running tap water for at least 5 minutes.

16. Dehydrate clear and mount in DPX.

REFERENCE: Mepham BL, Britten KJM. Immunostaining methods for frozen and paraffin
sections. In: Jones DB, Wright DH, eds. *Lymphoproliferative diseases.* London: Kluwer Academic Publishers; 1990;12,187-211.

Appendix vi: Antigen Retrieval -Heat-mediated -Microwave Pretreatment using Citrate or EDTA buffer

Laboratory: LE73 No of Pages: 2

Prepared by: J H D Williams (SOPJ4) Date: 24.12.96

PRINCIPLE: In fixed paraffin-embedded tissue sections demonstration of some antigens is enhanced after pretreatment in a microwave oven using a pre-selected buffer (citrate, EDTA or urea).

RISK ASSESSMENT:
1) Microwaves are dangerous.
2) The containers of buffer will become very hot during microwaving so a face shield and insulated gloves must be worn when removing the containers from the microwave (Step 5).
3) A face shield and insulated gloves are provided.
4) Observe relevant COSSH requirements for all chemicals and reagents .

REQUIREMENTS :

Equipment: Microwave oven eg Panasonic NN-6450 (800 watts), Plastic staining racks to hold up to 25 slides which are able to withstand temperatures of loo°C eg Merck, cat no:406/0238/00, Polythene boxes and lids (100x100x125mm) able to withstand temperatures of loo°C eg Merck, cat no: 125/1620/02. To allow steam to escape lids should be perforated using a hot needle before use (20-30 holes).

Reagents: 0.01M citrate buffer (pH6.0) prepared as follows:
Citric acid crystals 2.1g
RO water 1000ml
Adjust pH to 6.0 with 1M sodium hydroxide (approximately 25ml).

OR EDTA 1mM pH 8.0 prepared as follows:

EDTA 0.37gm
RO water 1000 ml
Adjust to pH 8.0 with 0.1M NaOH (approximately 8 mls) Beware pH very sensitive

NB : Consult antibody register for recommended buffer to be used for each antibody.

Related Protocols:
SOP 73/3c Antigen Retrieval -Heat-mediated -Microwave pretreatment using Urea to demonstrate the p80 antigen.
SOP 73/3b Antigen retrieval -Heat mediated -Pressure Cooker Pretreatment.

Quality Control: not applicable

METHOD/PROCEDURE:

Preparation of sections
a. Sections should be mounted on APES coated slides (SOP73/6) and dried for at least 24 hours at 37°C before staining.
b. Sections should be de-paraffinised and endogenous peroxidase blocked in the usual way and placed in water until required.

Method
1. Fill the plastic staining racks with 24 slides and place in the polythene box. To maintain a constant load 3 polythene boxes are always used, together with 72 slides. Blank slides, without sections, must be used to make up the number.

2. Fill each box with 330ml of prepared buffer, ie up to the black line marked on the box. Place the perforated lid firmly on the box.

3. The three boxes should always be placed in the same position on the plate of the oven. There are three raised lumps on the underside of the Panasonic oven plate which will act as markers.

4. Set the microwave to, a) medium power and b) 25 minutes. Start and allow to run.

5. When the time has elapsed, remove one box at a time (CARE AS IT WILL BE VERY HOT) .Remove the lid and fill quickly with cold running water. Leave all three racks in running water for 2- 3 minutes. .

6. Place the slides back in the staining trays, wash in TBS 2x5 minutes before continuing with the immunostaining technique (Step 5, SOP73/4).

REFERENCE: Modified from the method published by:
Cattoretti, G., Pileri, S., Parravicini, C., Becker, M.H.G., Poggi, S., Bifulco, C., Key, G., D' Amato, L. , Sabattini, E. , Feudale, E. , Reynolds, F. , Gerdes, J. and Rilke, F. (1993) Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *Journal of Pathology* 171, 83-98.

Appendix vii: Antigen Retrieval -Proteolytic Enzyme -Pronase Pretreatment

Laboratory: LE73 No of Pages: 1 Prepared by: J H D Williams (SOPJ7) Date: 2.1.97

PRINCIPLE: In fixed paraffin-embedded tissue sections demonstration of some antigens is enhanced after pretreatment with the proteolytic enzyme pronase. The effectiveness of proteolysis for antigen retrieval is related to the length of fixation and the type of fixative used. Pronase is not always as effective as trypsin but is often used in preference to trypsin because a) it is more readily available than trypsin, b) it works at room temperature and c) there is less batch to batch variation (see SOP73/2).

RISK ASSESSMENT: Observe relevant COSSH requirements for all chemicals and reagents.

REQUIREMENTS :

Equipment: no specialised equipment needed. Reagents:
1. Pronase -Dako cat no *S2013 2. TBSpH7.6*
3. The 1% Pronase Stock Solution is made up by dissolving the contents of one vial of pronase (100mg) in 10mls of TBS. It is then stored in 0.1m1 aliquots at -20°C. This stock solution is stable for at least one year. A void repeated freezing and thawing .

Related Protocols:
SOP 73/5a Antigen retrieval -Proteolytic enzyme -Trypsin pretreatment.

Quality control:
1. The activity of each new batch of pronase should not vary considerably but each new batch must be tested before use (see SOP73/2).
2. As the length of pretreatment is related to the length and type of fixative used it may be necessary to adjust the incubation time, from the standard time of 15-20 minutes, for individual tissue sections .

METHOD/PROCEDURE:

Preparation of sections
a. Sections should be mounted on APES coated slides (SOP73/6) and dried for at least 24 hours at 37°C before staining.
b. Sections should be de-paraffinised and endogenous peroxidase blocked in the usual way and placed in water until required.

Method
1. Prepare the 0.05% Pronase Working Solution by thawing one vial of 0.1ml of Pronase Stock Solution and adding 1.9mls of TBS. Mix well. The working solution is stable for 5 days at 2-8°C.

2. Drain slides and cover with 200,ul of 0.05% pronase. Incubate at room temperature for approximately 15-20 minutes. The optimal incubation time depends on the length and type of formalin fixation, this is determined by titration (see SOP 73/2).

3. Wash in TBS 2x5 minutes before continuing with the immunostaining technique (Step 5, SOP73/4).

REFERENCE: None given in data sheet.

Appendix viii: Blocking of Endogenous Avidin Binding Sites (EABS)

Laboratory: LE73 No of Pages: 2 Prepared by: JHD Williams (SOP15) Date: 3/1/97

PRINCIPLE: 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 StABC technique (SOP 73/4 and 73/10) and is a particular problem in frozen sections. EARS can be blocked by reacting the tissue with unconjugated avidin, which is then s:aturated with unlabelled biotin. This procedure effectively blocks further non-specific attachment of the enzyme-labelled avidin biotin complex.

RISK ASSESSMENT: Observe relevant COSHH requirement for all chemicals and reagents .

REQUIREMENTS :

Equipment: no specialised equipment needed.

Reagents:
1. Avidin/Biotin Blocking Kit -Vector Laboratories Cat no: SP-2001.
2. 2. Culture medium, made up a follows:
   10% foetal calf serum in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% bovine serum albumin (BSA). Stored in 1ml aliquots at -18°C.

Related Protocols:
SOP 73/12 Streptavidin-Biotin Peroxidase Complex (StABCPx) Immunostaining Technique for Frozen Sections, Cytospins and Smears using Monoclonal or Polyclonal Antibodies.
SOP 73/14 Streptavidin-Biotin Alkaline Phosphatase Complex (StABCM Phos) Immunostaining Technique for Frozen Sections, Cy to spins and Smears using Monoclonal or Polyclonal Antibodies.
SOP 73/4 Streptavidin-Biotin Peroxidase Complex (StABCPx) Immunostaining Technique for Fixed, Paraffin Embedded Sections using Monoclonal or Polyclonal Antibodies.
SOP 73/10 Streptavidin-Biotin Alkaline Phosphatase Complex (StABCAlk Phos) Immunostaining Technique for Fixed, Paraffin Embedded Sections using Monoclonal or Polyclonal Antibodies .

Quality Control: not applicable.

METHOD/PROCEDURE:

I. Before applying the primary antibody add the avidin solution undiluted from the kit and leave for 20 minutes.

2. Wash 3x2 minutes in TBS.

3. Apply the biotin solution undiluted from the kit and leave for 20 minutes.

4. Wash 3x2 minutes in TBS.

5. Apply the culture medium (Reagent 2) and leave for 20 minutes.

6. DO NOT W ASH OFF. Drain the culture medium from the slides, apply the primary antibody and continue the immunostaining technique as given in the relevant protocol.

REFERENCES:

Wood G.S. and Warnke R. (1981) Suppression of endogenous avidin-biotin activity in tissues and its relevance to biotin-avidin detection systems. *The Journal of Histochemistry and Cytochemistry* 29, 1196-1204.

Vilela M.J., Parrish E.P., Wright D.H. and Garrod D,R. (1987) Monoclonal antibody to Desmosomal Glycoprotein 1 -a new epithelial marker for diagnostic pathology. *Journal of Pathology* 153, 365-375.

AVIDIN/BIOTIN BLOCKING KIT: PRODUCT SPECIFICATIONS

Product BLOCKING KIT Catalog No. SP-2001

Amount 18 ml of each solution Storage Condition -!l::S,.

Blocking kit reagents may be used to block nonspecific binding of Biotin/Avidin System reagents.

Principle:

Some tissues may bind avidin, biotinylated horseradish peroxidase or other Biotin/Avidin System components without prior addition of biotinylated antibody.

This binding may be due to endogenous biotin or biotin-binding proteins, lectins, or nonspecific binding substances present in the section. If a high background is present using the ABC reagents (or other avidin conjugate) in the absence of biotinylated secondary antibody, pre-treatment of the tissue with avidin, followed by biotin (to

block the remaining biotin binding sites on the avidin), may be required.
The blocking kit consists of an Avidin D solution and a biotin solution. Pre-treatment of the section with the Avidin D solution should always be followed by incubation with the biotin solution. The Avidin D and biotin solutions should be used directly as supplied.

Suggested Protocol for Tissue Sections:

After incubation with normal serum, incubate section with Avidin D blocking solution for 15 minutes. Rinse briefly with buffer, then incubate for 15 minutes with the biotin blocking solution. These steps should be performed prior to the addition of primary antibody or lectin.

In many cases an alternative procedure has proved satisfactory. This method incorporates avidin/biotin blocking into the normal steps employed in labeling. Four drops of the Avidin D solution can be added to each I mI of the diluted normal blocking serum (preferably dialyzed to remove any free biotin from the serum). This reagent is used in place of the usual serum block step. After a brief rinse, the primary antibody is added, containing 4 drops of the biotin solution per I mI of primary antibody. This step not only introduces the primary antibody into the section, but blocks the available biotin binding sites on the avidin. Combining the biotin block step with the primary antibody step is not recommended if the primary antibody is biotinylated. When using biotinylated primary antibodies, the biotin solution should be added prior to the addition of primary antibody as a separate step.

Suggested Protocol for Transfer Blots:

After the initial blocking step with either TTBS or 10% nonfat dry milk, the membrane is immersed for 10 minutes in an avidin blocking solution prepared by dispensing 2 drops of the Avidin D solution into 10 mI of TTBS (using the Tween 20 protocol) or TBS (using the protein blocking protocol). Wash briefly with buffer. Incubate the membrane for 10 minutes in a biotin blocking solution, made by dispensing 2 drops of the Biotin solution into 10 mI ofTTBS or TBS. Proceed with the transfer blot detection procedure.

N Vector Laboratories. Inc., 30 Ingold Road, Burlingame, CA 94010 U.S.A., (415) 697-3600. Fax (415) 697-0339

Appendix ix: Demonstration of Enzyme Peroxidase using Biomen Liquid DAB Substrate Kit

Laboratory: LE73 No of Pages: 2

Prepared by: Mr R Lee/MrB Mepham (SOP15) Date: 4.10.97

Principle: The peroxidase label on the final stage reacts with hydrogen peroxidase and diaminobenzidine (DAB) to form an insoluble brown precipitate at the site ofantigen/antibody reaction.

Risk Assessment: DAB is a possible carcinogen, so handle with care. Observe relevant COSHH requirement for all chemicals and reagents.

Requirements: The reagents are supplied concentrated in a kit which is stored between 2-8°C. Reference (HK153-5K). This kit contains:-

2 x 4ml of3, 3'-diaminobenzidine chromogen solution.

20ml of lOX concentrated substate buffer.

2 x 3ml of hydrogen peroxide substrate solution.

Method/Procedure: To make 2.5ml ofworking solution (allow 200~1 per slide). Use in appropriate multiples for the number of slides to be stained. Prepare as follows:-

1. Add 0.25ml of lOX substrate solution to 2.25ml RO water. Add 2 drops of chromogen and mix. Add 1 drop of Hydrogen Peroxide (H2O2) substrate solution and mix.
2. Add enough working solution (2-5 drops) to entirely cover the tissue section only and incubate at room temperature for 3 -10 minutes. In most cases, colour development will be completed in 5 minutes. However, development time will vary due to the amount of specific antigen/antibody binding in the specimen and the ambient temperature of the room.
3. The working solution is stable and can be stored for over eight hours at room temperature.

Notes:

1. Caution DAB is a suspected carcinogen. Avoid contact with skin, use of gloves is recommended. Excess agent should be discarded appropriately.

2. DAB is a light-sensitive chromogen and, once mixed with buffer, the working solution should be stored in the dark or wrapped in foil for further use.

Appendix x: Reagent Standardisation for Immunocytochemistry Laboratory

LE 73 No of Pages: 2

Prepared by: JHD Williams (SOPJ20) Date: 3/1/97

PRINCIPLE :

a) Antibodies: With the exception of Streptavidin biotin kits (StABC), all new antibodies and new batches of antibody must be titrated before use for optimal staining to be achieved. This is to determine the dilution which gives the strongest specific staining but with the least amount of background staining. New primary antibodies, to be used on paraffin sections, must also be tested to determine the optimal method of antigen retrieval ie pronase, microwave, pressure cooker or no treatment (SOP 73/3a, 3b, 5b). New batches of StABC kits do not require titration as the concentration of the reagents supplied in each kit is the same each time.

b) Trypsin: the activity of trypsin can vary considerably from batch to batch and from manufacturer to manufacturer. Therefore each new batch of trypsin received must be tested at different incubation times to determine it's optimal activity , that is the activity which gives optimal staining of the antigen but with minimal tissue digestion. The incubation time for trypsin varies with both fixation time and type of fixative used; the optimal trypsin time is determined using tissue fixed in 10% NBF for 24 to 48 hours and therefore it may be necessary to test alternately fixed tissue blocks individually.

c) Pronase: in comparison with trypsin, the activity of pronase should not vary considerably from batch to batch. It should therefore only be necessary to test each new batch of pronase at three incubation times based around the optimal time for the previous batch. As with trypsin, the optimal incubation time can vary with both fixation time and type of fixative used.

d) Other reagents and chemicals: it is recommended that the activity of all new batches of chemicals and reagents should be checked before use.

RISK ASSESSMENT: Observe relevant COSHH requirements for all chemicals and reagents .

REQUIREMENTS : Equipment: depends on reagent being tested, consult relevant protocol. Reagents: depends on reagent being tested, consult relevant protocol.

Related Protocols: the standardisation procedure is relevant to all protocols.

METHOD/PROCEDURE:

a) Antibodies

1) Obtain sections of known positive control tissue, either frozen or in paraffin, depending on the tissue in which the antibody is to be used. Information concerning recommended positive control tissue is usually given in the datasheet supplied by the manufacturer, if not consult the relevant pathologist.

2) FOR NEW ANTIBODIES ON PARAFFIN SECTIONS ONL Y: test antibody on the positive control sections using all four different retrieval techniques: microwave, pronase or trypsin,pressure cooker or no treatment (see relevant SOP -73/4, 3a, 3b, 5b). When the optimal pretreatment has been determined continue with step 3) using the appropriate pretreatment.

3) Test the antibody on the positive control sections using a number of different dilutions starting around the dilution recommended in the datasheet supplied with thc antibody or if the antibody has been used before, start around the previous working dilution. If possible use 'double dilutions' eg 1/2, 1/4, 1/8, 1/16 and 1/32 (doubling the dilution each time). If no recommended dilution is given a guide for dilutions is as follows: i) polyclonal -1/500 to 1/8000 .

ii) monoclonal supernatant -1/2 to 1/64

iii) monoclonal purified -1/50 to 1/800

iv) monoclonal ascites -1/500 to 1/8000

4) From examination of the stained sections it should be possible to determine the optimal working dilution and pretreatment (paraffm only) for each antibody tested.

5) After titration all antibodies should be stored at their recommended storage temperature for maximum activity to be maintained:
i) Those requiring storage at 4 °C should be placed in a suitable position in one of the antibody trays in the fridge and this should be noted in the antibody file.
ii) those requiring storage below o°C must be aliquoted out and frozen immediately after titration as repeated freezing and thawing is detrimental to an antibody. Each antibody should be aliquoted into suitable volumes, from 50ul (minimum) to Iml. The volume , chosen will depend on the dilution of the antibody. The aliquots should be labelled with the antibody name and placed in a plastic bag labelled with the antibody name, dilut~on, pretreatment, aliquot size, date frozen, batch no (if appropriate) and the number of the box the aliquots are to be stored in. The bag should then be stored in the numbered box in the freezer and details recorded in the antibody file. One aliquot at a time can thefi be taken out and kept in the fridge (record in antibody file).

NOTE: Occasionally antibodies work at too high concentration or do not keep at all at fridge temperature. In this situation the antibody should be flrst diluted to an intermediate dilution eg 1/100 before aliquoting and freezing. An aliquot can then be removed from the freezer when required, further diluted to the working concentration and any diluted antibody not used on the day discarded.

b) Trypsin and Pronase

NOTE: Where possible pronase should be used in preference to trypsin as the activity of each new batch of pronase should not vary considerably so therefore does not require such extensive testing as trypsin.

1) Preferably using the polyclonal antibody to kappa light chains on two different blocks of tonsil (fixed for 24 to 48 hours), test the new batch of trypsin for the following incubations times: 2, 5, 8, 10, 12, 15, 20 and 30 minutes (pronase -15 and 20 minutes). The previous working batch should be run at the same time using it's optimal incubation time 'to act as a positive control.
NOTE: The kappa light chain antibody is used as it is particularly sensitive to the effects of trypsin.

2) From examination of the stained sections it should be possible to determine the optimal digestion time for 'routinely' fixed tissue ie 10% NBF for 24 to 48 hours. Alternatively fixed tissue may need to be tested individually.

c) Other chemicals and reagents

It is recommended that all new batches of chemicals and reagents used in immunocytochemistry should be tested before use. This can be carried out by testing the new batch at the same time as running the old, working batch, in the relevant protocol.

d) Obtaining positive control tonsil for immunocytochemistry

1) Obtain fresh tonsil from the operating theatres, cut up and fix in 10% NBF for 24 hours before processing to paraffin wax.

2) Test each block of tonsil by staining with a polyclonal antibody to both kappa light chains and to IgM using pronase pretreatment for 15 and 20 minutes.

3) All blocks which stain well for both antibodies can be used as positive control material for immunocytochemistry .

REFERENCE: N/A

Appendix xi: Antibody data sheets
Collagenases:

R&D SYSTEMS

Monoclonal Anti-human MMP-1 Antibody

ORDERING INFORMATION

Catalog Number: MAB901

Clone: 36665.111

Size: 200 µg

Formulation: 0.2 µm filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: pro/active human MMP-1

Immunogen: CHO cell-derived rhMMP-1

Ig class: mouse IgG,

Applications: Western blot
Immunohistochemistry
Neutralization
Immunoprecipitation

Preparation
This antibody was produced from a murine hybridoma elicited from a mouse immunized with purified, CHO cell-derived, recombinant human MMP-1 (rhMMP-1). The IgG fraction of ascites fluid was purified by Protein A affinity chromatography.

Formulation
Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS).

Reconstitution
Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 0.2 mg/mL.

Storage
Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 4° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity
This antibody has been selected for its ability to bind pro/active forms of human MMP-1. Based on western blot results, this antibody shows no cross-reactivity with rhMMP-3, rhMMP-2 or rhMMP-9.

Applications
Western Blot - This antibody can be used at 1 µg/mL with the appropriate secondary reagents to detect rhMMP-1. The detection limit for rhMMP-1 is approximately 20 ng/lane under both reducing and non-reducing conditions.

Immunohistochemistry - This antibody can be used at approximately 10 - 15 µg/mL with the appropriate secondary reagents to detect human MMP-1 on frozen or paraffin-embedded sections. For detection of labeling of paraffin-embedded human tissues, the ABC technique using chromogenic substrates (NovaRED, AEC, DAB, etc.) is recommended.[1]

Neutralization - This antibody can be used to neutralize the active MMP-1 cleavage of type I collagen α chains into classic ¾, ¼ fragments. 20 µg of antibody preincubated with 1 µg of active MMP-1 neutralizes the cleavage of type I collagen α chains by greater than 80%.

Immunoprecipitation - This antibody has been used to immunoprecipitate Pro and 45 kD active MMP-1 from conditioned media of transfected NS0 myeloma cells. The recovered rhMMP-1 can be detected by western blot analysis using R&D Systems' polyclonal antibody (Catalog # AF901).

[1] *Due to accumulation of autofluorescent pigment lipofucsin in neuronal tissues dissected from non-human primates or humans, the use of fluorescent probes such as FITC or Cy3 are not recommended if autofluorescence is not quenched (for example, by treating tissues after finishing IHC staining with 1% Sudan Black in 70% methanol for 10 minutes at room temperature). Residual autofluorescence may obscure specific labeling. Non-fluorescent enzymatic protocols (e.g. DAB, AEC, or immunogold-silver staining) may be used.*

Optimal dilutions should be determined by each laboratory for each application.

For immunohistochemistry images, please refer to our website at http://www.rndsystems.com/asp/c_immunohistochemistry_add.asp

R&D Systems, Inc.
1-800-343-7475

3/30/01

Anti-human MMP-8 Antibody

ORDERING INFORMATION

Catalog Number: AF908

Lot Number: DPK01

Size: 100 µg

Formulation: 0.2 µm filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: human pro and active MMP-8

Immunogen: NS0-derived rhMMP-8

Ig Type: human MMP-8 specific IgG

Applications: Western blot
Immunoprecipitation
Immunocytochemistry

Preparation

Produced in goats immunized with purified, NS0-derived, recombinant human MMP-8 (rhMMP-8). MMP-8 specific IgG was purified by human MMP-8 affinity chromatography.

Formulation

Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS).

Endotoxin Level

< 10 ng per 1 mg of the antibody as determined by the LAL method.

Reconstitution

Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 0.1 mg/mL.

Storage

Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 4° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity

This antibody has been tested for its ability to recognize recombinant human MMP-8 in western blots, dot blots, and immunoprecipitation. This antibody does not cross-react with recombinant human MMP-1, -2, -3, -7, -9, -10, -12 and –13 in dot blots.

Applications

Western blot - The antibody can be used at 0.1 µg/mL with the appropriate secondary reagents to detect MMP-8. The detection limits for recombinant human MMP-8 are approximately 5 ng/lane under both reducing and reducing conditions.

Immunoprecipitation - The antibody has been used to immunoprecipitate rhMMP-8 from conditioned media of transfected NS0 cells. The recovered rhMMP-8 can be detected by western blot analysis using a mouse monoclonal antibody (Catalog # MAB908).

Immunocytochemistry - The antibodies have been used at 5 µg/mL and 15 µg/mL to detect human MMP-8 in transfected cells.

Optimal dilutions should be determined by each laboratory for each application.

R&D Systems, Inc.
1-800-343-7475

11/1/00

Monoclonal Anti-human MMP-13 Antibody

ORDERING INFORMATION

Catalog Number: MAB511

Clone: 87512

Lot Number: DHD01

Size: 500 µg

Formulation: 0.2 µm filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: human pro and active MMP-13

Immunogen: CHO cell-derived rhMMP-13

Ig class: mouse IgG_1

Applications: Western blot
Immunoprecipitation
Immunopurification
Immunohistochemistry

Preparation

This antibody was produced from a murine hybridoma elicited from a mouse immunized with purified, CHO cell-derived, recombinant human MMP-13 (rhMMP-13). The IgG fraction of the ascites fluid was purified by Protein G affinity chromatography.

Formulation

Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS).

Reconstitution

Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 500 µg/mL.

Storage

Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 4° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity

This antibody has been selected for its ability to bind human MMP-13. This antibody has been shown to bind both pro and active forms of human MMP-13. This antibody does not cross-react with recombinant human MMP-1, -2, -3, -7, -8, -9, -10 and -12.

Applications

Western Blot - This antibody can be used at approximately 1.0 µg/mL with the appropriate secondary reagents to detect human MMP-13 under both reducing and non-reducing conditions. The detection limit for rhMMP-13 is approximately 20 ng/lane.

Immunoprecipitation/Immunopurification – Sepharose beads coupled with this antibody have been used to immunoprecipitate and immunopurify rhMMP-13 from condition media.

Immunohistochemistry - This antibody can be used at 25 µg/mL to detect rhMMP-13 in transfected cells by immunocytochemistry. This antibody can also be used at 8 - 25 µg/mL to detect human MMP-13 in 10 - 15 µm thick paraffin-embedded tissue sections. R&D Systems' Cell and Tissue Staining Kits (CTS Series) are recommended for optimal signal detection.

Optimal dilutions should be determined by each laboratory for each application.

For immunohistochemistry images, please refer to our website at http://www.rndsystems.com/cyt_cat/ihcprot.html

R&D Systems, Inc.
1-800-343-7475

9/13/00

R&D SYSTEMS

Monoclonal Anti-human MMP-2 Antibody

ORDERING INFORMATION

Catalog Number: MAB903

Clone: 1A10

Size: 200 µg

Formulation: 0.2 µm filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: pro/active hMMP-2

Immunogen: CHO cell-derived rhMMP-2

Ig class: mouse IgG_{2A}

Applications: Western blot
Histology

Preparation

This antibody was produced from a murine hybridoma elicited from a mouse immunized with purified, CHO cell-derived, recombinant human MMP-2 (rhMMP-2). The IgG fraction of the tissue culture supernatant was purified by Protein A affinity chromatography.

Formulation

Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS).

Reconstitution

Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 0.2 mg/mL.

Storage

Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 4° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity

This antibody has been selected for its ability to bind human pro/active forms of MMP-2. In dot blots, this antibody shows no cross-reactivity with rhMMP-1, -3, -7, -8, -9, -10, -12 and -13.

Applications

Western Blot - This antibody can be used at approximately 1 µg/mL with the appropriate secondary reagents to detect human MMP-2. The detection limit for rhMMP-2 is approximately 1 ng/lane and 100 ng/lane under non-reducing and reducing conditions, respectively.

Histology - This antibody can be used at approximately 3.3 µg/mL with the appropriate secondary reagents to detect human MMP-2 on paraffin embedded sections.

Optimal dilutions should be determined by each laboratory for each application.

R&D Systems, Inc.
1-800-343-7475

5/24/01

Monoclonal Anti-human MMP-9 Antibody

ORDERING INFORMATION

Catalog Number: MAB936

Clone: 36020.111

Lot Number: AFF01

Size: 200 g

Formulation: 0.2 m filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: pro/active hMMP-9

Immunogen: CHO cell-derived rhMMP-9

Ig class: mouse IgG_1

Application: Western blot
Immunoprecipitation
Immunohistochemistry

Preparation

This antibody was produced from a hybridoma resulting from the fusion of a mouse myeloma with B cells obtained from a mouse immunized with purified, CHO cell-derived, recombinant human matrix metalloproteinase 9 (rhMMP-9). The IgG fraction of ascites fluid was purified by Protein A affinity chromatography.

Formulation

Lyophilized from a 0.2 m filtered solution in phosphate-buffered saline (PBS).

Reconstitution

Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 0.2 mg/mL.

Storage

Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 8° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C **in a manual defrost freezer** for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity

This antibody has been selected for its ability to bind the pro (92 kDa) and active (82 kDa) forms of rhMMP-9. It does not react with the C-terminal truncated form (65 kDa) of rhMMP-9. Based on western blot results, this antibody shows no cross-reactivity with rhMMP-2, rhMMP-1 or rhMMP-3.

Applications

Western Blot- This antibody can be used at approximately 1 g/mL with the appropriate secondary reagents to detect human MMP-9. The detection limit for rhMMP-9 is approximately 20 ng/lane under non-reducing and reducing conditions.

Immunoprecipitation - This antibody has been used at 25 g/mL to immunoprecipitate rhMMP-9 from conditioned media of transfected CHO cells. The recovered rhMMP-9 can be detected by western blot analysis using a goat polyclonal antibody (R&D Systems, Catalog # AF909, BAF909 or AB909).

Immunohistochemistry - This antibody can be used with the appropriate secondary reagents to detect human MMP-9 in paraffin-embedded human tissues. Working dilutions for 5 - 15 m thick sections are 10 - 15 g/mL. For detection, the ABC technique using chromogenic substrates (NovaRED, AEC, DAB, etc.) is recommended.[1]

[1] *Due to accumulation of autofluorescent pigment lipofucsin in neuronal tissues dissected from non-human primates or humans, the use of fluorescent probes such as FITC or Cy3 are not recommended if autofluorescence is not quenched (for example, by treating tissues after finishing IHC staining with 1% Sudan Black in 70% methanol for 10 minutes at room temperature). Residual autofluorescence may obscure specific labeling. Non-fluorescent enzymatic protocols (e.g. DAB, AEC, or immunogold-silver staining) may be used.*

Optimal dilutions should be determined by each laboratory for each application.

For immunohistochemistry images, please refer to our website at http://www.RnDSystems.com/asp/c_immunohistochemistry_add.asp

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1-800-343-7475

2/21/02

R&D SYSTEMS

Anti-human MMP-3 Antibody

ORDERING INFORMATION

Catalog Number: AF513

Lot Number: DNA01

Size: 100 µg

Formulation: 0.2 µm filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: human pro and active MMP-3

Immunogen: NS0-derived rhMMP-3

Ig Type: human MMP-3 specific IgG

Applications: Western blot
Immunoprecipitation
Immunocytochemistry

Preparation

Produced in goats immunized with purified, NS0-derived, recombinant human MMP-3 (rhMMP-3). MMP-3 specific IgG was purified by human MMP-3 affinity chromatography.

Formulation

Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS).

Endotoxin Level

< 10 ng per 1 mg of the antibody as determined by the LAL method.

Reconstitution

Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 0.1 mg/mL.

Storage

Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 4° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity

This antibody has been tested for its ability to recognize recombinant human MMP-3 in western blots, dot blots, and immunoprecipitation. This antibody does not cross-react with recombinant human MMP-1, -2, -7, -8, -9, -12 and -13 in dot blots. It showed approximately 15% cross-reactivity with human MMP-10 in western blots.

Applications

Western blot - The antibody can be used at 0.1 µg/mL with the appropriate secondary reagents to detect MMP-3. The detection limits for recombinant human MMP-3 are approximately 1 ng/lane under both reducing and reducing conditions.

Immunoprecipitation - The antibody has been used to immunoprecipitate rhMMP-3 from conditioned media of transfected NS0 cells. The recovered rhMMP-3 can be detected by western blot analysis using a mouse monoclonal antibody (Catalog # MAB905).

Immunocytochemistry - The antibody has been used at 5 µg/mL and 15 µg/mL to detect human MMP-3 in transfected cells.

Optimal dilutions should be determined by each laboratory for each application.

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1-800-343-7475

11/1/00

Anti-human MMP-7 Antibody

ORDERING INFORMATION

Catalog Number: AF907

Lot Number: DPS01

Size: 100 µg

Formulation: 0.2 µm filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: human pro and active MMP-7

Immunogen: NS0-derived rhMMP-7

Ig Type: human MMP-7 specific IgG

Applications: Western blot
Immunoprecipitation

Preparation

Produced in goats immunized with purified, NS0-derived, recombinant human MMP-7 (rhMMP-9). MMP-7 specific IgG was purified by human matrix metalloproteinase 7 affinity chromatography.

Formulation

Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS).

Endotoxin Level

< 10 ng per 1 mg of the antibody as determined by the LAL method.

Reconstitution

Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 0.1 mg/mL.

Storage

Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 4° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity

This antibody has been tested for its ability to recognize recombinant human MMP-7 in western blots, dot blots, and immunoprecipitation. This antibody does not cross-react with recombinant human MMP-1, -2, -3, -8, -9, -10, -12 and -13 in dot blots.

Applications

Western blot - The antibody can be used at 0.1 µg/mL with the appropriate secondary reagents to detect MMP-7. The detection limits for recombinant human MMP-7 are approximately 20 ng/lane under both reducing and reducing conditions.

Immunoprecipitation - The antibody has been used to immunoprecipitate rhMMP-7 from conditioned media of transfected NS0 cells. The recovered rhMMP-7 can be detected by western blot analysis using a mouse monoclonal antibody (Catalog # MAB9071).

Optimal dilutions should be determined by each laboratory for each application.

R&D Systems, Inc.
1-800-343-7475

12/11/00

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custserv@chemicon.com • techserv@chemicon.com • www.chemicon.com

MOUSE ANTI-RECOMBINANT HUMAN TIMP-1 MONOCLONAL ANTIBODY

CATALOG NUMBER:	MAB3301
LOT NUMBER:	19050410
QUANTITY:	100 µg
CONCENTRATION:	2 mg/mL
SPECIFICITY:	The antibody specifically reacts with human TIMP-1. This is a purified mouse monoclonal antibody to recombinant human tissue inhibitor of metalloproteinases-1 (recombinant TIMP-1).
ISOTYPE:	IgG1/k
CLONE:	147-6D11
APPLICATIONS:	Immunoblotting Immunohistochemistry on paraffin-embedded tissue sections EIA
SPECIES REACTIVITY:	Does not cross-react with mouse and rat TIMP-1.
PRESENTATION:	Purified immunoglobulin
FORMAT:	Liquid in 0.1M Na-Phosphate buffer, pH 7.0 containing 2% protease free bovine serum albumin.
STORAGE/HANDLING:	Maintain frozen at -20°C in undiluted aliquots for up to 12 months.

REFERENCES:

1. Kodama, S. et al. (1987). Monoclonal antibodies to bovine collagenase inhibitor. *Collagen Rel. Res.* **7:** 341-350.
2. Okada, Y. et al. (1989). Immunolocalization of matrix metallo-proteinases 3 (stromelysin) in rheumatoid synovioblasts (B cells): correlation with rheumatoid arthritis. *Ann. Rheum. Dis.* **48:** 645-653.
3. Fukuda, Y. et al. (1991). Immunohistochemical study on tissue inhibitors of metalloproteinases in normal and pathological human livers. *Gastroenterol. Jpn.* **26:** 37-41.

For research use only; not for use as a diagnostic.

Important Note: *During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For products with volumes of 200 µL or less, we recommend gently tapping the vial on a hard surface or briefly centrifuging the vial in a tabletop centrifuge to dislodge any liquid in the container's cap.*

Manufactured by Fuji Chemical Industries, Ltd. Not available for sale in Japan.

07-98/MAB3301/GC

CHEMICON International, Inc. 28835 Single Oak Drive • Temecula, CA 92590
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custserv@chemicon.com • techserv@chemicon.com • www.chemicon.com

MOUSE ANTI-HUMAN-TIMP-2 MONOCLONAL ANTIBODY

CATALOG NUMBER:	MAB3310
LOT NUMBER:	19060939
QUANTITY:	100 µg
CONCENTRATION:	2 mg/mL
SPECIFICITY:	The antibody specifically reacts with human TIMP-2.
ISOTYPE:	IgG1/k
CLONE:	67-4H11
APPLICATIONS:	Immunoblotting Immunohistochemistry on paraffin-embedded at 5 µg/mL (see Tomita reference) or frozen tissues at 1 µg/mL (see Ohashi reference). EIA
SPECIES REACTIVITY:	Cross-reacts with mouse, rat, guinea pig, rabbit and bovine TIMP-2.
PRESENTATION:	Purified immunoglobulin
FORMAT:	Liquid in 0.1M Na-Phosphate buffer, pH 7.0 containing 2% protease free bovine serum albumin.
STORAGE/HANDLING:	Maintain frozen at -20°C in undiluted aliquots for up to 12 months.

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For research use only; not for use as a diagnostic.

Important Note: *During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For products with volumes of 200 µL or less, we recommend gently tapping the vial on a hard surface or briefly centrifuging the vial in a tabletop centrifuge to dislodge any liquid in the container's cap.*

Manufactured by Fuji Chemical Industries, Ltd. Not available for sale in Japan.

9-98/MAB3310/TL

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