MATRIX METALLOPROTEINASES IN COLORECTAL CANCER DEVELOPMENT AND PROGNOSIS

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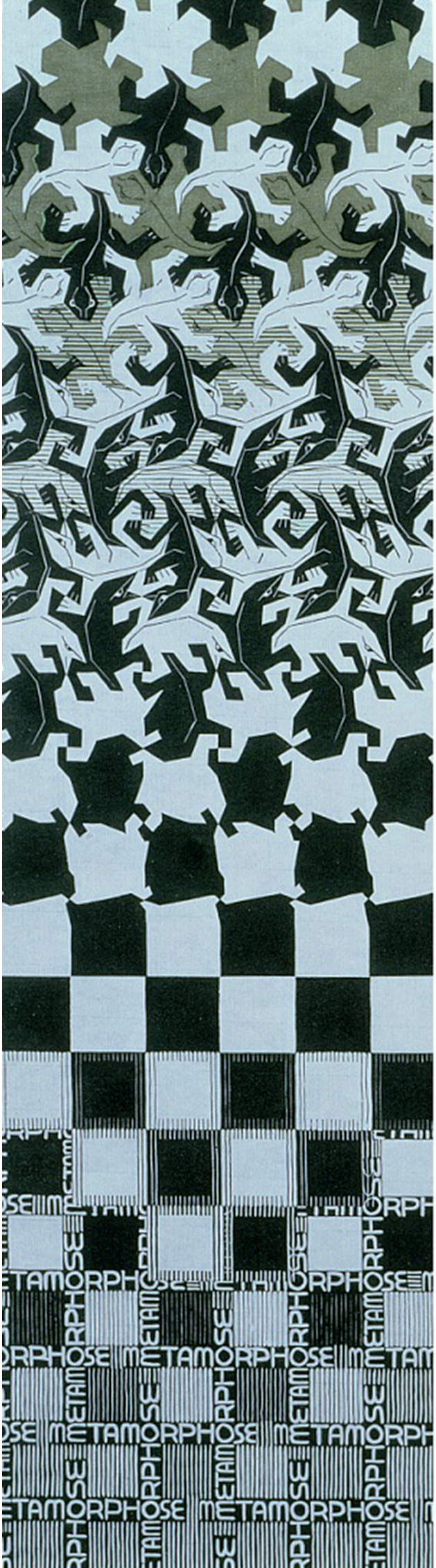
CONTENTS

ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AP-1	activator protein-1
APC	adenomatous polyposis coli
APMA	*p*-aminophenylmercuric acetate
ARMS	amplification refractory mutational system
BIA	bioactivity assay
BSA	bovine serum albumin
CEA	carcinoembryonic antigen
CI	confidence interval
CRC	colorectal cancer
EA	esophageal adenocarcinoma
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMMPRIN	extracellular matrix metalloproteinase inducer
EMT	epithelial-mesenchymal transition
ESCC	esophageal squamous cell carcinoma
FGF	fibroblast growth factor
GI	gastrointestinal
GWAS	genome-wide association study
HCC	hepatocellular carcinoma
HNL	human neutrophil lipocalin (*synonym* NGAL)
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
NFkB	nuclear factor kappa B
NGAL	neutrophil gelatinase-associated lipocalin (*synonym* HNL)
NS	non significant
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PMN	polymorph nuclear neutrophil
PUMP-1	putative matrix metalloproteinase-1 (*synonym* MMP-7)
RECK	reversion-inducing cysteine-rich protein with Kazal motifs
RFLP	restriction fragment length polymorphism
SEM	standard error of the mean
SD	standard deviation
SNP	single-nucleotide polymorphism

Sp-1	specificity protein-1
Tcf	T-cell factor
TIMP	tissue inhibitor of metalloproteinase
TNM	tumor node metastasis
tPA	tissue-type plasminogen activator
TGF	transforming growth factor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor



CHAPTER 1

General introduction

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer related death in The Netherlands. More than 12,000 new cases were diagnosed in 2008. Almost 50% of the patients die of the disease and the majority of the disease-associated deaths can be attributed to the presence of distant metastases. The incidence of colorectal cancer in The Netherlands has increased in the period between 1989 and 2008 by 1% per year (source: www.iknl.nl). In men, 14% of all malignant tumours are colorectal cancers, which makes it the third most frequent tumour (after lung cancer and prostate cancer); in women, colorectal cancer is the second most frequent tumour (13% of the total number of cancers, preceded only by breast cancer) (Source: www.oncoline.nl). In 10% to 15% of colorectal cancers there is familial clustering and in almost 5%, CRC is associated with an autosomal dominant cancer syndrome (like Lynch syndrome or Familial Adenomatous Polyposis). In these highly penetrant hereditary disorders the lifetime risk to develop colorectal cancer varies between 25% and 100%, if no preventive measures are taken. The remaining 85% to 90% are sporadic cases. Until the beginning of the century, all colorectal cancers were thought to originate from adenomatous polyps, a process that has been described as a stepwise sequence of events by Vogelstein *et al*[1-3]. In this model for colorectal cancer development, the adenomatous polyp plays a crucial role as the precursor lesion that undergoes genetic changes as it develops from an early adenoma, via an intermediate and high grade adenoma to colorectal cancer and eventually to metastatic disease (Figure 1)[4].

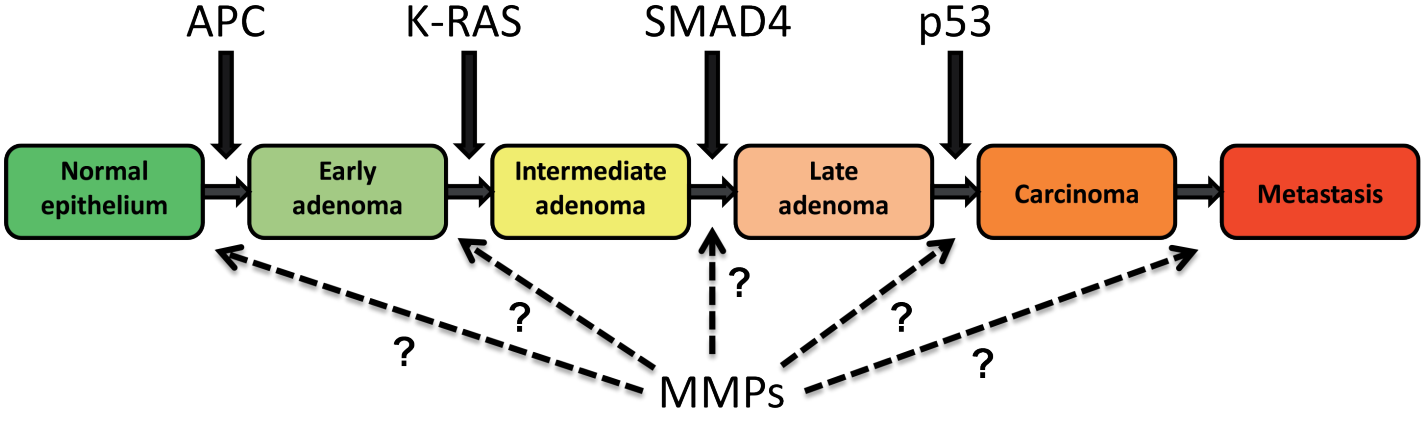


Figure 1. Schematic overview of the adenoma-carcinoma sequence of colorectal cancer development, according to Fearon and Vogelstein[1]. Some of the genes that are involved in the different phases of this multistep process are shown. Adapted from Ricci-Vitiani *et al.* (2008)[4].

A decade ago, an alternative route to colorectal cancer development was described[5, 6]. This alternative pathway, called the serrated pathway, describes the development of a different precursor lesion (*i.e.*, a sessile serrated polyp or sessile serrated adenoma) to cancer. In contrast to the traditional adenoma-carcinoma sequence, BRAF mutations and widespread DNA hypermethylation (CpG island hypermethylation phenotype or CIMP) play an important role in the serrated pathway[7-9]. Currently it is estimated that a majority of colorectal cancers develop according to the "classic" adenoma-carcinoma pathway (*i.e.*, according to the Vogelstein model), but a substantial part of cancers probably originate from a sessile serrated adenoma via the serrated pathway[10, 11].

Colorectal cancer treatment and prognosis is largely dependent on the cancer stage at the time of diagnosis. Whereas the 5-year survival of patients with a Dukes' A carcinoma[12], which is limited to the mucosa, is more than 90%, it decreases to less than 10% in Dukes' D patients who have distant metastases (Source: Surveillance, Epidemiology and End Results [SEER] program). Crossing of natural barriers, leading to invasive growth, and angiogenesis are crucial in the process of tumour progression and dissemination. At presentation, 25% of the patients with colorectal cancer already have distant metastases and this number increases up to 40% during the course of the disease. A shift towards detection of earlier stages of colorectal cancer can be achieved by population screening for colorectal cancer, which will start in The Netherlands in 2013. Especially in Dukes' B and C patients, who have an intermediate prognosis, the prognosis of individual patients is highly variable. Much research has been performed to identify markers involved in the malignant process that could be of additional value to determine prognosis and might identify patients eligible for adjuvant treatment.

Matrix metalloproteinases: structure and substrate specificity

Matrix metalloproteinases (MMPs) constitute a group of proteases that are involved in tissue remodelling and matrix turnover in many physiopathological conditions. The MMPs belong to the metzincin family, consisting of zinc- and calcium-dependent proteinases, that share a methionine at the C-terminal of the zinc-ligand which forms a Met-turn and a HEXXHXXGXXH zinc-binding motif[13]. Other members of the metzincin family, which share structural and functional features, are the subfamily of a disintegrin and metalloproteinase (ADAMs) and the group of a disintegrin and metalloproteinase with thrombospondin motifs (the ADAMTS family). The MMPs constitute a family of zinc-dependent endopeptidases, first described in 1962 by Gross and Lapière when they identified an enzyme that contributes to tail resorption during tadpole metamorphosis[14]. Since then, 27 MMPs have been identified of which 23 are present in humans.

All of the MMPs share the following features: 1) they are capable of degrading at least one of the extracellular matrix components, 2) they contain a zinc ion at the active site and are inhibited by chelating agents, 3) they are secreted as latent pro-enzymes (zymogens)

that need activation by plasmin, trypsin, thrombin or other proteases (including other MMPs), 4) they are inhibited by specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) and 5) they all share similar amino acid sequences[15]. The 23 human MMPs share a highly conserved structure including a N-terminal pro-peptide domain, a zinc-containing catalytic domain and, with the exception of MMP-7, MMP-23 and MMP-26, a C-terminal hemopexin-like domain[16]. According to their structure and major functions, MMPs can be subdivided in collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25), and a rest group. The global structure of the different subgroups of MMPs is shown in figure 2[17, 18]. Although every MMP has its own characteristic substrate profile, there is overlap between the different MMPs and most MMPs have a rather broad substrate specificity[19]. Under pathological conditions, like in cancer, almost all MMPs are present. Due to compartmentalization, a phenomenon that is discussed in the next paragraph, the catalytic activity of certain MMPs may be preferred over others[20].

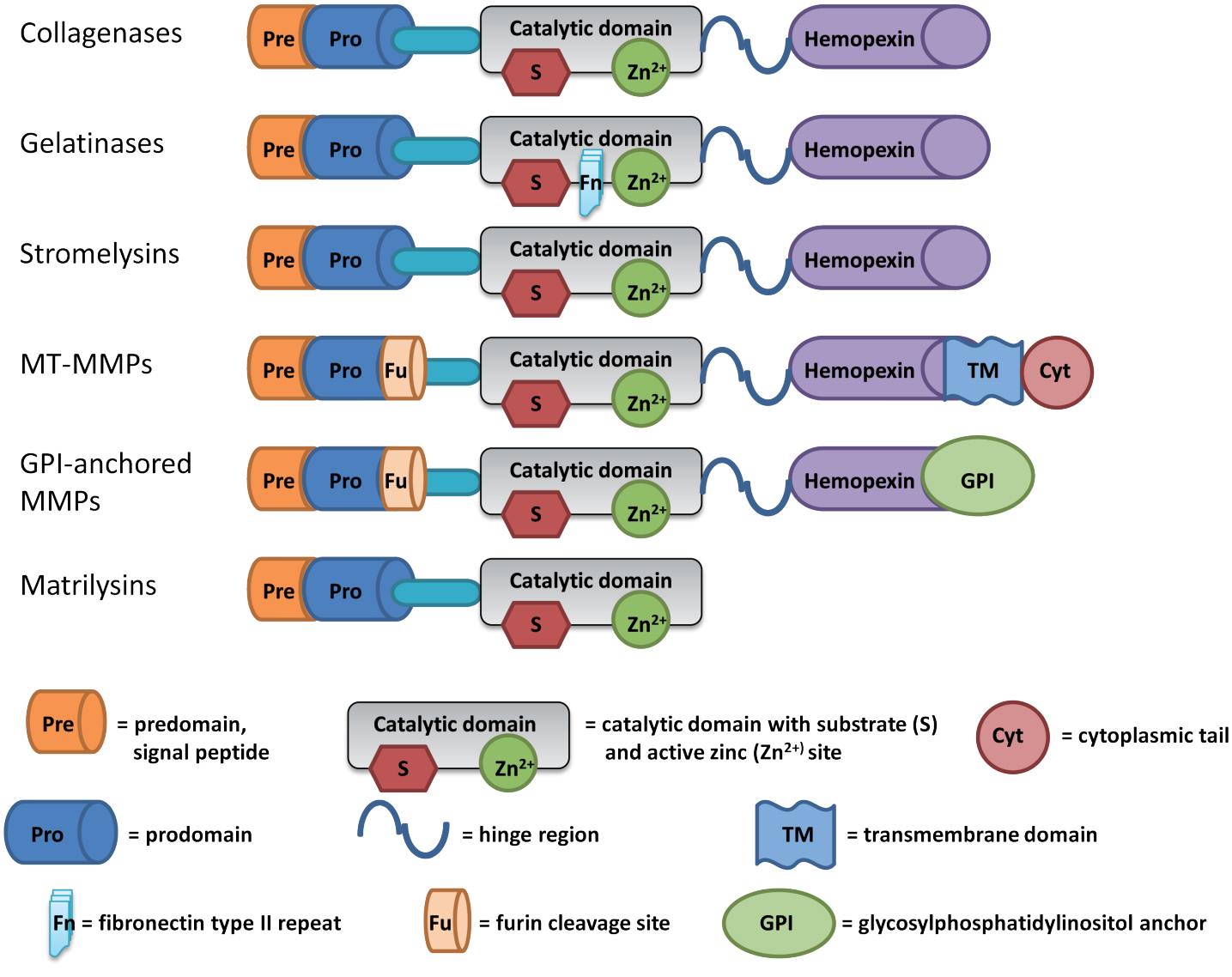


Figure 2. Domain structure of the major classes of MMPs. Adapted from Cauwe *et al.* (2007)[49], Busti *et al.* (2010)[17] and Brauer *et al.* (2006)[18].

Regulation of matrix metalloproteinases

Regulation of MMP activity takes place at different levels: regulation of gene expression, secretion and activation of latent MMPs, compartmentalization and regulation of enzymatic activity by endogenous activators and inhibitors.

1. *Regulation of gene expression*

The regulation of MMP gene expression is complex and is different under physiological (*e.g.*, embryonic development, childhood development, wound healing, bone turnover) and pathological (*e.g.*, cancer, arthritis, cardiovascular disease) circumstances. Transcription factors that bind to specific sites in the promoter region of the gene regulate transcriptional activity. Most of these transcription factors belong to the AP-1, SP-1, β-catenin/Tcf-4, NFκB and PEA3 families[21]. The levels of these transcription factors are influenced by cytokines (like interleukins, interferons, tumour necrosis factor alpha [TNF-α]) and growth factors (like transforming growth factor-beta [TGF-β], epidermal growth factor [EGF], vascular endothelial growth factor [VEGF] and platelet-derived growth factor [PDGF]) and hormones[22]. The end effect on the transcriptional level can be either stimulatory (*e.g.*, growth factors, proto-oncogenes) or inhibitory (*e.g.*, glucocorticoids and TGF-β)[15]. As the MMP-promoters show a high structural similarity, they are often co-regulated by the same transcription factors. On the other hand, and somewhat surprisingly, functionally similar MMPs (like the gelatinases) often have a distinct composition of the promoter region and are therefore regulated by different transcription factors[21].

Single nucleotide polymorphisms (SNPs) located in the promoter region of the genes may create or disrupt transcription factor binding sites and thereby influence transcriptional activity in either a positive or negative way. By these mechanisms, SNPs may be associated with cancer susceptibility and/or correlate with the disease phenotype or prognosis of patients who have already been diagnosed with cancer. The most common SNPs in the promoter region of the MMP genes and their correlation with either susceptibility for gastrointestinal (GI) cancer, or the course of disease of patients who already have developed cancer of the GI tract, are discussed in chapter 2.

2. *Secretion and activation of latent MMPs*

Except for MMP-11 and the membrane bound MT-MMPs, all MMPs are secreted as latent pro-enzymes, also called zymogens. These pro-enzymes are inactive due to the presence of a prodomain which binds to the zinc ion in the catalytic domain, thereby preventing the substrate from binding to the catalytic site. Proteinases (like trypsin, chymase but also MMPs themselves) are able to cleave the prodomain from the latent MMPs, creating a shorter and active MMP molecule. In this stepwise activation process, a $zinc^{2+}$ ion is first released from the cysteine complex. It was recently discovered that proteolytic removal of the prodomain is not a definite prerequisite for the activation process

to take place. Allosteric activation, which takes place when a thiol-binding reagent binds to the inactive MMP, resulting in displacement of the propeptide from the active site, is an alternative way of activating MMPs[23].

3. *Compartmentalization*

Specificity of proteolysis is regulated by the affinity of an enzyme for a certain substrate. Furthermore, where and how an MMP is released and kept in the pericellular environment, also referred to as compartmentalization, is at least as important as the enzyme-substrate interaction[20]. Similar to the membrane-bound MMPs, the nonmembrane-bound MMPs released from the cells are kept locally in a high concentration by specific cell-MMP interactions, for example the binding of MMP-2 to α*v*β3 integrin and MMP-9 to CD44[20, 24, 25]. Thereby, their proteolytic activity can be targeted towards specific substrates in the pericellular space.

4. *Endogenous regulators of enzymatic activity*

Tissue inhibitors of metalloproteinases (TIMPs) are the natural inhibitors of the MMPs. Currently, four TIMPs have been identified (known as TIMP-1 to -4) that reversibly bind to MMPs in a 1:1 ratio, forming binary non-covalent complexes which are resistant to heat-denaturation and proteolytic degradation[26]. All TIMPs are capable of binding at least five different MMPs (but not at the same time) and the substrate spectrum of TIMP-2 even includes all MMPs. However, each TIMP has its own substrate preference. TIMPs are also able to bind the proforms of MMPs, and this interaction is relatively specific: TIMP-2, TIMP-3 and TIMP-4 bind to proMMP-2 (but not proMMP-9), whereas TIMP-1 and TIMP-3 bind to proMMP-9 (but not proMMP-2)[27]. The binding of a TIMP to an MMP does not always have inhibitory effects, as illustrated by the binding of TIMP-2 in combination with MT1-MMP to proMMP-2, leading to the activation of proMMP-2[28-30]. Besides these endogenous MMP-specific inhibitors, other antiproteases like α2-macroglobulin, which is present in tissue fluids and plasma, also inhibit MMPs. Binding of this plasma protein to MMP leads to endocytosis, resulting in irreversible clearance of the MMPs from the circulation[31]. Furthermore, RECK (reversion-inducing cysteine-rich protein with Kazal motifs), a 110 kDa membrane-anchored glycoprotein with multiple EGF-like repeats and serine protease inhibitor-like domains[32], is an inhibitor of MMP-2, MMP-9 and MT1-MMP secretion and activity[32, 33]. On the other hand, endogenous activators of MMPs can also increase the MMP-production. EMMPRIN (extracellular matrix metalloproteinase inducer) is an MMP-inducer that is present in normal cells, but can be tumour-cell derived as well and is even up-regulated in cancer cells[34, 35]. This 58,000 kDa heavily glycosylated protein, member of the immunoglobulin superfamily, has been shown to stimulate production of MMP-1, -2, -3, and -9 by local endothelial cells and fibroblasts[36-39].

Functions of MMPs under physiological and pathological circumstances

Tissue remodelling is essential in physiological processes like growth and embryonic development. The nature and variety of the substrates of MMPs suggests a major role for this proteinase family in tissue morphogenesis and this assumption has been confirmed in various experimental models[40-44]. Also in reproductive processes like the menstrual cycle, ovulation, and the involution of the uterus, MMPs are overexpressed[44, 45]. Under physiological conditions, various MMPs are involved in the formation of new blood vessels[46] and in adults, their role in bone turnover and development as well as wound healing, in which processes take place that resemble those in malignant disease, like migration, ECM degradation and invasion, has been well established[47].

The proteolysis of extracellular matrix components is one of the first recognized functions of MMPs. Although substrate specificity is broad and most MMPs are capable of degrading more than one component of the extracellular matrix (ECM), each MMP has its specific substrate profile, which is not limited to ECM components (like collagens, laminin, fibronectin, vitronectin, tenascin, elastin, entactin and proteoglycans) but extends to cytokines, growth hormones and binding proteins[48]. In the last decade it has become clear that MMP function is much more comprehensive than regulation of cell growth by cleavage of cell surface-bound growth factors and receptors, and by release of sequestered growth factors from the ECM. This now also includes regulation of apoptosis, influencing cell motility by cleavage of adhesion molecules and revealing matrix signals, regulation of angiogenesis (in a pro- as well as anti-angiogenic way), interfering with the immune system and modulation of the bioactivity of chemokines[49, 50]. All these processes are relevant in different stages of cancer development and progression. The first step in cancer growth and metastases is to cross the natural barriers (*e.g.*, the basement membrane), a process in which the proteolytic properties of the MMPs are of importance. After the cancer has invaded into the deeper layers of the normal tissue, vascular invasion and angiogenesis as well as the ability for individual cancer cells to survive in the circulation become important. In most cases, the liver is the primary target organ for metastases of colorectal cancer. Before cancer cells are able to embed and to multiply in another organ, a so called “metastatic niche” needs to be created[51]. MMPs have been demonstrated to play an important role in the formation of this specific environment in the target organ that permits the growth and/or invasion of cancer cells at a distant site[52]. Figure 3 illustrates the different cell types that are implicated in the aforementioned processes and shows which MMPs are involved at the different stages of tumour growth, invasion and metastasis.

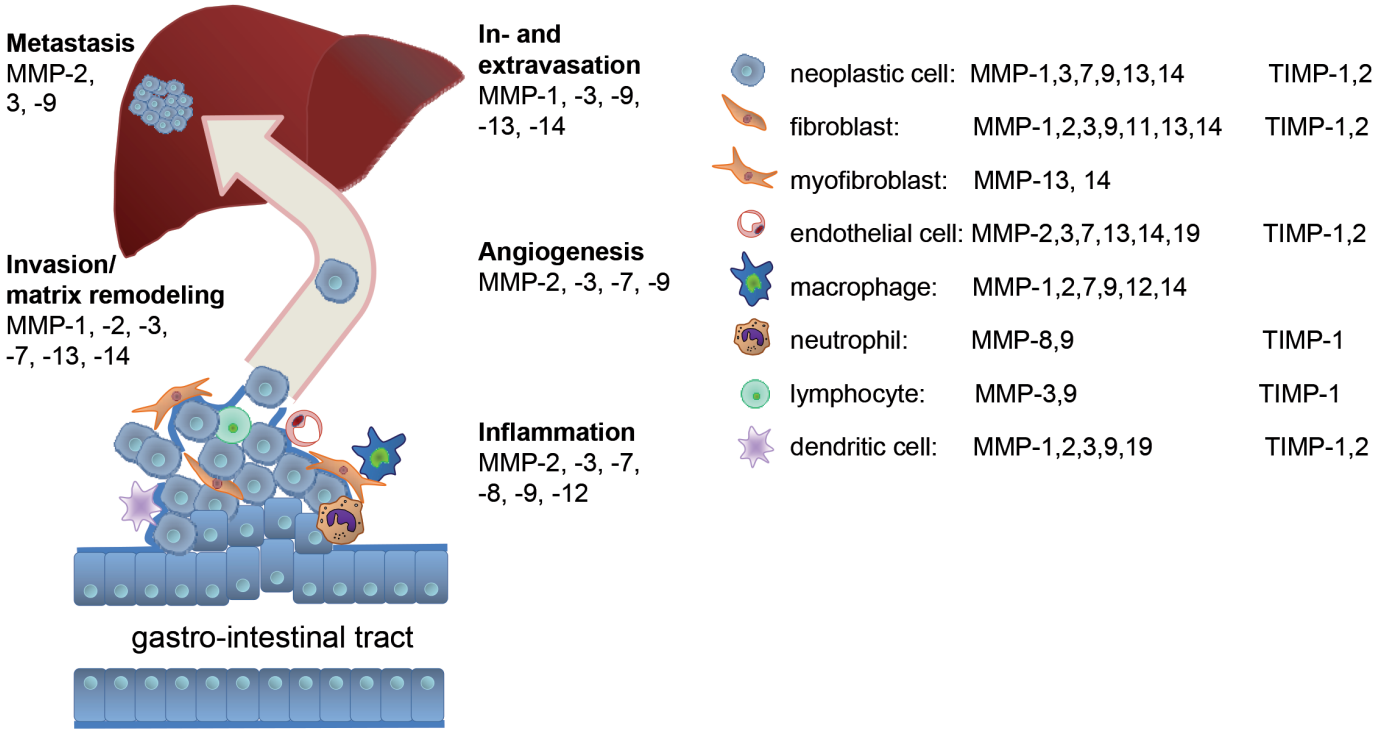


Figure 3. Overview of the different processes in tumour development and metastasis, and the MMPs that are involved in each of these processes. The right panel shows the cellular source of the MMPs. Most MMPs are produced mainly by stromal cells (like inflammatory cells, fibroblasts and endothelial cells) that are present abundantly in the tumour microenvironment. Only a small part of MMP production occurs in the tumour cells themselves. Some examples of the MMPs and TIMPs that are produced by the various stromal cells and neoplastic cells are shown. Adapted from Kessenbrock *et al.* (2010)[54].

CHARACTERISTICS OF MMPs STUDIED IN THIS THESIS

Matrix metalloproteinase -2 and -9

MMP-2 (gelatinase A or 72 kDa type-IV collagenase) and MMP-9 (gelatinase B or 92 kDa type-IV collagenase) are named after two of their major substrates. Both enzymes contain fibronectin type II-like repeats within their catalytic domains (Figure 2), which increases the affinity for binding to gelatin (denatured collagen) and elastin[53]. Although tumour cells have been reported to secrete MMP-2 and MMP-9, both gelatinases are produced mainly by stromal cells. MMP-2 is expressed constitutively by many different cell types including macrophages, endothelial cells, mast cells, fibroblasts, hematopoietic progenitor cells and dentritic cells[31, 54]. MMP-9 is produced mainly by inflammatory cells, like neutrophilic granulocytes, macrophages, mast cells and lymphocytes, but other cell types including dendritic cells and fibroblasts are also sources of MMP-9. In 1980, Liotta *et al*. demonstrated that the degradation of collagen type IV by MMPs leads to invasion of the cancer cells into the deeper layers of the bowel wall and subsequent metastasis[55]. Due to their capability to degrade type-IV collagen, which is a major component of the basement membrane, the gelatinases have been regarded with particular interest in cancer research.

As is the case for MMPs in general, the functional spectrum of MMP-2 and MMP-9 extends far beyond their collagenolytic activity. Besides collagen type IV and gelatin, other ECM components like elastin, laminin and aggrecan, but also non-ECM molecules like growth factors (*e.g.* TGF-β), chemokines and other proMMPs are targets for MMP-2 and/or MMP-9[56-67]. An important role in angiogenesis has been established for MMP-9, which triggers the angiogenic switch by releasing VEGF[68]. In an *in vivo* animal model, MMP-2 has been shown to be required for the switch to an angiogenic phenotype[69]. On the other hand, angiogenesis could be inhibited by the MMP-9 mediated formation of angiostatin, which is a breakdown product of plasminogen and a potent angiogenesis inhibitor[70]. MMP-9 is essential in the formation of a metastatic niche, in which its ability to break down basement membrane barriers and induce the release of VEGF are crucial[71]. Inflammation is another essential aspect of cancer progression[72]. One of the ways in which MMP-2 and MMP-9 are implicated in inflammation is by activation of pro-TNF-α, which is a pro-inflammatory cytokine that is produced in large quantities in cancer and has been shown to promote cancer cell survival[73, 74]. Proteolysis of chemokines by MMP-2 or MMP-9 can also lead to inactivation of pro-inflammatory cytokines and therefore the role of these MMPs in inflammation is not only pro-inflammatory, but they serve as regulators of inflammation[75]. The influx of tumour-infiltrating inflammatory cells, like macrophages, neutrophils and mast cells into the tumour microenvironment will lead to a local increase of MMP-9 production, as these cells are the major source of MMP-9.

Matrix metalloproteinase-7

MMP-7 (also known as matrilysin or putative metalloproteinase [PUMP]-1) is the smallest member of the MMP-family, consisting of a predomain, prodomain and catalytic domain only, lacking a C-terminal hemopexin domain (figure 2). The substrate specificity of MMP-7 resembles that of the stromelysins and includes ECM components (like fibronectin, gelatins, collagen type IV, laminin and elastin), proMMPs -1, -2 and -9 and other proteins (like transferrin, urokinase plasminogen activator (uPA), e-cadherin and TNF-α precursor), although the structure of the catalytic domain is more similar to that of the interstitial collagenases[76-83]. It is the only MMP, apart from membrane-type 1-MMP, that is secreted mainly by the tumour epithelial cells themselves. Under physiological circumstances, MMP-7 is expressed in the wound-edges from injured epithelium of mucosal surfaces in the lung and intestine, where it is required for re-epithelization[84]. Loosening of cell-cell contacts (and thereby increased cell migration) through MMP-7 mediated shedding of e-cadherin ectodomains is probably the underlying mechanism in this process[85, 86]. Also in healthy, non-injured epithelium MMP-7 is constitutively expressed and regulates apoptosis and the defence against bacteria by regulating the production of defensins. In a mouse model, MMP-7 activates intestinal pro-α defensins, which fight pathogenic intestinal bacteria like *Escherichia coli* and *Salmonella Typhimurium*[87, 88]. The various functions of MMP-7 make it

a good candidate to be studied in a cancer environment, and it has indeed been demonstrated that MMP-7 is involved at different levels of cancer progression and metastasis.

In colorectal cancer, MMP-7 has been reported to induce angiogenesis by accelerating the proliferation of endothelial cells[89] and it was found to activate the EGF receptor by the release of TGF-α, which is an EGF ligand[90]. MMP-7 also increased tumour cell survival by interfering with at least two pathways. Firstly, it's binding to insulin-like growth factor binding protein (IGFBP)-3 resulted in proteolysis of this protein, leading to increased bio-availability of IGF-1 and increased tumour cell survival[91]. Secondly, MMP-7 increased resistance to programmed cell death by shedding membrane-bound Fas ligand and thereby creating an apoptosis-resistant phenotype[92].

Matrix metalloproteinase-8 (MMP-8) and neutrophil gelatinase-associated lipocalin (NGAL)

The members of the subfamily of MMPs referred to as collagenases, to which MMP-8 belongs, share the ability to cleave the fibrillar collagens type I, II and III. The substrate range of MMP-8 extends beyond that and includes a wide range of collagens (collagen type VII, X) and non-collagenous ECM components (like laminin, proteoglycans) as well as non-structural substrates like angiotensin, IGFBP-5, chemokines and serine protease inhibitors[93]. MMP-8 is also named collagenase-2 or neutrophil collagenase, the latter of which suggests that MMP-8 is derived from polymorphonuclear neutrophils (or PMNs), which have indeed long be assumed to be the only source of MMP-8. It has been shown, however, that not only other inflammatory cells, like T-cells and plasma cells but also non-inflammatory cells, like epithelial cells, colonic mucosal cells and myofibroblasts, express MMP-8, albeit mainly in acute or chronic inflammatory conditions[93].

Neutrophil gelatinase-associated lipocalin (NGAL), also known as human neutrophil lipocalin (HNL) or lipocalin-2, is a 25 kDa protein that belongs to the lipocalin superfamily. This family consists of about 20 small lipoproteins that share a similar 3D-structure and the capability to bind to small hydrophobic ligands like hormones, fatty acids, prostaglandins and retinoids[94]. Lipocalin-2 has first been described to be present in the granules of human neutrophilic leucocytes[95], but was later discovered to be expressed by certain epithelial cells, mainly under circumstances of inflammation or malignancy[96, 97]. It is able to covalently bind to matrix metalloproteinase-9 (MMP-9) and inhibit its degradation, thereby sustaining MMP-9 proteolytic activity[98, 99]. Furthermore, lipocalin-2 is implicated in the process of iron trafficking and metabolism. By binding to bacterial siderophores ("iron carriers" in Greek language), which are small molecules with a high affinity for Fe^{3+}, lipocalin-2 acts as a bacteriostatic agent as it blocks the growth of the bacteria[100]. Mammalian cells produce siderophore-like molecules that also bind to lipocalin-2, which might lead to either iron deposition in the cell (in case of iron-loaded siderophores) or, in case of binding to a siderophore not complexed with iron, to cellular uptake of an iron-free siderophore-lipocalin-

2 complex which induces a state of iron depletion and presumably stimulates apoptosis[101-103]. The exact role of lipocalin-2 in cancer seems to be contradictory and dependent of the cellular origin of the tumour, as pro-tumour effects (*e.g.*, inhibition of apoptosis) as well as anti-tumour effects (*e.g.*, stimulation of apoptosis, inhibition of adhesion and invasion of cancer cells, and the arrest of VEGF synthesis) have been reported in various tumour types[96, 104].

Matrix metalloproteinases in colorectal cancer

In the year 2000, Hanahan and Weinberg defined six hallmarks of cancer which consist of six biological capabilities that are of importance during the development of human cancer[105]. These hallmarks are: 1) sustaining proliferative signalling by production of autocrine growth signals, 2) escaping growth suppressors, 3) insensitivity to apoptosis, 4) enabling replicative immortality by loss of senescence, 5) induction of angiogenesis and 6) tissue invasion and metastasis. The different functions of MMPs that have been described in this chapter demonstrate that they are implicated in some way in all of these processes which suggests that they are probably involved in virtually all different types of cancers. Over expression of MMP-1, -2, -3, -7, -9, -10, -11, -12, -14 (MT1-MMP), -21 and -25 (MT6-MMP) has been described in human colorectal cancer and often the MMP-levels in cancer tissue were associated with the clinical outcome of the patients[106-152]. Also in colorectal adenomas, MMP-2, -7 and -9 and their inhibitors TIMP-1 and TIMP-2 have been demonstrated to be up-regulated[118, 139, 148, 153-156]. The exact pattern of up-regulation of MMPs in the different stages of colorectal cancer development and progression has only been partially uncovered, but insight in this process is crucial for the development of MMP-inhibiting therapeutical strategies.

Outline of the studies described in this thesis

The studies described in this thesis were focused on the possible impact of MMP-2, MMP-7, MMP-8, MMP-9 and NGAL on colorectal cancer development, metastasis and survival of the patients. In order to put the different studies in perspective, **chapter 1** first gives a short introduction on colorectal cancer, MMPs and their specific functions in a cancer microenvironment.

The adenomatous polyp is the most common precursor lesion of colorectal cancer. A number of genetic alterations are needed before an adenomatous polyp evolves to colorectal cancer. In each step of this process, specific mutations can be identified as described earlier in this chapter and illustrated in figure 1. There is evidence that MMPs are involved in the early phase of the neoplastic process. Once a cancer has developed, it has the potential to metastasize to distant organs. In colorectal cancer, the liver is most often the target organ for distant metastases. Throughout the process from adenoma formation, via the development of colorectal cancer and eventually the occurrence of distant

metastases, MMPs might be of pivotal importance. We speculated that the role of different MMPs might vary throughout this process. **Chapter 2** describes the analysis of how MMP-2, MMP-8, MMP-9 and NGAL could be involved in the different steps of the adenoma-carcinoma-metastasis sequence.

Subsequently a study on the use of MMP-2 and MMP-9 measurements in body fluids, as a putative reflection of the malignant process in the intestine, for the prediction of the course of disease in colorectal cancer patients is described in **chapter 3**. Besides the (labour-intensive) technique of quantitative gelatin zymography, also a bio-activity assay (BIA) and an enzyme-linked immunosorbent assay (ELISA) were used to measure the MMP-levels in urine and peripheral blood. The levels of MMP-2 and MMP-9 in plasma and urine were evaluated for their prognostic impact in patients with colorectal cancer.

Single-nucleotide polymorphisms (SNPs) are the most common form of genetic variation and consist of the replacement of a single base pair in the human genome. This replacement can occur in coding and non-coding regions of the genome. These subtle genetic variations may influence the level of production of the respective protein and affect the susceptibility to develop a certain disease, but may also influence the course of a disease. Most of the SNPs of MMPs that have been studied in cancer of the digestive tract are located in the promoter region of the gene and might therefore influence gene expression. **Chapter 4** describes a comprehensive evaluation of the literature on SNPs in the MMP genes in gastrointestinal cancer (*i.e.*, esophageal, gastric, hepatocellular and colorectal carcinoma).

MMP-2 and MMP-9 were previously demonstrated to be implicated in a variety of cancers. Because of their capability to degrade collagen type IV, a major component of the basement membrane, they have been in the centre of interest for a long time. In most tumours, high levels of MMP-2 and MMP-9 are associated with an unfortunate outcome of the patients. The study we performed on the impact of MMP-2 and MMP-9 protein levels and their corresponding genotypes in 215 colorectal cancer patients is described in **chapter 5**. Particularly the correlation of the genotypes and the phenotypical expression of the gelatinases in homogenates of colorectal carcinoma tissue with survival were evaluated in this study.

Changes in normal mucosa distant from a tumour have been previously associated with disease outcome. From 198 of the 215 patients described in chapter 3, normal colon tissue at 5-10 centimetres distance from the malignancy was available in our biorepository. This permitted a look into the MMP-2 and MMP-9 levels in normal colorectal tissue of patients suffering from colorectal cancer in relation to their 5-year survival. The results of this study are described in **chapter 6**.

The results of a study on MMP-7 expression and activity in 174 colorectal carcinomas and 52 colorectal adenomas are reported in **chapter 7**. Two single nucleotide polymorphisms of the MMP-7 gene, located in its promoter region and known to modify

gene transcription activity *in vitro* (MMP-7 -153 C/T and -181 G/T SNP) were studied in the 174 colorectal cancer patients, with special attention to their correlation with tissue levels of MMP-7 and survival. Furthermore, this chapter focuses on MMP-7 expression and activity during the different stages of colorectal cancer development and the correlation between the tumour levels of (active) MMP-7 and outcome of the colorectal cancer patients.

Finally the different studies described are summarized and discussed in **chapter 8** of this thesis. In addition, some possible directions for further research and clinical implications are given.

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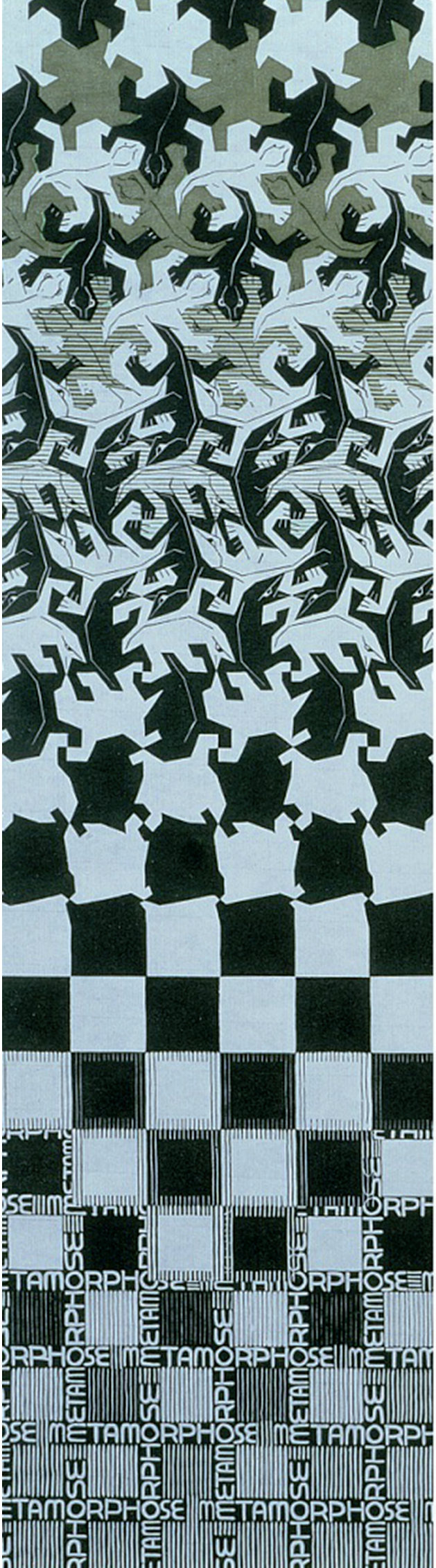

CHAPTER 2

MMP-2, MMP-8, MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL) in the colorectal cancer sequence

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ABSTRACT

Background and aim of the study: Matrix metalloproteinases (MMPs) have been implicated in colorectal cancer progression and prognosis. However, the role of MMPs in early cancer development and in the process of dissemination of the disease has been studied less extensively. In the present study, we investigated the expression and activity of MMP-2, -8 and -9 and neutrophil gelatinase-associated lipocalin (NGAL) in tissue from various stages of colorectal cancer progression.

Methods: The expression of NGAL, MMP-8, -2 and -9 was measured by ELISA in a series of normal colorectal mucosa, adenomatous polyps, adenocarcinomas, liver metastasis and normal liver (n= 20 of each group), and the activity of MMP-9 was measured by Bio–Immuno-Activity Assay (BIA) and quantitative gelatin-zymography of tissue homogenates. The degree of inflammation was assessed by myeloperoxidase (MPO) activity.

Results: There was a consistent and significant step-wise increase in the MMP-8 level associated with the progression from normal mucosa to adenoma (2-fold) and from adenoma to carcinoma (8-fold), with similar high levels in the liver metastases as in the primary carcinomas. We found a similar, though less pronounced, increase (up to 3-fold) in MMP-9 in this malignancy sequence, with a good correlation between the ELISA and zymography results ($r = 0.60$). NGAL also showed an increase in the normal mucosa - adenoma (4-fold) - carcinoma (6-fold) - liver metastasis (3-fold) sequence. The MMP-2 level only showed an increase (up to 1.5-fold) in the carcinomas. Interestingly, the primary colorectal carcinomas contained a significantly ($P < 0.05$) higher percentage of both MMP-2 and MMP-9 in the active enzyme form than adenomas and liver metastases (respectively 55 ± 6 *vs* 29 ± 3 and 30 ± 3, and 21 ± 1 *vs* 14 ± 2 and 10 ± 3). Normal liver tissues had low MMP levels.

Conclusion: In conclusion, the development of colorectal cancer, as illustrated by the normal mucosa - adenoma - carcinoma - liver metastasis sequence, is accompanied by a concurrent increase in the NGAL and MMP levels, particularly MMP-8 and MMP-9, providing evidence that they are causatively involved in colorectal cancer progression.

INTRODUCTION

Colorectal cancer is the third most frequent cancer worldwide, with more than 1,2 million new cases each year and 608.000 deaths in 2008 (source: World Health Organization, www.WHO.int). Up to 15% of these cancers are clustered within families, while the other 85% are classified as sporadic colorectal cancer. In both familial and sporadic cases, the majority of these cancers develop from a precursor lesion, the adenomatous polyp, in which sequential changes eventually lead to the development of cancer[1, 2].

Matrix metalloproteinases (MMPs) are metal-dependent proteolytic enzymes that play an important role in various biological processes as they determine the rate of remodelling of the extracellular matrix and are implicated in the regulation of angiogenesis, migration, invasion and cancer-cell growth and cell death[3, 4]. Approximately 25 members of the MMP family have so far been identified, which have been numbered in the order of their discovery and subdivided based on the characteristics of their substrate specificity in collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs[5]. Altogether, the members of the MMP family are able to degrade virtually all components of the extracellular matrix, such as collagens, laminin, fibronectin, vitronectin, entactin and proteoglycans[5]. Most MMPs are up-regulated in malignancy and, in most cases, are thought to contribute to cancer progression and dissemination. However, this is not always the case, as illustrated by the protective effects of overexpression of some MMPs (*e.g.*, MMP-8 or neutrophil collagenase) that has been described for several tumours[6-8].

Lipocalins, together with the fatty-acid-binding proteins (FABPs) and avidins, belong to the calycins[9]. These lipocalins are involved in the regulation of cell homeostasis, modulation of the immune response, and clearance of endogenous and exogenous compounds[9]. Neutrophil gelatinase-associated lipocalin (NGAL) is a 25 kD protein of human neutrophils, that is in part covalently complexed with MMP-9 (gelatinase B, 92 kDa type IV collagenase). It is located in specific granules apart from gelatinase granules in human neutrophils[10].

In colorectal carcinomas, increased levels of active MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 have been reported compared to normal mucosa[11-13]. In addition, a role in early carcinogenesis has been suggested for MMP-9, but not for MMP-2[14-16].

The aim of the present study was to investigate the presence and activity of MMP-2, MMP-8, MMP-9 and NGAL throughout the sequence from normal tissue, via adenomas and colorectal cancer, to liver metastases from colorectal origin. We used several techniques, including bio-immuno-activity assay (BIA), ELISA and quantitative zymography, to quantify the total and active amount of the proteinases.

PATIENTS, MATERIALS AND METHODS

Patients and Tissues

Adenomas (n=20) were freshly obtained by endoscopic snare polypectomy at the Department of Gastroenterology and Hepatology of the Leiden University Medical Center (LUMC). Fresh tissue samples of the adenomas were frozen and stored at -70°C until extraction. Part was routinely formalin fixed and embedded in paraffin for histological evaluation. Fourteen adenomas were found to be tubulovillous, five were tubular and one was villous. The degree of epithelial cell dysplasia was low grade in 14 adenomas and high grade in 6 adenomas.

In addition, fresh tissue was obtained from 20 patients who underwent resection for primary colorectal carcinoma at the Department of Surgery of the Leiden University Medical Center as previously described[17]. Representative samples of the carcinoma and macroscopically normal mucosa, taken 5-10 cm from the tumour, were frozen and stored at -70°C until extraction and parallel samples were routinely fixed and embedded in paraffin. Using the Dukes' classification as modified by Astler and Coller[18], the carcinomas were classified in stage B1 (n=4), B2 (n=8), C1 (n=2), C2 (n=3) and D (n=3). Differentiation grade was poor in 2, moderate in 13 and well in 5 carcinomas. Four out of the 20 carcinomas were of the mucinous type.

Finally, fresh tissue was collected from 20 patients who underwent partial liver resection because of colorectal liver metastasis as previously described[19]. Representative samples of the carcinomas and of macroscopically normal liver tissue were frozen and stored at -70°C, other parts were routinely formalin fixed and embedded in paraffin. In all cases, the diagnosis metastasis of an adenocarcinoma from colorectal origin was confirmed by histopathological examination.

Tissue extraction and protein concentration

Tissue specimens were homogenized in 0.1 M Tris-HCl, 0.1% (v/v) Tween 80 buffer (pH 7.5), as extensively described previously[17, 20, 21]. Briefly, the samples were homogenized in 1 ml buffer per 60 mg wet tissue at 0°C. The homogenates were centrifuged at 8000 g for 2.5 min at 4°C and the supernatant was stored at -70°C until analysis. The protein concentration of the supernatant was determined by the method of Lowry[22].

MMP-2 and MMP-9 gelatin zymography

Presence of active and latent forms of matrix metalloproteinases was analyzed by zymography on 10% SDS-polyacrylamide gels containing 2% gelatin and overnight incubation at 37°C, as described previously (12, 24). Sample volumes were adjusted to obtain a uniform protein content of 10 µg per sample. The gels were stained with Coomassie brilliant blue R-250, dried between sheets of cellophane, and the degree of

gelatin digestion was quantified using a LKB Ultroscan XL enhanced laser densitometer (633 nm). Two amounts (12 and 24 µg protein of an internal standard preparation, *i.e.* an homogenate of a colonic carcinoma containing both MMP-2 and MMP-9, were included on each gel for correction of intergel variation and as reference for the expression in arbitrary units (AU). This zymographic analysis was highly linear over an at least 20-fold range (*i.e.* 2-40 µg protein per sample).

NGAL, MMP-2, MMP-8 and MMP-9 ELISAs

The total amount of NGAL, MMP-2, MMP-8 and MMP-9 was determined, in appropriate dilutions of the homogenates, by established sandwich-ELISAs, as described previously[23-25]. Results were expressed in ng MMP per mg protein.

MMP-2 and MMP-9 Bio-Immuno-Activity Assay (BIA)

Total MMP-2 and MMP-9 was also measured using a colorimetric assay[26]. Briefly, antibodies to MMP-2 and MMP-9, as in the ELISAs, were used as catching antibody for the respective MMPs in the homogenates, during overnight incubation. These MMPs were then activated by incubation with 0.5 mM p-aminophenyl-mercuric acetate at 37°C and their activity measured with modified pro-urokinase (Ukcol) and S-2444 (0.6 mM), as chromogenic substrate, in assay buffer at 37°C. Reactions were performed in 96-well flat-bottomed microtitre plates, and a Titertek Multiskan photometer was used to follow the absorbance change at 405 nm. Results were calculated from a standard curve and expressed as units MMP per mg protein.

Myeloperoxidase activity

Myeloperoxidase (MPO) in tissue homogenates was assessed according to the procedure described by Kruidenier *et al*[27]. In short, 50 µl tissue homogenate was incubated in buffer with 0.026% ortho-dianisidine dihydrochloride and 0.02% hydrogen peroxide. The reaction kinetics were followed in colorimetrically at 450 nm in microtitre plates. MPO was expressed in Arbitrary Units (AU) per mg protein.

Statistical analysis

Group means are given as mean ± standard error of the mean (SEM). Differences between dependent samples from groups were tested for significance using paired Student's t-test with separate variance estimate if the standard deviations were significantly different according to the F-test. For testing the significance of a difference in means for independent samples the independent-samples Student’s t-test was used. Differences were considered significant when $P < 0.05$ (SPSS for Windows Release 7.0, 1995, SPSS Inc., Chicago, U.S.A.).

RESULTS

Myeloperoxidase activity

Inflammation, as assessed by the myeloperoxidase activity, was significantly higher ($P \leq 0.001$) in adenomas (4.72 ± 0.35) compared to normal mucosa (1.99 ± 0.28), colorectal cancer (2.14 ± 0.30), liver metastases (1.92 ± 0.37) and normal liver tissue (0.51 ± 0.12). No relevant difference was observed between carcinomas and metastases.

MMP-8

There was a consistent and significant step-wise increase in the level of MMP-8 in the normal mucosa-adenoma (2-fold)-carcinoma (8-fold) sequence, with a similar level in the liver metastases compared to the primary carcinomas (Figure 1). Even after correction for inflammation by the MPO content, the increase of MMP-8 in carcinoma and liver metastasis persisted (Figure 2).

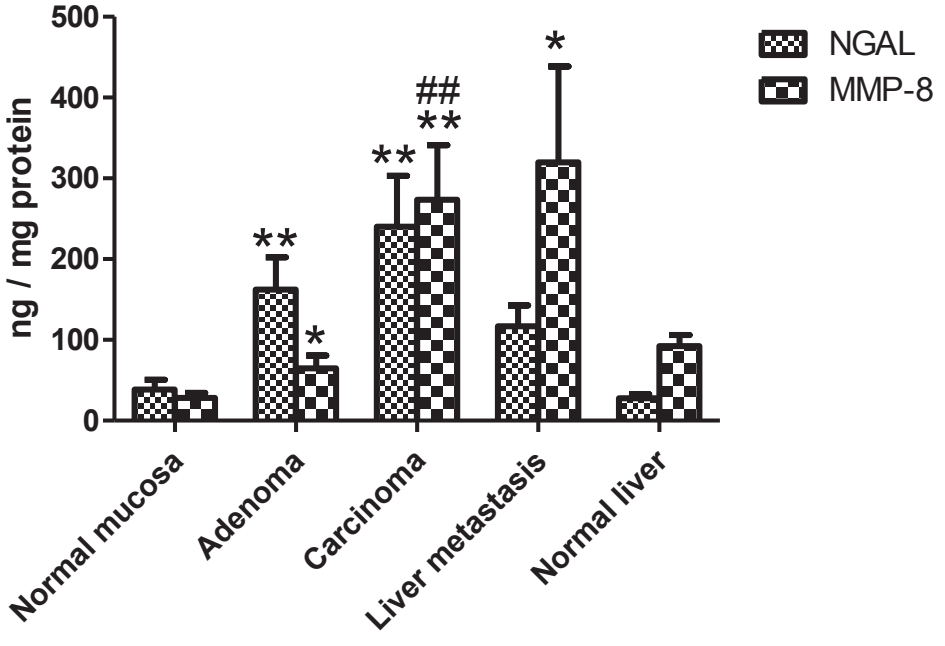

Figure 1. MMP-8 and NGAL, as measured by ELISA, in the colorectal cancer sequence. * $P \leq 0.05$ *vs*. normal mucosa; ** $P \leq 0.01$ *vs*. normal mucosa; ## $P \leq 0.01$ *vs*. adenoma.

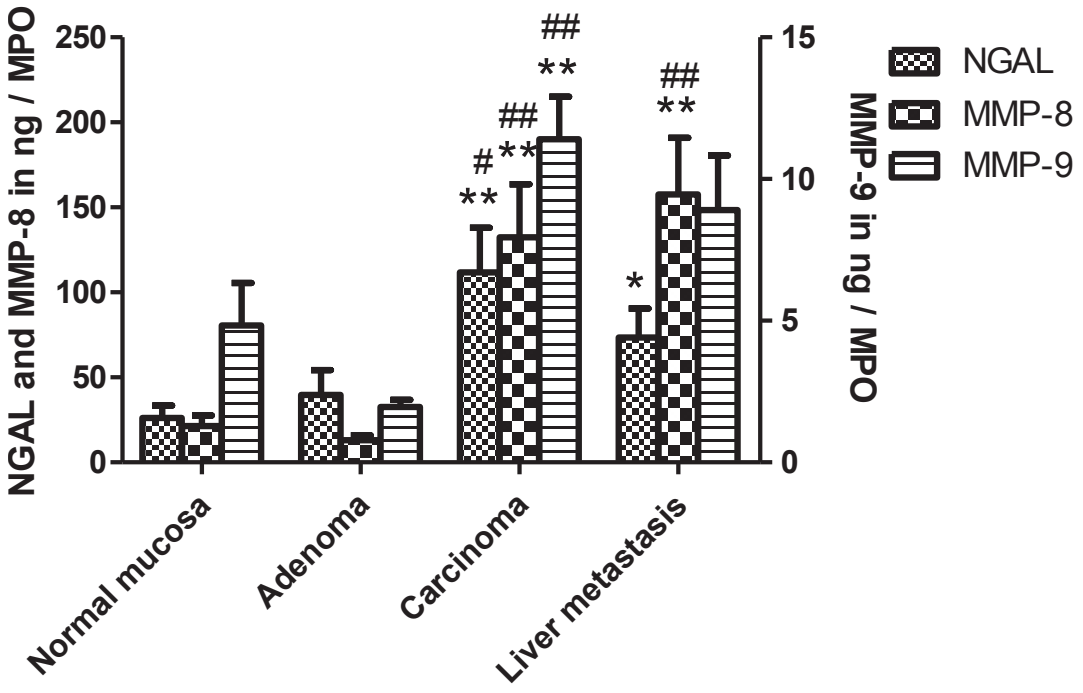

Figure 2. Levels of MMP-8, NGAL (left axis), and MMP-9 (right axis), as determined by ELISA, in colorectal neoplasia, corrected for MPO activity. Values are expressed in ng NGAL or MMP/MPO. * $P \leq 0.05$ *vs*. normal mucosa; ** $P \leq 0.01$ *vs*. normal mucosa; # $P \leq 0.05$ *vs*. adenoma; ## $P \leq 0.01$ *vs*. adenoma.

Neutrophil granulocyte-associated lipocalin (NGAL)

Adenomas (4-fold) and carcinomas (6-fold) showed a marked increase in NGAL content compared to normal mucosa and resembled the trend of MMP-9 (Figure 1). Liver metastases were found to contain more NGAL than normal liver. After correction for inflammation the primary colorectal carcinomas still contained 4 times - and liver metastases 2 times - the amount of NGAL present in normal colonic mucosa (Figure 2).

MMP-9

The MMP-9 ELISA showed a comparable, though less pronounced, increase (up to 3-fold) in the sequence normal mucosa-adenoma-carcinoma (Figure 3). The MMP-9 content in liver metastases was lower than in the primary tumours and comparable to that in adenomas. After correction for inflammation, the colorectal carcinomas still contained consistently higher amounts of MMP-9 (Figure 2). Quantitative zymography showed significantly higher amounts of pro-, active- and total MMP-9 in carcinomas compared to the other tissues. The percentage of active MMP-9 in the carcinomas (21 ± 1%) was considerably higher compared to adenomas and liver metastases (14 ± 2% and 10 ± 3%, respectively), but lower than in normal colon mucosa (30 ± 4%).

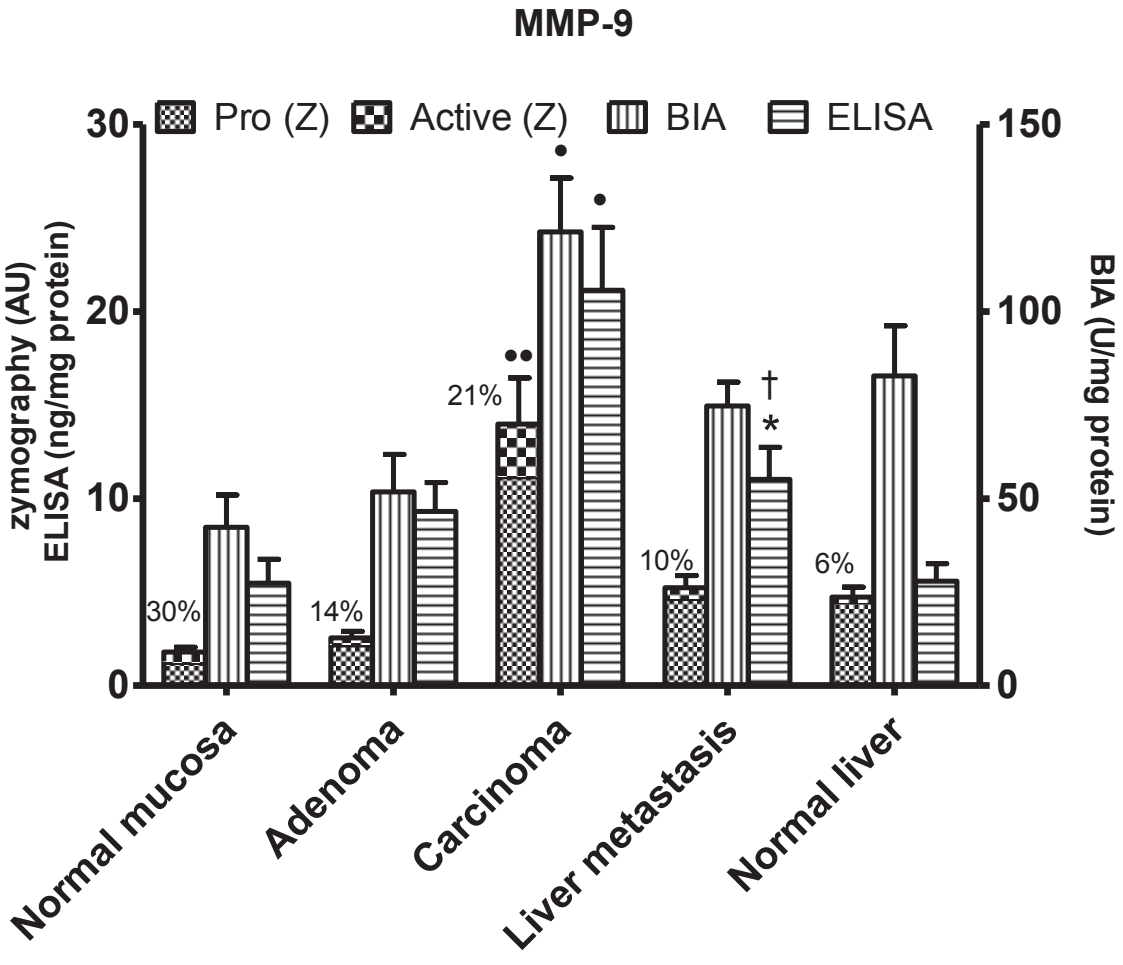

Figure 3. MMP-9 in the colorectal cancer sequence. Left axis: quantitative gelatin zymography (Z), results expressed in activity units (AU) and ELISA, results expressed in ng per mg protein. Right axis: BIA, results expressed in U/mg protein. * $P \leq 0.05$ *vs*. normal mucosa; • $P \leq 0.05$ *vs*. other tissues; •• $P \leq 0.01$ *vs*. other tissues; † $P \leq 0.05$ *vs*. normal liver. The percentages indicate the percentage active MMP-9 of the total amount of MMP-9 in the zymographic analyses.

BIA results were comparable to ELISA and quantitative zymography data (Figure 3). The highest MMP-9 content was found in the primary colorectal carcinomas. Liver metastases showed a lower MMP-9 content, comparable to adenomas. The overall correlation between ELISA, quantitative zymography and BIA was good, ranging from 0.60 *(*$P < 0.001$) between ELISA and zymography to 0.69 ($P < 0.001$) between BIA and zymography.

MMP-2

The MMP-2 levels showed only an increase (up to 1.5-fold) in the carcinomas compared to normal mucosa (Figure 4). The MMP-2 level in liver metastases showed a comparable increase to the carcinomas when assessed by zymography, but not by the other techniques. The percentage of active enzyme in carcinomas (55 ± 6%) was significantly higher than in adenomas (29 ± 3%) and liver metastases (30 ± 3%). Normal liver tissue homogenates were found to contain lower levels of MMP-2 than any of the other tissues, although this difference was significant only in the ELISA results. The correlation between ELISA and gelatin zymography for MMP-2 was good (r = 0.57; $P < 0.001$), between BIA and ELISA the correlation was moderate (r = 0.32, $P < 0.005$), and relatively weak between the BIA and quantitative gelatin zymography (r = 0.24, $P < 0.05$).

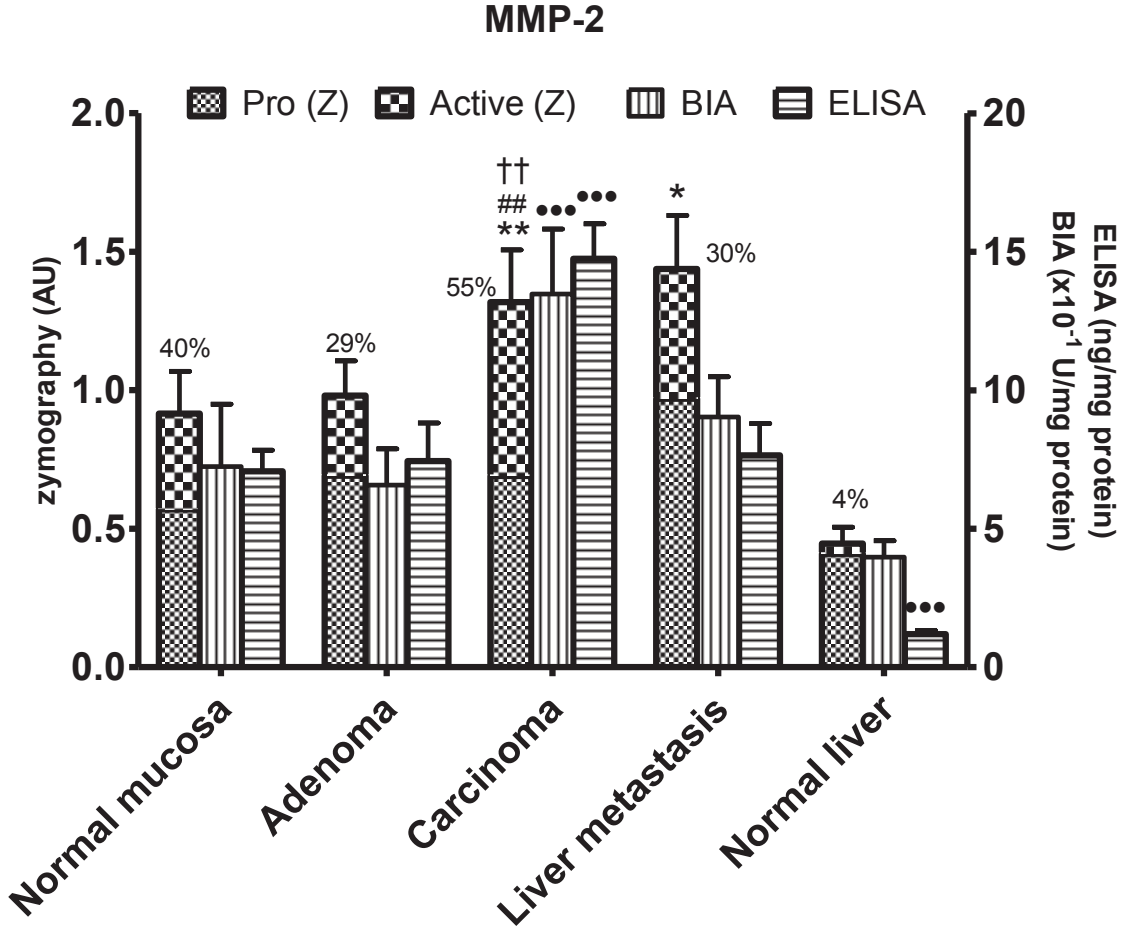

Figure 4. MMP-2 in the colorectal cancer sequence. Left axis: quantitative gelatin zymography (Z), results expressed in activity units (AU). Right axis: ELISA and BIA, results expressed in ng per mg protein (ELISA) and $x10^{-1}$ U/mg protein (BIA). * $P \leq 0.05$ *vs*. normal mucosa (total enzyme); ** $P \leq 0.01$ *vs*. normal mucosa (active enzyme); ## $P \leq 0.01$ *vs*. adenoma (active enzyme); †† $P \leq 0.01$ *vs*. normal liver (active enzyme); ••• $P \leq 0.001$ *vs*. other tissues. The percentages indicate the percentage active MMP-2 of the total amount of MMP-2 in the zymographic analyses.

DISCUSSION

Several *in vitro* and *in vivo* studies, reviewed by Mook *et al.*[28], have shown the relevance of MMP-2 and MMP-9 in colorectal cancer progression and metastasis. Both gelatinases are in principle secreted as inactive pro-enzymes. Activation of these pro-MMPs takes place through interaction with other MMPs and/or other proteases[28]. Quantitative zymography has been shown previously to be an extremely reliable and sensitive technique for the detection of these gelatinases and to distinguish between the proenzyme and active form[29, 30]. Using this technique, we found that pro-, active- and total MMP-2 and -9 levels are higher in carcinomas compared to normal colonic mucosa. These findings are in agreement with several other reports[12, 16, 31-35].

In the present study, we have also looked into the expression of MMPs in the early and later stages of colorectal carcinogenesis. We found that tissue levels of MMP-2, -8, and -9 and NGAL are increased in carcinomas and liver metastases of colorectal carcinomas, even after correction for inflammation, and that MMP-8, MMP-9 and NGAL are increased in colorectal adenomas as well.

Several studies already suggested a role for MMP-9 in early carcinogenesis, whereas MMP-2 seems to be of importance later in the adenoma-carcinoma sequence, once a carcinoma has developed. An up-regulation of MMP-9 in colorectal adenomas compared to the normal mucosa was found in two other zymography studies[14, 16]. Up-regulation of the latent or active form of MMP-2 has never been observed in precancerous lesions. A trend towards a step-wise increase of MMP-9 in the colorectal adenoma-carcinoma sequence was also demonstrated in immunohistochemical studies of adenoma and carcinoma tissue[15, 36] and these results were confirmed by performing real time RT-PCR on the same tissue specimens[15]. MMP-9 expression in adenomas appears to be correlated with severity of dysplasia, but not with histological subtype[14, 16, 37, 38]. In accordance with the abovementioned studies, we found a significant increase of total and active MMP-9 in colorectal carcinoma versus normal tissue measured either by ELISA, BIA or zymography. A trend towards increased expression of MMP-9 was observed in adenomas compared to normal mucosa, but this did not reach statistical significance. MMP-2 was only moderately up-regulated in colorectal cancer but not adenomatous tissue.

The formation of adenomas was further studied in APC-Min mice[39] after knocking out either the MMP-2 or the MMP-9 gene. Ablation of the MMPs did not influence adenoma size; however, adenoma number was reduced by 40% and proliferation was reduced by 50% in the MMP-$9^{-/-}$ mice compared to their MMP-$9^{+/+}$ littermates[40]. Knockout of MMP-2 did not reduce adenoma number or the number of proliferating cells. These findings support the hypothesis that there is a role for MMP-9, but not for MMP-2, early in the adenoma-carcinoma sequence.

MMP-8 (neutrophil collagenase, collagenase-2) is a 75 kDa neutrophil-derived matrix metalloproteinase that degrades type I, II en III collagen and a wide range of non-collagenous substrates like serine protease inhibitors and chemokines[41]. Several studies in breast cancer, lung cancer and melanoma have shown that increased expression of MMP-8 can play a protective role in the progression of metastasis of cancer, probably through regulation of the inflammatory process induced by carcinogens and the modulation of tumour cell adhesion and invasion[42, 43]. These findings illustrate that the influence of MMP-8 on cancer varies between cancer types and throughout the cancer process[41], which makes it unsuitable as a target for anticancer drugs. Little is known about its role in colorectal cancer. Our results show that MMP-8 is significantly increased in adenomas, carcinomas and liver metastases compared to normal mucosa. The consequence of MMP-8 overexpression in the colorectal cancer sequence, *i.e.*, protection or contribution to carcinogenesis, needs to be further explored.

NGAL (or neutrophil gelatinase-associated lipocalin, also called lipocalin-2 or human neutrophil lipocalin [HNL]) is a 25 kDa glycosylated protein that forms homo-dimers in neutrophil granules and heterodimers with MMP-9. It prevents inactivation of MMP-9 by binding to the gelatinase and thereby preventing its degradation, is a carrier of small lipophilic ligands, *e.g.* N-formyl peptides, and is involved in migration processes[10]. NGAL was significantly enhanced in adenomas and carcinomas compared to normal mucosa. In liver metastases, NGAL levels were lower than in the carcinomas.

Neutrophil granulocytes are a major source of NGAL, MMP-8 and MMP-9 secretion. After correction for inflammation, the levels of both MMPs and NGAL were not significantly different in normal colon tissue and adenomas, suggesting that the increased expression in adenomas can mainly be attributed to an increased neutrophil influx. In carcinomas and liver metastases, however, the increase in MMP-8, MMP-9 and NGAL levels persisted even after correction for inflammation. These findings demonstrate that an influx of neutrophils into the tumour microenvironment is only partially responsible for the increase in MMP-8 and MMP-9 in the tumour and indicate that there is a true increase in the production of these proteins within the malignant tissues. In a series of gastric cancer patients, MMP-9 and NGAL-staining was present in a substantial part of epithelial cells[44]. Also in colorectal cancer specimens, intensive epithelial NGAL staining was seen in the transitional mucosa between normal and malignant tissue, whereas occasionally MMP-9 positivity was observed in endothelial cells and incidentally in muscle cells, macrophages and fibroblasts[44]. Several studies suggest that NGAL protects MMP-9 from auto-degradation by the formation of a NGAL-MMP-9 complex and thereby increases MMP-9 activity[44-46]. With and without correction for inflammation, we found that NGAL showed -not surprisingly- the same pattern in tissue values as MMP-9.

Pyke *et al.*[47] demonstrated the presence of mRNA of MMP-2 in fibroblasts/fibroblast-like cells and of MMP-9 mRNA in macrophages in cancer cell surrounding stromal tissue, but

not in the cancer cells themselves. In an experimental mouse model, using the short hairpin RNA interference technology (shRNA), Gerg *et al.*[48] were able to demonstrate that MMP-2 and -9 are also expressed by colorectal cancer cells themselves. Furthermore, distinct roles were identified for the two gelatinases in the development of colorectal cancer metastases; MMP-9 is important in the process of extravasation and invasion, whereas MMP-2 influences the outgrowth of metastases but not their formation.

The liver is the main site of metastasis formation in colorectal cancer. The presence of MMP-2 and MMP-9 in colorectal liver metastasis has already been demonstrated by immunohistochemistry, in situ hybridization and zymography[49-54]. In almost all cases, expression of MMP-2 and MMP-9 in metastases was higher than in normal liver tissue. We found that the MMP-9 and NGAL content of liver metastases from colorectal origin are lower than in the primary tumour, whereas MMP-2 and MMP-8 were expressed at an equal level. The percentage of active MMP-2 and MMP-9 was found to be lower in liver metastases than in the primary colorectal carcinoma and equalled the activity percentage found in adenomas. We speculate that the higher percentage of active enzyme reflects an increased proteolytic activity in the primary colorectal cancer, contributing to their invasive capacity. Few studies have compared the MMP-2 and -9 expression in liver metastases and in the primary tumour. In partial concordance with our results, Gentner *et al.*[55] found lower mRNA expression of all measured MMPs (including MMP-2, -8 and -9) in liver metastases compared to the primary colorectal cancers derived from the same patients. These findings were not confirmed by Asano *et al*.[56] who also used RT-PCR to quantify the mRNA expression of several MMPs in liver metastases; and although MMP-1, -10 and -11 expression was found to be decreased in the liver metastases compared with primary colorectal cancers, no difference in the expression of MMP-2, MMP-8 and MMP-9 was found. A dot blot hybridization study also showed no difference between mRNA levels of MMP-2 and MMP-9 in colorectal cancer or liver metastases[57]. Gelatinase-activity was increased in colorectal cancer in comparison to normal colorectal tissue, but the activity was lower in hepatic metastases[58]. It is therefore possible that up-regulation of these MMP-proteins occurs at a posttranscriptional level.

In conclusion, our results show that the development of colorectal cancer, as illustrated by the normal mucosa – adenoma – carcinoma – liver metastasis sequence, is accompanied by a concurrent increase in the MMP levels which might contribute to the invasive process. Our findings suggest different roles for MMP-2, -8 and -9 throughout this process, which may have implications for the use of anti-MMP therapies in different stages of colorectal cancer.

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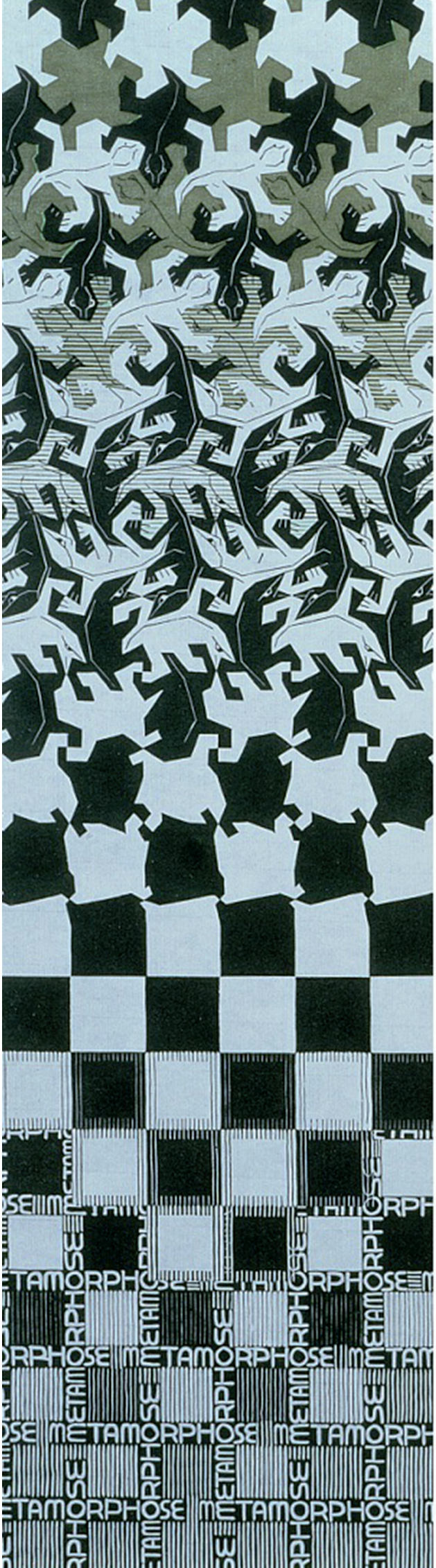

CHAPTER 3

Urinary and plasma MMP-2 and MMP-9 levels in relation to surgical treatment and outcome of colorectal cancer

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ABSTRACT

Background and aim of the study: There is considerable evidence for the involvement of matrix metalloproteinases (MMPs) in colorectal cancer progression and MMP-2 and MMP-9 have consistently been shown to be increased in colorectal cancer tissue. Less is known about the relation between circulating and urinary levels of these MMPs and the outcome of colorectal cancer. The aim of the present study was to investigate whether levels of the gelatinases MMP-2 and MMP-9 in tissue, plasma and/or urine were related to the clinical outcome in patients with colorectal cancer.

Methods: MMP-2 and MMP-9 levels in cancer tissue and normal mucosa, and in pre- and postoperatively obtained plasma and urine of 49 colorectal cancer patients were analysed with gelatin-zymography, BIA and ELISA.

Results: Significantly higher levels of MMP-2 and MMP-9 were detected in carcinoma tissue compared to tumour-free adjacent mucosa. Plasma and tumour levels of MMP-9 were found to be higher in patients with recurrent disease or irresectable disease at presentation, compared to patients without evidence of disease after resection. High MMP-2 levels in urine, when measured by zymography, were associated with higher risk of recurrent disease or distant metastases.

Conclusion: This is the first study to demonstrate that patients with colorectal cancer not only have measurable levels of MMP-2 and MMP-9 in their plasma, but also in their urine. Furthermore, high zymographic levels of MMP-2 in the urine, as well as high levels of MMP-9 in plasma, were found to be associated with poor surgical and clinical outcome of these colorectal cancer patients.

INTRODUCTION

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependant proteolytic enzymes that are involved in many physiological and pathological processes. One of their major functions is the remodelling of extracellular matrix (ECM), which takes places under normal circumstances in embryogenesis, bone remodelling and wound healing[1]. MMP-2 and MMP-9, also referred to as the gelatinases, are characterized by their ability to degrade denatured collagen and type-IV collagen, present in the basal membranes, which was one of the first recognized functions of these MMPs. Nowadays, we know that their functional repertoire extends far beyond the degradation of ECM components and involves regulation of cell proliferation and differentiation, apoptosis, angiogenesis and immune surveillance[2]. The first reports about the implication of MMP-2 and MMP-9 in cancer dissemination appeared in the late eighties[3-7]. Since then, their involvement in colorectal cancer progression, invasion and metastasis has been well established[8]. Gelatinases have also been shown to have an impact on the prognosis of colorectal cancer patients. Most of the publications on this subject concern MMP-levels in carcinoma tissue, where high MMP-2 levels are almost invariably identified as an unfavourable prognostic factor[9-14]. The results for MMP-9 are less uniform, possibly due to the anti-angiogenic effects of high levels of MMP-9, but the cell type overexpressing MMP-9 seems to be important for the outcome of cancer patients as well[11, 15-23].

The correlation between levels of MMP-2 and MMP-9 in the peripheral blood and the presence of cancer or the outcome of cancer patients has been investigated in various types of cancer, including colorectal carcinoma[24-30]. Results have not been unequivocal, which may possibly be explained by differences in detection techniques (zymography, ELISA or BIA), the type of material evaluated (serum or plasma) and the collection methods used[31, 32]. In the last couple of years, more information has also become available about urinary levels of MMPs in various kinds of tumours. It has even been suggested that the use of urine as a source of biomarkers has several advantages over blood, since it seems to contain less interfering proteins, is relatively clean and easier to collect than a blood sample[33]. Furthermore, MMPs were reported to be hardly present in the urine of healthy people, so the presence of MMPs in human urine might provide strong evidence for the presence of a pathological condition. Up to now, no data are available about urinary MMPs in colorectal cancer. In the present study, we investigated whether MMP-2 and MMP-9 levels in tumour and normal tissue, citrate-plasma and urine are related to the clinical outcome of patients with colorectal cancer. The samples were analysed by gelatin-zymography, enzyme-linked immunosorbent assay (ELISA) and a bioactivity assay (BIA).

MATERIALS AND METHODS

Patients and study design

Forty-nine patients, who were scheduled to undergo a surgical resection of colorectal cancer at the Leiden University Medical Center, were included in the study. Urine and citrate plasma samples were collected before resection (0 days-1.5 months), and if possible approximately 3 weeks (1 week- 2 months) and 4.5 months (2.5-12 months) after the resection. None of the patients had received radiotherapy or chemotherapy prior to surgery. The urine and citrate plasma samples were collected before 9:00 a.m. under fasting conditions. To correct for dilution effects, standardization by urinary creatinine concentration was obtained by dividing the MMP-concentration by the creatinine level of the corresponding urine sample. Data collection could be completed at all three time points in 16 patients. From 17 patients only preoperative urine and plasma samples could be collected, from 4 patients preoperative and 3 weeks after resection, and from 12 patients preoperative and 4.5 months after resection urine and plasma were obtained. From the resection specimens, representative samples of the carcinoma and macroscopically normal mucosa (5-10 cm from the tumour) were frozen and stored at -70 °C. From 5 patients, only preoperative biopsy material of tumour and normal tissue was available, from 3 patients no tissue samples were available and from 1 patient we only obtained biopsies from the carcinoma. Major clinical and pathological data were registered. All patients entered the study at the date the first urine and plasma samples were collected, prior to surgery. The follow-up period was at least 3.7 years and ended in the event of death or at the last follow-up date (follow-up range 0-92 months). The carcinomas were staged using the Dukes' classification modified by Astler and Coller[34]. Patients who had irresectable disease at presentation or who developed recurrent disease and/or distant metastases during follow-up ("tumour-positive" patients) were compared to patients with no evidence of disease after resection (NED) with respect to their tissue, plasma and urinary MMP-2 and MMP-9 levels. In some of the analyses patients with a Dukes D colorectal cancer undergoing a "palliative or no resection", *i.e.*, with inoperable disease were compared to patients who had undergone a resection with curative intent and were still alive at the end of the study period ("curative resection, alive"-group) and to patients who had a resection with curative intent and died during follow-up ("curative resection, dead"-group). Only one of the patients that were still alive and the end of follow-up had a recurrence during the follow-up period, none had distant metastases. Of the patients that died after surgery with curative intent, 8 patients had died because of a recurrence or distant metastases, 6 patients died from complications shortly after surgery or unrelated illness (*e.g.* breast cancer, lung cancer) and in 5 patients the cause of death was unknown.

The study was performed according to the instructions and guidelines of the LUMC Medical Ethics Committee and in accordance with the Helsinki declaration.

Tissue extraction and protein concentration

Extracts were prepared from 10-100 mg wet tissue samples as previously described[35-37]. The samples were homogenised in 1 ml 0.1 M Tris-HCl, 0.1% Tween-80 buffer (pH 7.5) per 60 mg wet tissue at 0°C. The homogenates were centrifuged twice at 8000 g for 2.5 minutes at 4°C. The supernatants were stored at -20°C until analysis. Protein concentration of the supernatant was determined by the method described by Lowry *et al*[38].

Gelatin-zymography

The presence of active and latent forms of the matrix metalloproteinases MMP-2 and MMP-9 was analysed by gelatin-zymography as described previously[39-41]. Briefly, gels containing 10% polyacrylamide and 0.2 % gelatine were loaded with 10 μl sample and 5 μl sample buffer (20% SDS, 8% saccharose, 0.5 M Tris-HCl (pH 6.8) and 0.05% bromophenol blue) and electrophorised during 2-2.5 hour, to separate the MMP isoforms on molecular weight and activate the proenzymes. The gels were subsequently incubated overnight at 37°C, stained and analysed for gelatine digestion by laser densitometry. An internal standard of 5 µg / 10µl and 10 µg / 10µl of one tumour homogenate with MMP-2 and MMP-9 in active and pro-form was present at each gel for correction of intergel variations. Results were expressed in arbitrary units (AU).

Enzyme-linked immunosorbent assay (ELISA)

The total amount of MMP-2 and MMP-9 was determined, after a 1:10 dilution of the urine and homogenates and a 1:33 dilution of the plasma, by sandwich ELISAs as previously described[40, 42]. Results were expressed in ng MMP per mg protein.

Bio-Immuno-Activity assay (BIA)

Latent and active MMP-2 and MMP-9 were measured according to the Bio-Immuno-Activity assay technique previously described[43]. Briefly the same antibodies to MMP-2 or MMP-9 that were used in the ELISA were used as catching antibody for the respective MMPs in the samples during overnight incubation at 4 °C. The MMP-2 or -9 was then activated by incubation with 0.5 mM p-aminophenyl-mercuric acetate at 37°C and the activity measured with modified pro-urokinase (Ukcol) and S-2444 (0.6 mM), as chromogenic substrate, in assay buffer at 37°C. Reactions were performed in 96-well flat-bottomed microtitre plates, and a Titertek Multiskan photometer was used to follow the absorbance change at 405 nm. Results were expressed as units MMP-2 or MMP-9 per mg protein. In urine, the BIA for MMP-2 was not successful as there were no measurable levels of MMP-2 in this assay.

Statistical analyses

Group means are given as mean ± standard error of the mean (SEM). Differences between 3 groups were first tested for significance with the Kruskal-Wallis test before the exact significance between the groups was tested with the Mann-Whitney U-test (unpaired test) or with the Wilcoxon Signed-Rank test (paired test) by SPSS 16.0 statistical package. Differences were considered significant when $P \leq 0.05$.

RESULTS

Patient characteristics

The characteristics of the 49 patients are shown in table 1. The mean age (± SEM) of the patients was 65.5 (± 1.8) years (range 31-85). During follow-up, 27 patients died and 20 patients had a metastasis or recurrence. All patients (n=8) who had a palliative resection or no surgery at all had Dukes D carcinoma at presentation and died during the follow-up period. Forty-seven patients had a single primary colorectal cancer; one patient had developed a cancer in the sigmoid colon after curative surgery for a rectal cancer six years earlier and one patient had two synchronous tumours in the ascending colon and rectum. In one patient with a Dukes B2 carcinoma, the pathology specimen contained pathologic lymph nodes suggestive of prostate cancer. This diagnosis was confirmed postoperatively but was not known before surgery.

Table 1. Patient characteristics

	Category	n	%
Gender	Female	18	36.7
	Male	31	63.3
Age	<65 yrs	20	40.8
	≥65 yrs	29	59.2
Modified Dukes' stage	A	4	8.2
	B1	4	8.2
	B2	19	38.8
	B3	1	2.0
	C1	2	4.1
	C2	11	22.4
	D	8	16.3
End of follow-up; tumour yes/no	NED	29	59.2
	Recurrence/metastasis	20	40.8
Resection	Palliative / no resection	8	16.3
	Curative intention and alive	22	44.9
	Curative intention and dead	19	38.8

NED; no evidence of disease

MMP levels in carcinomas

MMP-2 and MMP-9 were significantly higher in carcinoma tissue compared to tumour-free adjacent colon mucosa (Figure 1). Particularly the pro-MMP-9 and active MMP-2, measured by zymography, were found to be dramatically higher (8-fold and 14-fold, respectively) in the carcinomas. These results were confirmed by the ELISA and BIA for both MMPs (MMP-2 ELISA: tumour 17.7 ± 2.1 *vs.* normal mucosa 5.4 ± 0.4 ng/mg protein; MMP-2 BIA [latent enzyme]: tumour 93.0 ± 11.6 *vs.* normal mucosa 77.9 ± 15.7 U/mg protein. MMP-9 ELISA: tumour 31.7 ± 5.4 *vs.* normal mucosa 7.1 ± 0.7 ng/mg protein; MMP-9 BIA [latent enzyme]: tumour 267 ± 75.2 *vs.* normal mucosa 92.9 ± 9.5 U/mg protein).

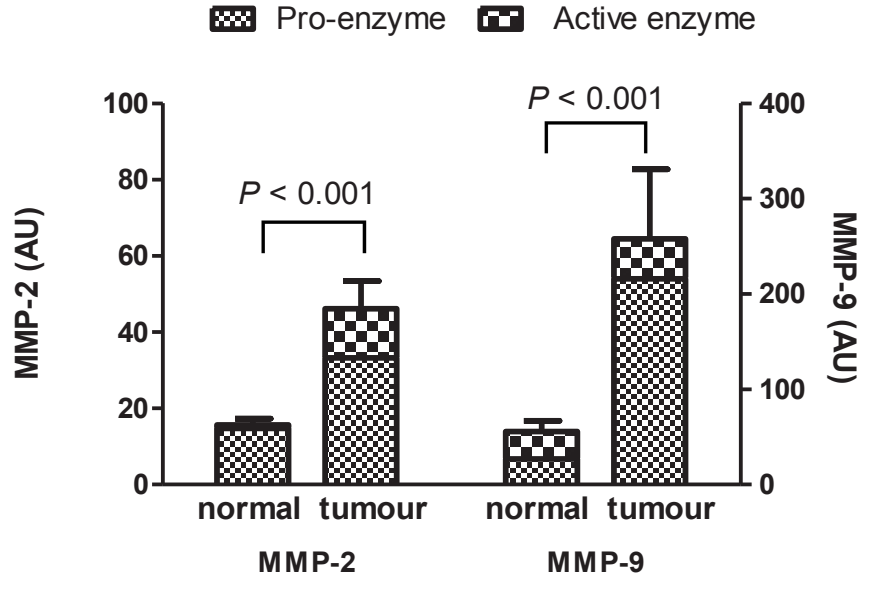

Figure 1. MMP-2 and MMP-9 levels in normal mucosa and carcinomas of 45 patients with colorectal cancer. Left axis: MMP-2 zymography, right axis: MMP-9 zymography; results are expressed in AU/10 µg protein. $P \leq 0.001$ pro-, active and total MMP-2 and pro-, total MMP-9 in tumour *vs.* normal mucosa; $P \leq 0.05$ active MMP-9 in tumour *vs.* normal mucosa.

The effect of tumour resection on MMP levels in plasma and urine

The plasma-levels of MMP-9, predominantly pro-enzyme as measured by gelatin-zymography, increased significantly 3 weeks after the resection and decreased to the preoperative level 4.5 months after resection (Figure 2). A similar pattern for MMP-9 in urine, that is, an increase 3 weeks after resection and a decrease 4.5 months after resection, was observed but these changes did not reach statistical significance (MMP-9 in urine preoperatively 310 ± 190, 3 weeks postoperatively 447 ± 432 and 4.5 months after resection 34.8 ± 29.1 AU/10µl urine, respectively). Plasma and urine MMP-2 levels did not fluctuate significantly during this perioperative period (Figure 2).

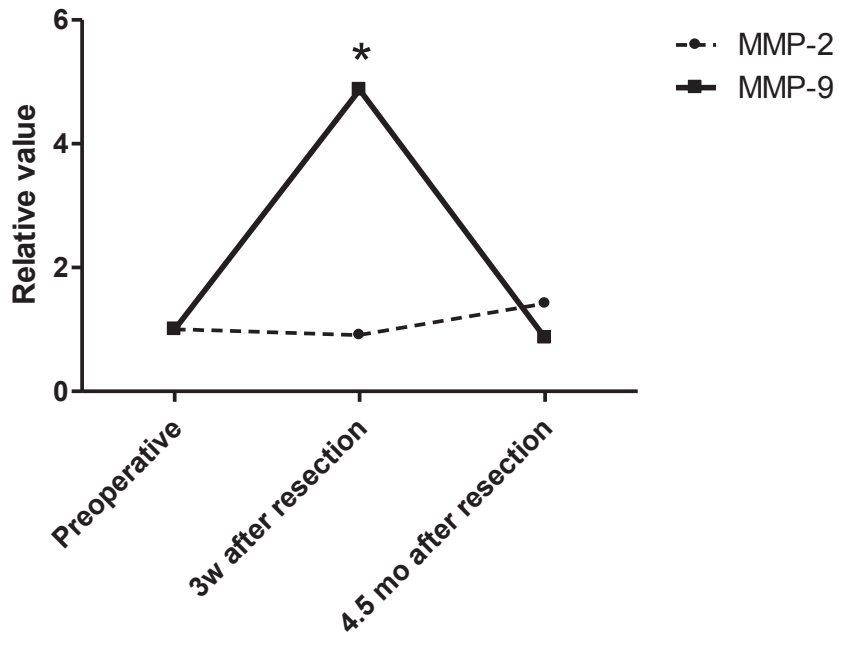

Figure 2. MMP-2 and MMP-9 levels in plasma collected preoperatively, 3 weeks or 4.5 months after resection expressed in relative values, where reference value =1 at t=preoperative. * $P < 0.05$ *vs.* preoperative and t=4.5 months after resection.

Relation between MMP levels and tumour recurrence and/or metastasis

Tumour MMP-9 levels were considerably higher in the patients with tumour recurrence/ metastasis than in the patients without evidence of disease after resection (NED), as shown in figure 3. Tumour MMP-2 levels only showed a trend towards higher MMP-2 levels in tumour positive patients compared to the patients with NED. Also in the plasma samples obtained before resection, pro/latent-MMP-9 was modestly (1.2-1.3 fold) higher in tumour positive patients compared to patients with NED ($P \leq 0.05$) (Figure 4). The plasma MMP-2 levels, however, were not significantly different in the patients with and without recurrent disease or distant metastasis (Figure 4).

In the analysis of the urine samples we excluded the patient with lymph node positive prostate cancer as these urinary MMP-values are probably for the greater part attributable to his prostate cancer. The urinary MMP-2 and MMP-9 levels of this patient were 38 and 63 times higher than the average MMP-levels of the patients with NED. In urine collected before colorectal cancer resection, zymography results show a significantly higher level of pro-, active and total MMP-2 in tumour positive patients as opposed to the NED patients ($P \leq 0.01$ for pro- en total MMP-2, $P \leq 0.05$ for active MMP-2; figure 5). These results could not be confirmed, however, by the MMP-2 ELISA (tumour positive 8.3 ± 2.4 *vs.* NED 17.2 ± 3.5 ng/ml urine; ns). The BIA of MMP-2 in urine yielded negative results in all patients.

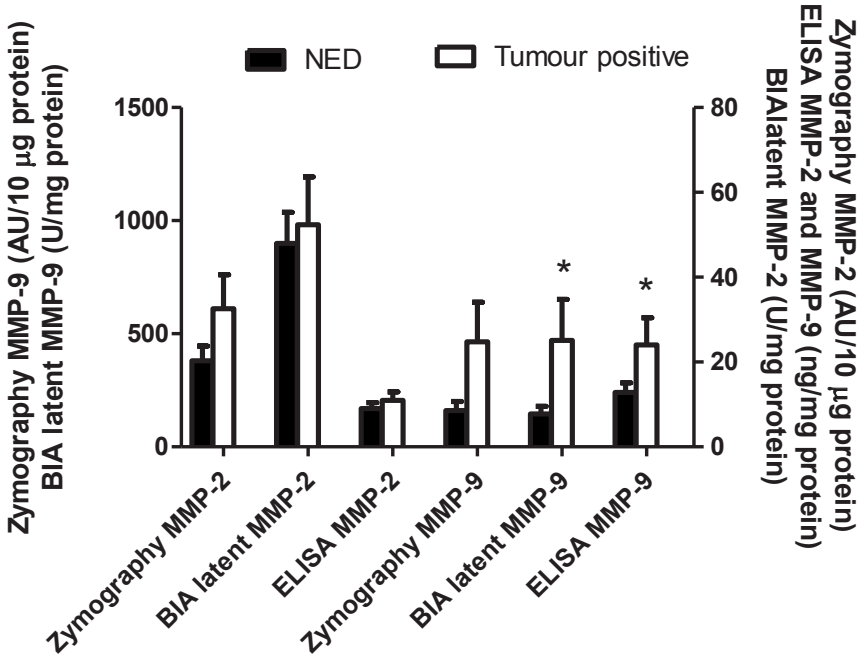

Figure 3. MMP-2 and MMP-9 levels in carcinomas of patients with no evidence of disease after resection (n=28) *vs.* tumour positive patients (n=18). Left axis: MMP-9 zymography and MMP-9 BIA, results are expressed in AU/10 µg protein (zymography) and U/mg protein (BIA). Right axis: MMP-2 zymography, MMP-2 and MMP-9 ELISA, MMP-2 BIA, results are expressed in AU/10 µg protein (zymography), in ng/mg protein (ELISA) and in U/mg protein (BIA). * $P < 0.05$ tumour positive *vs.* NED. NED; no evidence of disease.

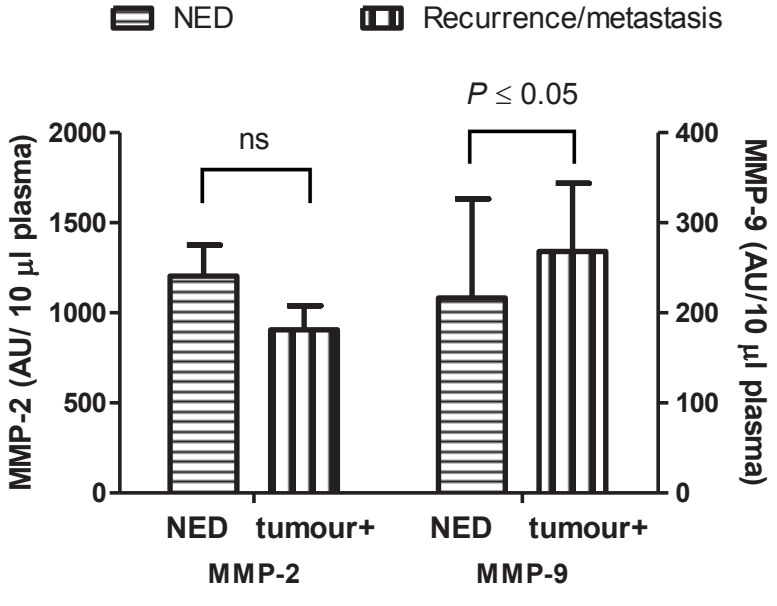

Figure 4. MMP-2 and MMP-9 levels in plasma collected preoperatively in patients with no evidence of disease after resection (NED) and patients with recurrent disease or distant metastases (tumour positive). Results of zymography, expressed in AU/10µl plasma. Left axis: MMP-2 zymography. Right axis: MMP-9 zymography. $P \leq 0.05$ NED *vs.* tumour positive for MMP-9

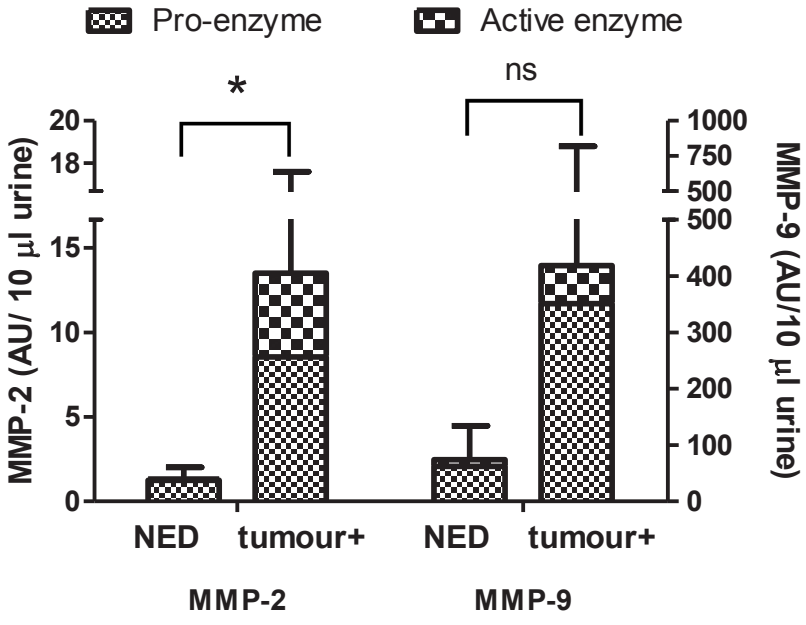

Figure 5. Pro, active and total MMP-2 and MMP-9 levels in urine collected preoperatively in patients with no evidence of disease after resection (NED) and patients with recurrent disease or distant metastases (tumour positive). Results of zymography, expressed in AU/10µl urine. Left axis: MMP-2 zymography. Right axis: MMP-9 zymography. * $P \leq 0.05$ NED *vs.* tumour positive for active MMP-2; $P \leq 0.01$ NED *vs.* tumour positive for pro- and total MMP-2

Zymography results for MMP-9 levels in the urine showed a similar trend towards higher urinary MMP-9 levels in tumour positive patients, that failed to reach significance due to large variations in the results obtained (Figure 5). The BIA and ELISA did not show any correlation between urinary MMP-9 levels and presence or absence of malignancy (ELISA: tumour positive 34.0 ± 27.8 *vs.* NED 33.4 ± 20.1 ng/ml urine and BIA: tumour positive 209 ± 162 *vs.* NED 249 ± 151 ng/ml urine). In addition, one of the patients with a Dukes D carcinoma had incurable disease due to liver metastases and local invasion into the urine bladder. The urinary MMP-levels were more than 10- to 100- fold higher than in the rest of the patients, most probably due to the presence of tumour tissue in the bladder. Excluding this patient from the analysis did not affect the trend of the results, however.

MMP levels in relation to surgery and outcome

Carcinoma tissue from the patients that were alive after a curative resection had the lowest MMP-9 levels compared to the patients with irresectable disease and the patients who had undergone a curative-intent resection but died during follow-up. Results of the zymography are shown in figure 6 and these were corroborated by the ELISA and BIA results (data not shown). For MMP-2, the same trend was observed but this did not reach statistical significance (Figure 6).

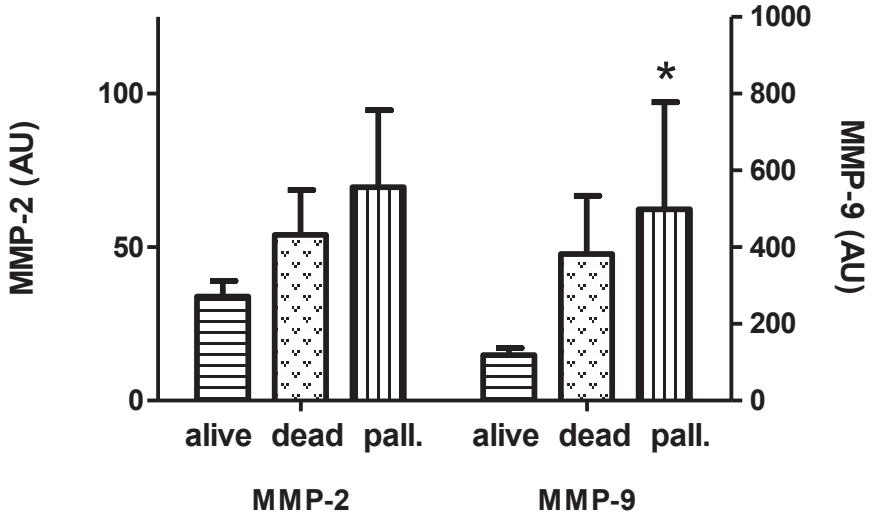

Figure 6. The MMP-2 and MMP-9 levels in cancer tissue, comparison between patients who had had a curative resection and were alive at the end of follow-up (n=22), patients who had had a curative resection and died (n=19) and patients who had no or a palliative resection (n=8), measured by zymography. The results are expressed as AU/ 10 µg protein. * $P \leq 0.05$ pro-, active and total MMP-9 *vs.* curative resection, alive.

In the plasma specimens obtained before resection, the MMP-9 levels were significantly higher in the Dukes D patients compared to patients who had undergone a resection with curative intent (Table 2). In the patients who had undergone surgery with curative intent and had died during follow-up, the plasma MMP-9 levels were found to be somewhat lower than those in patients who were still alive. These results were independent of the method of detection. Plasma MMP-2 levels were not related to clinical outcome at all.

The level and variation of both MMP-2 and MMP-9 in the urine of patients with irresectable disease was very large, even with exclusion of the patient with prostate cancer, but much higher than in the other patient groups. Urinary pro- and total MMP-2 levels, measured by zymography, of patients who underwent a curative resection but died during follow-up were found to be higher than in patients who underwent a resection with curative intent and were alive at the end of follow-up (Table 3). The ELISA results of the urine samples did not support these zymography findings and even showed higher MMP-2 values in the urine of patients who had undergone a curative resection and were still alive at the end of the study period (12.5 ± 1.9 ng/ml urine) compared to patients with irresectable disease (5.0 ± 3.3 ng/ml urine, $P < 0.05$). Patients who died after a resection with curative intent were found to have the highest urinary MMP-2 levels when measured by ELISA (18.2 ± 5.3 ng/ml urine). Urinary levels of MMP-9 appeared to be lower in the patients who underwent surgery with curative intent and were still alive as compared to those that had died during follow-up, but due to the large variation these differences did not reach statistical significance (Table 3).The same trend was observed in the ELISA and BIA results

Table 2. MMP-2 and MMP-9 in plasma

PLASMA	Curative resection, alive (n=22)	Curative resection, dead (n=19)	Palliative or no resection (n=8)
MMP-2 Total (Z)	1202 (± 206)	1044 (± 160)	846 (±209)
MMP-2 ELISA	254 (± 27.7)	341 (± 44.7)	311 (± 41.3)
MMP-2 BIA latent	960 (± 57.4)	863 (± 59.2)	880 (± 278)
MMP-9 Total (Z)	279 (± 146)[b]	111 (± 16.1)[a]	455 (± 158)[a,b]
MMP-9 ELISA	48.2 (± 16.3)[b]	25.8 (± 2.2)[a]	55.4 (± 5.7)[a,b]
MMP-9 BIA latent	373 (± 70.4)[c]	340 (± 35.4)[a]	533 (± 62.0)[a,c]

Mean (± SEM) levels of total MMP-2 and MMP-9 levels in plasma collected preoperatively in patients who underwent a resection with curative intent and were alive at the end of follow-up ("curative resection, alive", n=22), patients who underwent a resection with curative intent but died during follow-up ("curative resection, dead", n=19) and patients with incurable disease at presentation who did not undergo surgery with curative intent ("palliative or no resection", n=8). (Z) =Zymography results, data expressed in arbitrary units (AU) per 10 µl plasma. ELISA results are expressed in ng MMP per ml plasma. BIA results are expressed in U/ml plasma. [a] $P \leq 0.05$ palliative or no resection *vs*. curative resection, dead. [b] $P \leq 0.005$ palliative or no resection *vs*. curative resection, alive. [c] $P \leq 0.05$ palliative or no resection *vs*. curative resection, alive.

Table 3. MMP-2 and MMP-9 in urine

URINE	Curative resection, alive (n=21)	Curative resection, dead (n=19)	Palliative or no resection (n=8)
MMP-2 Pro	0.6 (± 0.3)[a]	3.6 (± 1.3)[a]	15.4 (± 13.1)
MMP-2 Active	0.1 (± 0.1)	2.7 (± 2.5)	6.0 (± 5.0)
MMP-2 Total	0.7 (± 0.3)[a]	6.3 (± 3.4)[a]	21.4 (± 18.1)
MMP-9 Pro	3.4 (± 1.6)	92.8 (± 79.3)	866 (± 841)
MMP-9 Active	2.2 (± 1.3)	19.4 (± 11.3)	162 (± 157)
MMP-9 Total	5.6 (± 2.9)	112 (± 88.3)	1027 (± 998)

Mean (± SEM) levels of MMP-2 and MMP-9 levels in urine collected preoperatively in patients who underwent a resection with curative intent and were alive at the end of follow-up ("curative resection, alive", n=21), patients who underwent a resection with curative intent but died during follow-up ("curative resection, dead", n=19) and patients with incurable disease at presentation who did not undergo surgery with curative intent ("palliative or no resection", n=8). The patient with prostate cancer is excluded from this analysis. Zymography results (after correction for creatinine levels) are expressed in arbitrary units (AU) per 10 µl urine. [a] $P \leq 0.05$ curative resection, dead *vs*. curative resection, alive.

for MMP-9 in the urine (ELISA: 4.6 ± 1.1, 27.1 ± 21.7, and 76.3 ± 69.4 ng/ml urine and BIA: 28.5 ± 9.8, 228 ± 183, and 458 ± 405 ng/ml urine in patients with “curative resection, alive”, “curative resection, dead” and “palliative or no resection”, respectively).

DISCUSSION

In the present study we found that patients with colorectal cancer not only have increased MMP-2 and MMP-9 levels in the carcinomas but also to have readily detectable levels of MMP-2 and MMP-9 in their urine and plasma. High carcinoma MMP levels were found to be indicative for a worse surgical and clinical outcome. Zymographic MMP-2 levels in the urine also correlated strongly with clinical and surgical outcome, whereas high plasma MMP-9 values showed a more moderate but significant association with clinical outcome. Whereas both gelatinases were increased in cancer tissue compared to normal mucosa, MMP-9 was found to be a stronger predictor of outcome than MMP-2 in this cohort of 49 patients. High levels of MMP-9 in cancer tissue were associated with the presence of malignancy at the end of the follow-up period and the highest MMP-9 levels were found in patients who had irresectable disease at presentation. MMP-2 levels in cancer tissue showed a trend towards higher levels in patients who had a tumour recurrence and/or distant metastases, but these differences were not significant.

Plasma levels of MMP-9 in these colorectal cancer patients were raised three weeks after surgery and decreased to preoperative levels in the following months, whereas MMP-2 plasma levels did not fluctuate in the perioperative period. These results confirm the findings of De Hingh *et al.* and probably reflect the response to major surgery and the process of wound healing[44]. The active form of MMP-9 could only be detected in plasma from 5 out of 49 patients, whereas the active form of MMP-2 was detected in none of the patients. These results are in accordance with others and suggest that the active forms of the gelatinases are only present in very limited amounts in the peripheral blood[45-47]. Plasma levels of pro-MMP-2 and pro-MMP-9 were detectable in almost all patients with colorectal cancer. When the patients were subdivided in patients with no evidence of disease after surgery and patients with irresectable disease at presentation or recurrent disease, plasma MMP-9 levels were modestly but significantly higher in the patients with a recurrence or distant metastases compared to the patients with NED. Plasma MMP-2 levels did not correlate with surgical outcome. Similar results were obtained when the patients were divided into three groups in relation to their clinical outcome, that is, resection and survival. Again, patients with the worst prognosis (who had incurable disease at presentation) had increased plasma MMP-9 levels compared to patients with the best clinical outcome (*i.e.* surgery with curative intent and still alive at the end of the follow-up period). Interestingly, several studies have shown that serum and/or plasma MMP-2 levels are lower in patients

with colorectal cancer compared to healthy controls[46, 48]. Furthermore, these studies report an inverse relationship between circulating MMP-2 levels and prognostic parameters. For example, lower MMP-2 levels in the peripheral blood were associated with more advanced tumour stage, lymph node involvement and higher CEA levels. We could not confirm these results in our study. MMP-9 levels in the peripheral blood have been reported to be increased in colorectal cancer[24-29, 49]. There have been varying reports on the correlation between circulating MMP-9 levels and the outcome of colorectal cancer patients. In some studies the correlation between MMP-9 levels and prognostic parameters was absent, while others reported a correlation between higher MMP-9 levels in the peripheral blood and Dukes stage, T-stadium and size of the tumour.

With the development of personalized medicine, the need for biomarkers which are predictive of the outcome of the disease increases. Furthermore, the treatment options for recurrent and even metastatic colorectal cancer have increased over the last decades[50] and because early detection of limited liver metastases creates the possibility of surgical metastasectomy and increases long-term disease-free survival, there is an increasing demand for biomarkers that can predict the presence of recurrent disease at an early stage. Waas and co-workers have followed-up patients who underwent curative resection for colorectal cancer for two years and monitored plasma MMP-2 and MMP-9 levels in order to predict a recurrence[51]. They did not find a correlation but noticed an increase in proMMP-9 that was non-significant due to large variations between the patients. The collection of blood, handling of the samples and the choice whether to use serum of plasma is pivotal for the interpretation of the results, especially for MMP-9. The level of circulating MMP-9 is significantly higher in serum than in plasma due to non-specific high background MMP-9 signal in serum and also the choice of anticoagulant in the tube is important, as citrate inhibits the release of gelatinases by blood cells and is therefore suggested to be more suitable than heparin or EDTA for measuring circulating MMPs[31, 52-57]. Although a time-dependant variation in the MMP-9 levels in heparin plasma has been reported[54], the dynamics of the MMP-levels in the different analytes show a similar pattern over time[58]. MMP-2 levels are not affected at all by the use of serum or plasma and type of anticoagulant.

Not surprisingly, most of the research concerning MMPs in urine has been performed in patients with cancer of the urinary tract, *e.g.* bladder cancer, prostate cancer and renal cell cancer[59-70]. However, elevated levels of MMPs are also found in the urine of patients with non-urogenital tumours like breast cancer and brain tumours[71-75] and even in preneoplastic lesions of the breast, urinary MMP-9 and ADAM12 were increased and suggested to potentially serve as tools to predict breast cancer risk[76]. The increase in gelatinase levels in the urine is not restricted to (pre-)malignancy and occurs also in benign diseases, like endometriosis and coronary artery disease[77, 78].

Our present study is the first to provide evidence for the presence of measurable amounts of MMP-2 and MMP-9 in the urine of colorectal cancer patients. Furthermore, we even observed a correlation with outcome, *i.e.*, the zymography results suggest that high MMP-2 levels in the urine predict an unfavourable outcome, illustrated by a higher chance of recurrent disease and/or distant metastases. These results were not confirmed by the ELISA measurements, which yield the lowest levels of urinary MMP-2 in patients with irresectable disease. The reason for the inconsistency between the ELISA and zymography results is as yet unclear but might be due to the difference in detection techniques, as the zymography detects active and pro-enzyme based on their enzymatic activity, while the ELISA detects the total amount of MMP, whether bound to an inhibitor complex or not. Urea, which is present in large quantities in urine, is known to interfere with the antigen-antibody binding from the immunoaffinity chromatography protocols and might therefore have influenced the ELISA results of the urine. In addition other cross-reactive urinary components may have interfered within the ELISA assay. Although we corrected for urinary creatinine levels, we did not perform dialysis or apply urease treatment to the urine, which might make the ELISA determinations more reliable. MMP-9 levels in urine do not correlate with the surgical or clinical outcome in our cohort of colorectal cancer patients. An association between urinary levels of MMP-2 and MMP-9 and outcome has been reported in bladder cancer, where both gelatinase levels in urine correlate with cancer stage and prognosis[62, 66-68, 79].

Analysis of the results of individual patients revealed two patients that had 10- to 100-fold higher MMP-2 and/or MMP-9 levels in urine than the rest of the patients. One of these patients had a synchronous (but previously undiagnosed) prostate cancer, a malignancy previously shown to be accompanied by increased gelatinase levels in urine[63, 80]. The other patient with extremely elevated urinary MMP-2 and MMP-9 values was found to have local invasion of his sigmoid cancer into the bladder. In bladder cancer, MMP-2 and MMP-9 in the urine are elevated in a substantial portion of the patients, probably due to a direct release of MMP-2 and -9 from the tumour in the bladder wall into the urine[66, 68, 79, 81-85]. The same mechanism seems to be responsible for the high levels of urinary MMPs in this CRC patient.

A major drawback to the use of circulating MMPs as a marker of recurrent disease is the aspecificity of MMPs for a particular illness. Plasma MMP levels are elevated not only in a number of malignant disorders, but also benign conditions and even in patients with irritable bowel syndrome plasma MMP-2 levels were reported to be elevated compared to patients with colorectal adenomas and healthy controls[24]. The same problem occurs with the use of gelatinase levels in urine as a marker of recurrent disease. Although MMP-2 and MMP-9 are hardly detectable in the urine of healthy controls, they can be found in patients with endometriosis, juvenile (hamartomatous) polyps, kidney disease and a number of other benign and malignant diseases[78, 81, 86, 87]. Still, in patients without comorbidity MMP-2 and

MMP-9 in urine and/or plasma might to some extent serve as a biomarker in the follow-up of (colorectal) cancer patients.

In conclusion, patients with colorectal cancer have detectable levels of MMP-2 and MMP-9 in their urine and plasma. Zymography results show that high MMP-2 levels in the urine correlate strongly with clinical and surgical outcome, whereas high plasma MMP-9 values show a more moderate but significant association with clinical outcome. High risk groups of colorectal cancer patients could thus be identified based on MMP measurements, which could have implications for potential (neo-)adjuvant therapy. Furthermore, these results suggest that circulating and urinary gelatinases might serve as biomarkers in the follow-up of colorectal cancer patients, although future investigations will be needed to confirm this hypothesis.

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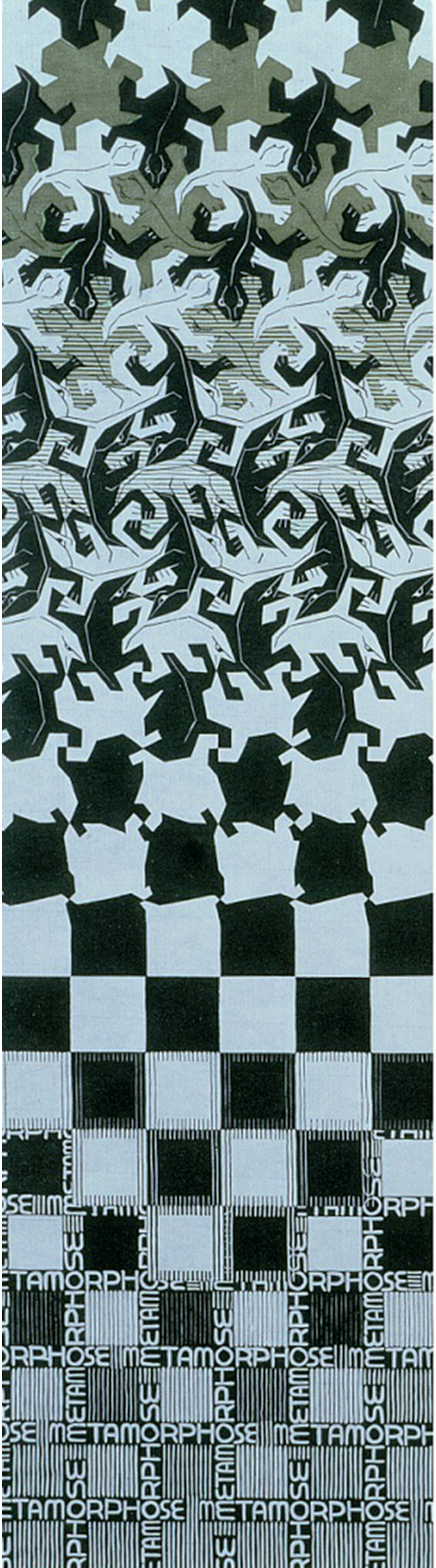

CHAPTER 4

Single-nucleotide polymorphisms of matrix metalloproteinases and their inhibitors in gastrointestinal cancer

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ABSTRACT

Matrix metalloproteinases (MMPs) are implicated in cancer development and progression and are associated with prognosis. Single-nucleotide polymorphisms (SNPs) of MMPs, most frequently located in the promoter region of the genes, have been shown to influence cancer susceptibility and/or progression. SNPs of MMP-1, -2, -3, -7, -8, -9, -12, -13 and -21 and of the tissue inhibitor of metalloproteinases (TIMPs) TIMP-1 and TIMP-2 have been studied in digestive tract tumors. The contribution of these polymorphisms to the cancer risk and prognosis of gastrointestinal tumors are reviewed in this paper.

INTRODUCTION

The matrix metalloproteinases (MMPs) belong to a metzincin superfamily of zinc-containing proteinases. Other members of this superfamily are the ADAM (a disintegrin and metalloproteinase) family and the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family, which have also been reported to be implicated in cancer progression[1,2]. MMP as well as ADAM/ADAMTS family members are inhibited by tissue inhibitors of metalloproteinases (TIMP)s. RECK (reversion-inducing, cysteinerich protein with Kazal motifs) is a membrane-anchored inhibitor of MMPs and ADAMs[3]. Even though ADAM, ADAMTS, and RECK are closely related to the MMPs and TIMPs, their single-nucleotide polymorphisms (SNPs) in gastrointestinal cancer have not been studied and are therefore not included in this review.

MMPs have proven to be of relevance for cancer development and prognosis in various organ systems. The 23 members of this family of endopeptidases all share a catalytic domain, a pro-peptide and a hemopexin-like C-terminal domain. According to their structure and major function or substrates, the MMPs are subdivided in the following subgroups: collagenases (MMP-1, -8 and -13), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and -26), gelatinases (MMP-2 and -9), membrane-type MTMMPs (MMP-14, -15, -16, -17, -24 and -25), and others[4-6]. The most firmly established function of MMPs is the degradation/remodeling of extracellular matrix. By cleavage of receptors and their ligands they also influence various growth and signaling pathways in normal and pathological conditions[6]. In cancer, MMPs are involved in angiogenesis by regulating the bio-availability of vascular endothelial growth factor (VEGF) (e.g. MMP-9) and the cleavage of matrix-bound VEGF (MMPs -3, -7, -9 and -16)[7]. On the other hand, cleavage of plasminogen by MMP-2, -9 and -12 leads to the production of angiostatin, an inhibitor of angiogenesis[8,9]. Furthermore, MMPs have been suggested to interfere in the balance between growth signals and growth-inhibiting signals e.g. by modulating the transforming growth factor-β (TGF-β) pathway and activation of the epidermal growth factor (EGF) receptor, to regulate the induction of apoptosis by cleavage of Fas ligand (by MMP-7), to play a role in the creation of a metastatic niche (MMP-3, -9 and -10), and to control inflammation (MMP-2, -3, -7, -8, -9, -12) and invasive processes (MMP-1, -2, -3, -7, -13 and -14), see Figure 1[6,10]. It has become increasingly clear that MMPs are not always detrimental since they also show anti-tumor effects, as illustrated by the inhibiting effects on angiogenesis described before[11].

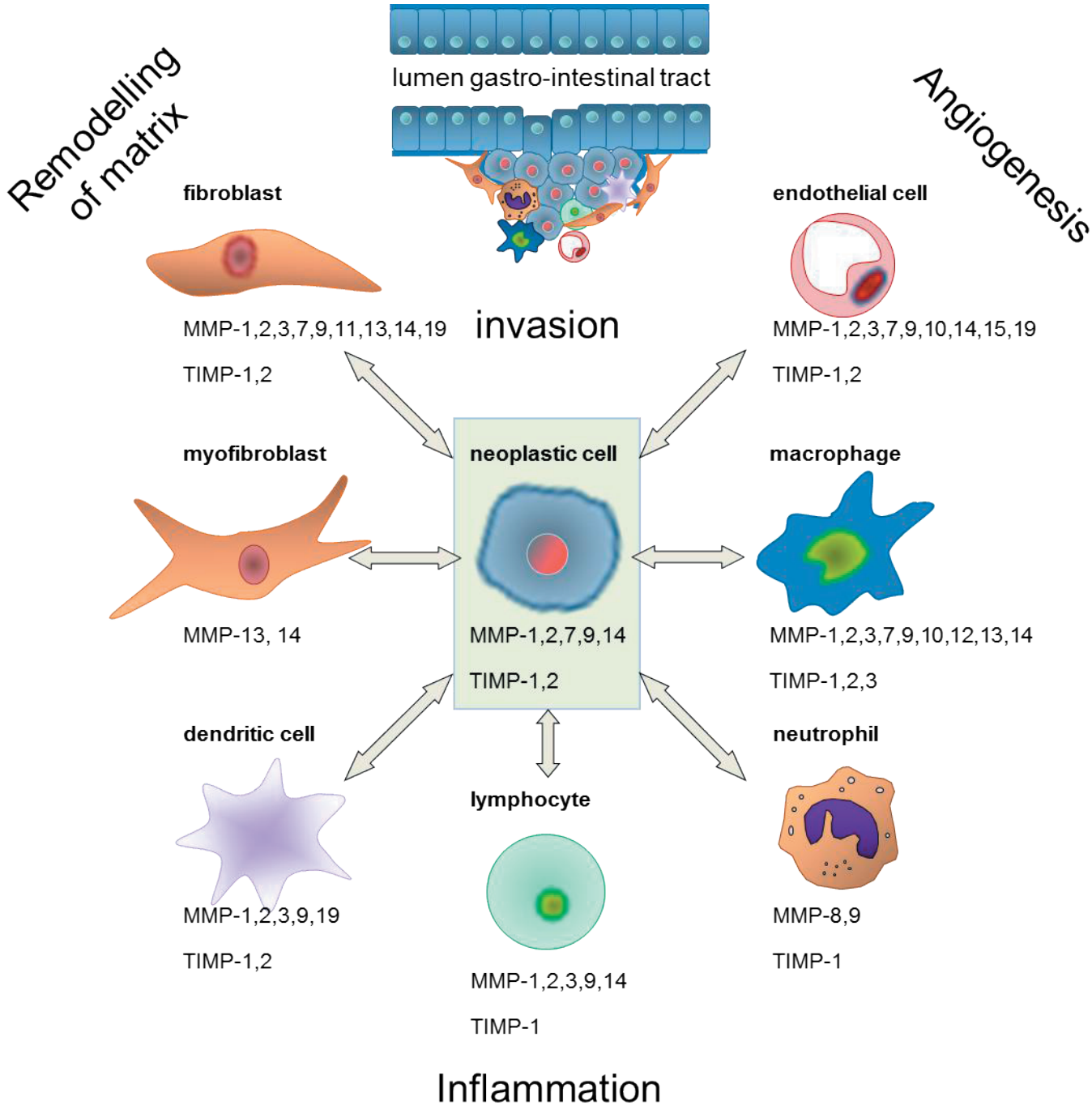

Figure 1. Schematic overview of the various types of matrix metalloproteinase-producing cells that are involved in the different processes during the various stages of cancer. MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.

MMPs are secreted as inactive pro-enzymes that need activation to exert their proteolytic properties. Their actions can be counteracted by specific inhibitors, i.e. the TIMPs.

Single-Nucleotide Polymorphism is the most common type of genetic variation. The estimated number of SNPs in the human genome is 10 million, but only a small part of these polymorphisms are functionally relevant. Most of the functional SNPs are located in the promoter region of the gene and are therefore expected to influence gene expression (Table 1)[12-30]. In this paper, we review the association of SNPs in the MMP and TIMP genes with the risk, the phenotype, and prognosis of gastrointestinal tumors.

Table 1. Effects of matrix metalloproteinases single-nucleotide polymorphisms on promoter activity

MMP	SNP	Effect of mutation	Influence on promoter activity *in vitro*	Ref.
MMP-1	-1607 1G/2G	Extra Guanine (2G) creates a binding site for transcription factor Ets-1	Increased in 2G allele (4-fold)	Rutter *et al*[18]
MMP-2	-1306 C/T	C to T substitution disrupts Sp-1 binding site	Decreased in T-allele	Price *et al*[22]
MMP-2	-735 C/T	C to T substitution influences Sp-1 binding site	Decreased in T-allele	Yu *et al*[15]
MMP-2	-790 T/G	Three transcription factors[1] bind to T (but not G) allele sequence	Decreased in G allele[2]	Vasku *et al*[28]
MMP-2	-955 A/C	Unknown	Effect unclear	Price *et al*[22]
MMP-2	-1575 G/A	G to T substitution decreases estrogen receptor α binding	Decreased in A allele	Harendza *et al*[27]
MMP-3	-1171 5A/6A[3]	Transcription suppressor binds with higher affinity to 6A allele	Decreased in 6A allele (2-fold)	Ye *et al*[19]
MMP-3	Lys45Glu	Lys to Glu substitution in exon 2 of gene	Effect unclear	Ouyang *et al*[16]
MMP-7	-181 A/G	Nuclear proteins bind with higher affinity to G allele	Increased in G allele[4] (2- to 3- fold)	Jormsjö *et al*[17]
MMP-7	-153 C/T	T allele binds additional nuclear proteins compared with C allele affinity for proteins that bind to both alleles higher in C allele	Increased in T allele[4] (2- to 3- fold)	Jormsjö *et al*[17]
MMP-8	-799 C/T	Influences binding of transcription factor?	Increased in T allele[5]	Wang *et al*[23]
MMP-8	17 C/G	Influences binding of transcription factor?	Increased in G allele[5]	Wang *et al*[23]
MMP-9	-90 CA(n)	Number of repeats influences strength of nuclear binding	Increased in n = 21 *vs* n = 14, n = 18	Shimajiri *et al*[12]
MMP-9	-1562 C/T	C to T substitution disrupts nuclear protein binding site	Increased in T allele	Zhang *et al*[20]
MMP-9	R279Q	Arg to Gln substitution in fibronectin type II domains	Effect unclear[6]	Wu *et al*[13]
MMP-9	P574R	Pro to Arg substitution in hemopexin domain	Effect unclear[6]	Wu *et al*[13]
MMP-9	R668Q	Arg to Gln substitution in hemopexin domain	Effect unclear[6]	Wu *et al*[13]
MMP-12	-82 A/G	A to G substitution results in decreased affinity for transcription factor AP-1	Decreased in G allele	Jormsjö *et al*[21]
MMP-12	1082 A/G	Asn to Ser substitution at coding region of hemopexin domain	Effect unclear	Joos *et al*[25]
MMP-13	-77 A/G	A to G substitution results in decreased affinity for transcription factor AP-1	Decreased in G allele (2-fold)	Yoon *et al*[24]
MMP-21	C572T	Ala to Val substitution in enzymes catalytic domain	Effect unclear	Shagisultanova *et al*[29]
TIMP-1	372 C/T	Unknown, located in exon 5, no effect on transcription or amino-acid sequence	Effect unclear	Hinterseher *et al*[26]
TIMP-2	-418 G/C	G to C substitution results in disruption of Sp-1 binding site	Decreased in C allele[2]	Hirano *et al*[30]
TIMP-2	303C/T	Unknown, located in exon 3, no effect on transcription or amino-acid sequence	Effect unclear	Kubben *et al*[14]

[1]The three transcription factors are: GKLF (Gut-enriched Krueppel-like factor), S8 and Evi1 (ectopic viral integration site 1 encoded factor); [2]Not confirmed; [3]Formerly known as -1612 5A/6A; [4]Only in combination MMP-7 -181G/-153T; [5]Only in combination MMP-8 -799T/-381G/+17G, in cells resembling chorion cytotrophoblasts; [6]Probably influences substrate binding and inhibitor binding. MMP: Matrix metalloproteinase; SNP: Single-nucleotide polymorphisms; TIMP: Tissue inhibitor of metalloproteinase.

LITERATURE SEARCH

Data sources

Electronic literature searches using PubMed, Embase and Web of Science were used to identify published papers concerning SNPs of MMPs, TIMPs, ADAMs, ADAMTS and RECK in gastrointestinal cancer up to September 2010. Search terms used included the MeSH heading “digestive system neoplasm” as well as all different types of gastrointestinal tumors mentioned separately, in combination with the MeSH heading “matrix metalloproteinases”, as well as all individual MMPs mentioned separately, combined with the MeSH heading “polymorphism, genetic” or synonyms of the term SNP. Papers were included when written in English or in any other language, provided that an English abstract was available. Full papers, as well as letters and abstracts, were included in this review. Publications concerning *in vitro* or animal studies only were excluded. Results are arranged by tumor type.

Software

To generate the forest plot, IBM SPSS statistics 17.0 was used.

ESOPHAGEAL CANCER

The incidence of esophageal cancer shows great geographical variation. This tumor is more common in Southern Africa and Eastern Asia than in Europe and Northern America (source: GLOBOCAN; http://globocan.iarc.fr). In the Asian population almost all cases of esophageal cancer are squamous cell cancers, whereas in the Western world adenocarcinoma occurs more often and its incidence has risen over recent decades. Because the pathophysiology and risk factors of squamous cell cancer and adenocarcinoma are different, we will discuss these two tumor types separately. An overview of the studies included in this paragraph is shown in table 2[31].

ESOPHAGEAL ADENOCARCINOMA

Only two papers describe the relationship between polymorphisms of MMPs and esophageal adenocarcinoma (EA). One of these studies focused on the protective effect of *Helicobacter pylori* (*H. pylori*) infection in patients with different genotypes of MMP-1 (-1607 1G/2G), MMP-2 (-1306 C/T), MMP-3 (-1171 6A/5A) and MMP-12 (-82 A/G)[32]. In individuals with an MMP-2 -1306 CC (wildtype) genotype, *H. pylori* infection (at any time during life) strongly protects against EA (adjusted odds ratio (OR) 0.29, 95% confidence interval (CI) 0.1-0.7). In persons with a CT or TT genotype, the esophageal cancer risk was not influenced by *H. pylori* infection. To a lesser extent, the protective effect of *H. pylori* infection on the development of EA was also seen in carriers of the MMP-3 wildtype (6A/6A) and MMP -12 wild -type (-82 AA). However, no association between any of the studied

Table 2. Polymorphisms of matrix metalloproteinases in esophageal cancer

Gene	SNP	Ref.	Cancer type	Ethnicity	Case/control	Outcome parameter	Results	Parameter OR	OR	95% CI	*P* value
MMP-1	-1607 1G/2G	Bradbury[33]	EA	Caucasian	313/455	Cancer risk	Increased cancer risk in 1G/2G and 2G/2G	2G/2G *vs* 1G/1G	1.83	1.2-2.8	0.005
						Overall survival	No difference in overall survival				
		Fruh[32]	EA	Caucasian	101/101	Cancer risk	No difference in cancer risk				
		Jin[42]	ESCC	Chinese	234/350	Cancer risk	No difference in cancer risk				
						LN metastases	No difference in LN metastases				
MMP-2	-735 C/T	Li[39]	ESCC	Chinese	335/624	Cancer risk	No difference in cancer risk				
		Yu[15]	ESCC	Chinese	527/777	Cancer risk	Increased cancer risk in CC (trend)	CC *vs* CT+TT	1.30	1.04-1.63	0.056
		Sun[31]	ESCC	Chinese	335/624	Cancer risk	No difference in cancer risk				
		Chen[40]	ESCC	Mongolian	188/324	Cancer risk	Increased cancer risk in TT	TT *vs* CC	4.82	1.59-14.60	
MMP-2	-1306 C/T	Fruh[32]	EA	Caucasian	101/101	Cancer risk	No difference in cancer risk				
						Cancer risk and HP	HP infection protects against EA in CC	CC *vs* CT+TT	0.29	0.1-0.7	
		Chen[40]	ESCC	Mongolian	188/324	Cancer risk	No difference in cancer risk				
		Li[39]	ESCC	Chinese	335/624	Cancer risk	Increased cancer risk in CC	CC *vs* CT+TT	1.57	1.10-2.23	0.010
		Yu[15]	ESCC	Chinese	527/777	Cancer risk	Increased cancer risk in CC	CC *vs* CT+TT	1.52	1.17-1.96	0.001
		Sun[31]	ESCC	Chinese	335/624	Cancer risk	Increased cancer risk in CC	CC *vs* CT+TT	1.57	1.10-2.23	
MMP-3	-1171 6A/5A	Bradbury[33]	EA	Caucasian	313/455	Cancer risk	Increased cancer risk in 6A/5A and 5A/5A	5A/5A *vs* 6A/6A	1.61	1.0-2.5	0.030
						Overall survival	No difference in overall survival				
		Fruh[32]	EA	Caucasian	101/101	Cancer risk	No difference in cancer risk				
						Cancer risk and HP	HP infection protects against EA in 6A/6A	6A/6A *vs* 5A/5A + 5A/6A	0.04	0.002-0.9	0.040
		Zhang[44]	ESCC	Chinese	234/350	Cancer risk	No difference in cancer risk				
						Cancer in smoker	Increased cancer risk in 5A allele in smokers	5A/5A + 5A/6A *vs* 6A/6A	1.95	1.08-3.53	
						LN metastases	Increased risk of LN metastases in 5A allele	5A/5A *vs* 6A/6A	2.24	1.07-4.69	0.030
						Infiltration depth	No difference in infiltration depth				
MMP-3	Lys45Glu	Ouyang[16]	ESCC	Chinese	227/378	Cancer risk	No difference in cancer risk				
MMP-7	-181 A/G	Zhang[45]	ESCC	Chinese	258/350	Cancer risk	Increased cancer risk in AG and GG	AG+GG *vs* AA	1.83	1.12-2.99	
						LN metastases	No difference in LN metastases				
MMP-9	R279Q	Wu[13]	ESCC	Chinese	132/132	Cancer risk	No difference in cancer risk				
MMP-9	P574R	Wu[13]	ESCC	Chinese	132/132	Cancer risk	Increased cancer risk in RR	RR *vs* PP	4.08	1.58-10.52	0.000
MMP-9	R668Q	Wu[13]	ESCC	Chinese	132/132	Cancer risk	No difference in cancer risk				
MMP-9	-1562 C/T	Xia[43]	ESCC	Chinese		Cancer risk	No difference in cancer risk				
MMP-12	-82 A/G	Bradbury[33]	EA	Caucasian	313/455	Cancer risk	No difference in cancer risk				
						Overall survival	No difference in overall survival				
		Fruh[32]	EA	Caucasian	101/101	Cancer risk	No difference in cancer risk				
						Cancer risk and HP	HP infection protects against EA in AA	AA *vs* AG/GG	0.44	0.2-0.8	0.020
MMP-12	1082 A/G	Bradbury[33]	EA	Caucasian	313/455	Cancer risk	No difference in cancer risk				
						Overall survival	No difference in overall survival				
MMP-12	-82 A/G	Li[39]	ESCC	Chinese	335/624	Cancer risk	No difference in cancer risk				
						Cancer risk	No difference in cancer risk				
MMP-13	-77 A/G	Zhang[41]	ESCC	Chinese	316/609	Cancer risk	No difference in cancer risk				

SNP: Single nucleotide polymorphism; EA: Esophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; LN: Lymph node; HP: *Helicobacter pylori*; OR: Odds Ratio; 95% CI: 95% confidence interval.

MMP polymorphisms and overall risk of EA was found.The second paper, published by the same group, investigated the polymorphisms of MMP-1 (-1607 1G/2G), MMP-3 (6A/5A), and MMP-12 (-82 A/G) in relation to the risk and overall survival of EA[33]. In a cohort of 313 cancer patients and 455 controls, they found an increased cancer risk in 2G-allele carriers of the MMP-1 -1607 1G/2G polymorphism [2G/2G *vs* 1G/1G: adjusted OR 1.83 (95% CI: 1.2-2.8), *P* = 0.005]. 5A-allele carriers of the MMP-3 polymorphism also had an increased risk of developing EA in the same patient population (5A/5A *vs* 6A/6A, OR 1.61, 95% CI: 1.0-2.5, *P* = 0.03). The various genotypes of the MMP-12 -82 A/G polymorphism were not associated with an EA risk. There was no difference in survival of the patients in relation to any of the above mentioned polymorphisms.

ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Matrix metalloproteinase-2 is overexpressed in esophageal squamous cell cancer (ESCC)[34-37]. There are two known functionally important SNPs in the promoter region of the MMP-2 gene, MMP-2 -1306 C/T and MMP-2 -735 C/T. The C to T transition at the -1306 position disrupts a Sp-1 transcription factor binding site and thereby reduces promoter activity[22] (Table 1). The allele frequency of the minor (T) allele is significantly lower in the Asian population (13.6%) than in the European population (23.3%)[38]. Increased risk of developing ESCC in individuals with -1306 CC genotype (compared to CT+TT genotype) has been reported in two large cohorts of Chinese patients (Figure 2)[15,39]. In Mongolian patients, the association between the different genotypes and incidence of ESCC did not reach statistical significance[40]. A recent meta-analysis showed that the -1306 CC genotype, which is the genotype with the highest transcriptional activity[22], is associated with an increased overall cancer risk and this association was maintained in the subgroup analysis of ESCC patients[38]. These findings suggest an important role for the MMP-2 -1306 C/T polymorphism in cancer development, which led us to the idea of plotting the results for this MMP-2 polymorphism derived from all the publications included in this review. Figure 2 illustrates that in gastrointestinal cancers the association between MMP-2 -1306 C/T polymorphism and cancer risk is not unidirectional.

The reports on the association of the MMP-2 -735 C/T polymorphism are more dispersed. T allele carriers of this polymorphism show a lower transcriptional activity[15], which could explain the trend towards increased cancer risk in CC carriers compared to CT+TT carriers, which was reported in a Chinese population (OR 1.30, 95% CI: 1.04-1.63, *P* =0.056)[15]. However, these results were not confirmed in another large Chinese cohort[39] and a Mongolian study even found the opposite: higher ESCC cancer risk in TT carriers compared to CC, OR = 4.82, 95% CI: 1.59-14.60[40].

No association between the different promoter polymorphisms of MMP-1 (-1607 C/T), MMP- 9 (-1562 C/T), MMP-12 (-82 A/G), MMP-13 (-77 A/G) or between a polymorphism in the catalytic domain of MMP-9 (R279Q) or R668Q and the occurrence of ESCC has been

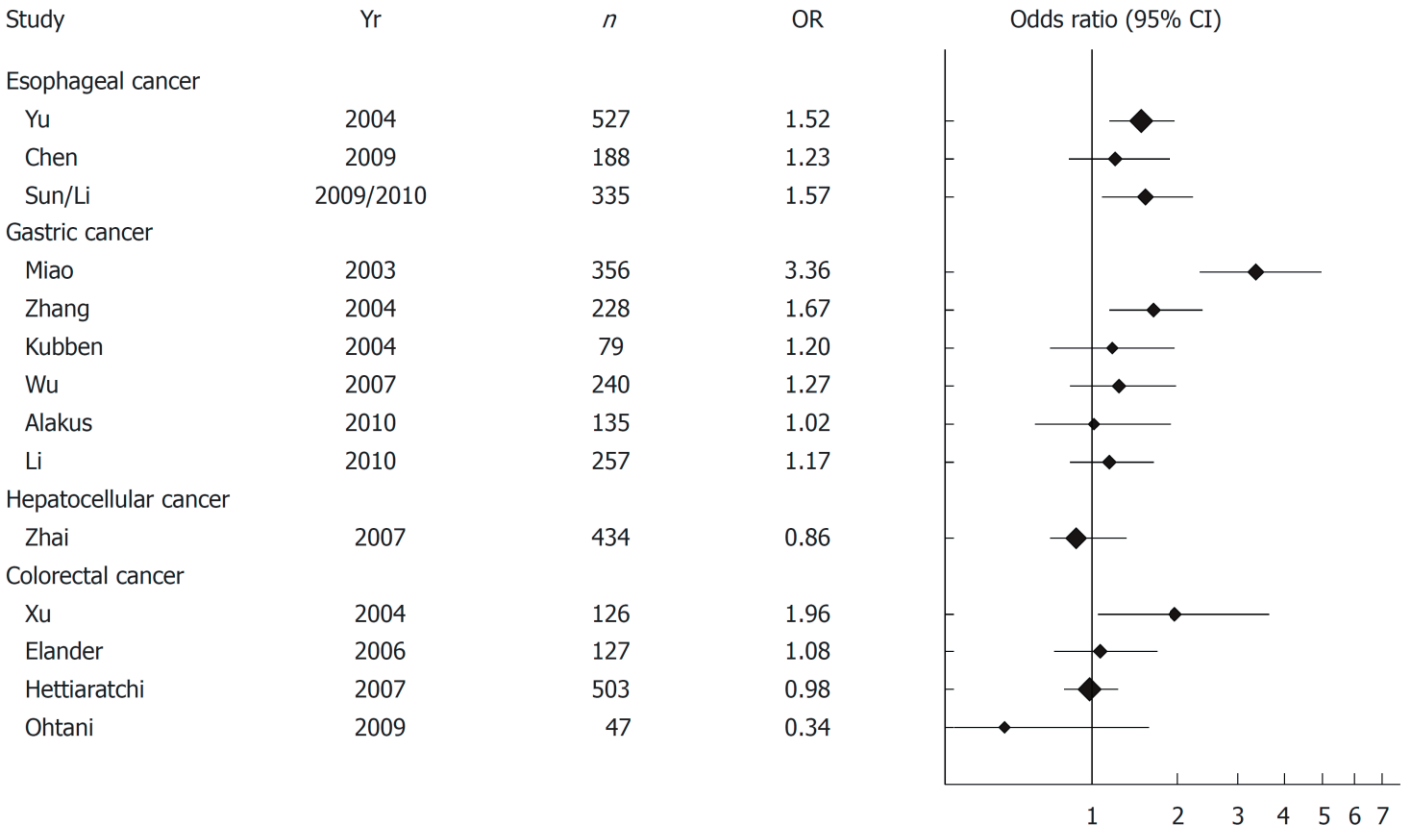

Figure 2. Forest plot of gastrointestinal cancer risk associated with the MMP-2 -1306 C/T polymorphism. Results are expressed as Odds ratios ± 95% confidence interval (CI) for CC *vs* CT+TT. The size of the diamonds indicates the size of the study cohort.

found[13,39,41-43]. A polymorphism of MMP-3, located in the promoter region at position -1171 (5A/6A) was not associated with the overall risk of developing ESCC. However, the cancer risk was lower in smokers with a 6A/6A genotype (cancer risk 5A/6A *vs* 6A/6A: OR = 2.12, 95% CI: 1.16-3.90) and the risk of lymph node metastases was lower in 6A allele carriers (risk of lymph node metastases 5A/6A *vs* 6A/6A: OR = 2.24, 95% CI: 1.07-4.69)[44]. An increase in ESCC was also observed in AG and GG carriers of the MMP-7 -181 A/G polymorphism (AG+GG *vs* AA: OR = 1.83, 95% CI: 1.12-2.99)[45]. MMP-7 is one of the smallest MMPs and has the capability of degrading a variety of extracellular matrix components, including elastin, type IV collagen, fibronectin, vitronectin, aggrecan and proteoglycans[46]. In various cancer types, including esophageal cancer, MMP-7 is over-expressed and associated with worse prognosis[47-49]. The G-allele of -181A/G is associated with higher basal transcriptional activity *in vitro*[17], which could explain the contribution of MMP-7 over-expression to prognosis.

In summary, the association between genotype and esophageal cancer susceptibility is most prominent for the MMP-2 -1306 C/T polymorphism with an increased risk of squamous cell cancer and an *H. pylori* protection against adenocarcinoma in the CC genotype carriers.

In an Asian population, ESCC risk is increased in G-allele carriers of the MMP-7 -181 A/G polymorphism. No clear association was found between any of the investigated SNPs and disease progression or prognosis.

GASTRIC CANCER

Gastric cancer is the second largest cause of global cancer related mortality. The World Health Organization reported 803.000 deaths worldwide in 2004. There is a male preponderance and known risk factors are *H. pylori* infection and tobacco smoking[50]. Most of the data concerning polymorphisms of MMPs in gastric cancer concern the Asian population, reflecting the much higher incidence of gastric tumors in the Eastern world compared to Western Europe and the United States. Table 3 gives an overview of the studies discussed in this paragraph.

The gelatinases MMP-2 and MMP-9 are upregulated in gastric cancer and increased MMP-2 and MMP-9 protein levels in tumor tissue of gastric cancer patients are associated with poor prognosis[51,52]. Two papers reported a significant increase in gastric cancer risk in -1306 CC carriers of the MMP-2 SNP[53,54], while four other studies did not find such a correlation (Figure 2)[14,39,55,56]. In a recent meta-analysis, the MMP-2 -1306 CC genotype was associated with a significant increase in gastric cancer susceptibility[38], but this meta-analysis did not include two studies that reported no difference in cancer risk between the different genotypes[14,56]. Survival was not influenced by the -1306 C/T polymorphism in any of these studies. However, Wu *et al* detected an increase in lymphatic and venous invasion in individuals with a CC genotype[55]. A second functional polymorphism of MMP-2 is a C to T transition at position -735 in the promoter region of the gene. This substitution influences a Sp1 transcription factor binding site, resulting in lower promoter activity in T allele carriers[15], similar to the C to T transition at position -1306. There is a trend towards increased cancer risk in MMP-2 -735 CC individuals, and this correlation is particularly significant in smokers.
The C to T substitution at position -1562 of the promoter region of MMP-9 results in the loss of binding to this region of a repressor nuclear protein, resulting in an increase in transcriptional activity in macrophages[20]. T-allele carriers of this polymorphism had deeper submucosal infiltration, more frequent lymphatic invasion and more advanced stage cancer compared to non-T allele carriers[57]. Nevertheless, none of the four publications describing the MMP-9 -1562 C/T polymorphism in gastric cancer found an association between the various genotypes and cancer risk[14,53,57,58]. In addition, Kubben *et al* did not find an association between the MMP-9 polymorphisms and tumor-related survival[14]. Two non-synonymous SNPs located in an exon of MMP-9, R279Q and P574R, were both associated with the risk of lymph node metastases in gastric cancer (higher risk in the RR and PP genotype, respectively), but did not show a relationship with gastric cancer risk[59].

MMP-7 over-expression has been demonstrated in various forms of cancer. In gastric cancer, MMP-7 expression has been linked to cancer progression and survival[60-62]. The

Table 3. Polymorphisms of matrix metalloproteinases in gastric cancer

Gene	SNP	Ref.	Cancer type	Ethnicity	Case/control	Outcome parameter	Results	Parameter OR	OR	95% CI	*P* value
MMP-1	-1607 1G/2G	Matsumura[65]	Gastric	Japanese	215/166	Cancer risk	No difference in cancer risk				
						Clinicopathol. par.	No correlation with any clinicopath. par.				
		Jin[42]	GCA	Chinese	183/350	Cancer risk	No difference in cancer risk				
						LN metastases	No difference in LN metastases				
MMP-2	-1306 C/T	Zhang[53]	Gastric	Chinese	228/774	Cancer risk	Increased cancer risk in CC	CC *vs* CT/TT	1.67	1.17-2.38	
		Wu[55]	Gastric	Taiwanese	240/283	Cancer risk	No difference in cancer risk				
						LN metastases	Increased risk of lymphatic invasion in CC	CC *vs* CT+TT	2.77	1.27-6.04	0.01
						Venous invasion	Increased risk of venous invasion in CC	CC *vs* CT+TT	2.93	1.27-6.78	0.012
						Survival	No difference in survival				
		Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	No difference in tumor-related survival				
		Alakus[56]	Gastric	Caucasian	135/58	Cancer risk	No difference in cancer risk				
						MMP-2 protein expression	No correlation with protein expression				
						Overall survival	No difference in overall survival				
		Li[39]	GCA	Chinese	257/624	Cancer risk	No difference in cancer risk				
		Miao[54]	GCA	Chinese	356/789	Cancer risk	Increased cancer risk in CC	CC *vs* CT+TT	3.36	2.34-4.97	
						Distant metastases	No difference in metastases				
MMP-2	-735 C/T	Li[39]	GCA	Chinese	257/624	Cancer risk	Trend towards increased cancer risk in CC	CC *vs* CT+TT	1.36	0.99-1.87	0.06
						Cancer risk in non-smoker	Increased cancer risk in CC in non-smoker	CC *vs* CT+TT	1.7	1.07-2.68	0.02
MMP-3	-1171 6A/5A	Zhang[44]	GCA	Chinese	183/350	Cancer risk	No difference in cancer risk				
						Cancer risk in smoker	No difference in cancer risk in smoker				
						LN metastases	No difference in LN metastases				
						Infiltration depth	No difference in infiltration depth				
MMP-8	-799 C/T	Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	No difference in tumor-related survival				
MMP-7	-181 A/G	Sugimoto[63]	Gastric	Japanese	160/434	Cancer risk	Increased cancer risk in G-allele carriers	AG+GG *vs* AA	2.32	1.24-4.35	0.009
						Cancer stage	Increased cancer stage in G allele carriers	AG+GG *vs* AA	3.66	1.54-8.73	0.003
		Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	More AA and less AG in cancer group	AG+GG *vs* AA	0.50	0.28-0.87	< 0.04
						Tumor-related survival	No difference in tumor-related survival				
		Li[64]	Gastric	Chinese	338/380	Cancer risk	Increased cancer risk in G allele carriers	AG+GG *vs* AA	1.95	1.24-3.05	0.004
						LN metastases	Increased risk of LN metastases in G allele	AG+GG *vs* AA			0.040
						Cancer stage	More advanced cancer stage in G allele	AG+GG *vs* AA			0.007
		Alakus[56]	Gastric	Caucasian	135/58	Cancer risk	No difference in cancer risk	AG+GG *vs* AA	1.06	0.69-1.64	0.79
						Overall survival	No difference in overall survival				
		Zhang[45]	GCA	Chinese	201/350	Cancer risk	Increased cancer risk in G allele carriers	AG+GG *vs* AA	1.96	1.17-3.29	
						LN metastases	No difference in LN metastases				
MMP-7	-153 C/T	Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	No difference in tumor-related survival				
MMP-8	17 C/G	Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	No difference in tumor-related survival				
MMP-9	R279Q	Tang[59]	Gastric	Chinese	74/100	Cancer risk	No difference in cancer risk				
						LN metastases	Increased risk of LN metastases in RR	RR *vs* QQ+RQ	5.74	1.59-13.43	
MMP-9	P574R	Tang[59]	Gastric	Chinese	74/100	Cancer risk	No difference in cancer risk				
						LN metastases	Increased risk of LN metastases in PP	PP *vs* RR+PR	4.17	1.39-11.78	
MMP-9	-1562 C/T	Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	No difference in tumor-related survival				
		Matsumura[57]	Gastric	Japanese	177/224	Cancer risk	No difference in cancer risk				
						Infiltration depth	Deeper submucosal infiltration in T allele	CT+TT *vs* CC	2.61	1.07-6.34	0.034
						Lymphatic invasion	Increased lymphatic invasion in T allele	CT+TT *vs* CC	2.27	1.09-4.74	0.028
						TNM classification	More advanced stage cancer in T allele	CT+TT *vs* CC	2.26	1.12-4.55	0.022
						Venous invasion	No difference in venous invasion	CT+TT *vs* CC	1.98	0.99-3.97	0.053
		Zhang[53]	Gastric	Chinese	228/774	Cancer risk	No difference in cancer risk				
		Wu[58]	Gastric	Taiwanese	263/354	Cancer risk	No difference in cancer risk				
MMP-12	-82 A/G	Li[39]	GCA	Chinese	335/624	Cancer risk	No difference in cancer risk				
						Cancer risk in smoker	No difference in cancer risk in smoker				
MMP-13	-77 A/G	Li[39]	GCA	Chinese	257/624	Cancer risk	No difference in cancer risk				
						Cancer risk in smoker	Decreased cancer risk in AG in smoker	AG *vs* AA/AG	0.47	0.28-0.8	0.01
		Zhang[41]	GCA	Chinese	243/609	Cancer risk	No difference in cancer risk				
						Cancer risk in smoker	Decreased cancer risk in AG in smoker				
TIMP-1	372 C/T	Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	No difference in tumor-related survival				
TIMP-2	-418 G/C	Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	No difference in tumor-related survival				
		Wu[55]	Gastric	Taiwanese	240/283	Cancer risk	No difference in cancer risk				
						LN metastases	Increased risk of LN metastases in GG	GG *vs* CG+GG	2.87	1.22-6.76	0.16
						Venous invasion	Increased venous invasion in GG	GG *vs* CG+GG	2.65	1.08-6.49	0.033
						Survival	No difference in survival				
		Yang[66]	Gastric	China	206/206	Cancer risk	More C-alleles in cancer patients	CC+CG *vs* GG	1.51	1.00-2.26	0.049
						Clinicopathol. par.	No correlation with any clinicopath. par.				
TIMP-2	303 C/T	Kubben[14]	Gastric	Caucasian	79/169	Cancer risk	No difference in cancer risk				
						Tumor-related survival	Better survival in CC patients	CC *vs* CT/TT	4.45	1.81-10.9	0.001
		Alakus[56]	Gastric	Caucasian	135/58	Cancer risk	No difference in cancer risk				
						LN metastases	More LN metastases in CC				0.01
						Distant metastases	Increased risk of distant metastases in CC				0.022
						Survival	No difference in survival				

SNP: Single nucleotide polymorphism; GCA: Gastric Cardia Adenocarcinoma; LN: Lymph node; OR: Odds ratio; 95% CI: 95% confidence interval; Clinicopath. par.: Clinicopathological parameters. [1]Except histological subtype.

genotype distribution of the -181 A/G polymorphism of MMP-7 is significantly different in various parts of the world; the frequency of the minor G-allele being 8.8% in the Asian population and 42.0% in the European population[38]. An increased risk of gastric cancer in G-allele carriers of the MMP-7 -181A/G polymorphism, who have a higher transcriptional activity, was reported in three studies[45,63,64]. These findings are in line with the observations in esophageal squamous cell cancers, as described above. Patients with the GG and AG genotype had a more advanced cancer stage. Interestingly, these findings are in contrast with two other papers, where either no correlation between the MMP-7 polymorphisms and gastric cancer risk was found[56] or there was even an inverse correlation, i.e. a higher percentage of MMP-7 -181 AA genotype in the gastric cancer group compared to the control group[14]. The discrepancy between these findings might be explained by a difference in ethnicity: in the first three papers all patients had an Asian background, whereas the latter two papers concern Caucasian patients.

The SNPs of MMP-1 (-1607 1G/2G), MMP-3 (-1171 5A/6A), MMP-7 (-153 C/T), MMP-8 (17 C/G) and MMP-8 (-799 C/T) are reported not to be associated with gastric cancer risk or prognosis[14,42,44,65]. Smokers with the AG genotype of the MMP-13 -77A/G polymorphism were reported to have a decreased risk of developing gastric cancer[39,41]. A trend towards increased cancer risk was observed in individuals with the AG genotype of the MMP-12 -82 A/G polymorphism[39,41].

One of the mechanisms that regulates MMP activity, in addition to promoter polymorphisms, is the interaction with TIMPs. The contribution of gene polymorphisms of TIMP-1 and TIMP-2 has only been studied sporadically in gastric cancer. The 372 C/T polymorphism of TIMP-1 did not correlate with cancer risk or cancer-related survival[14]. The G to C substitution at position -418 in the promoter region of the TIMP-2 gene has been suggested to disrupt a Sp-1 binding site, presumably leading to decreased TIMP-2 transcription[30]. Yang *et al* studied this TIMP-2 polymorphism in a group of 206 gastric cancer patients and 206 controls. The gastric cancer risk was elevated in C-allele carriers (CC + GC *vs* GG, adjusted OR = 1.51, 95% CI: 1.00-2.26, $P = 0.049$)[66]. Two other papers that described the contribution of this polymorphism on gastric cancer occurrence, did not find an association between the different genotypes and cancer risk[14,55]. In a cohort of 240 Taiwanese gastric cancer patients, Wu *et al*[55] found increased lymph node metastases, increased serosal invasion and increased venous invasion in patients with the TIMP-2 GG genotype. Despite these results, neither in the Taiwanese study, nor in a Dutch study, was an association with survival reported[14,55]. The function of the 303C/T polymorphism of TIMP-2, located in exon 3 of the gene, is unclear. Both Kubben *et al*[14] and Alakus *et al*[56] found no correlation between genotype and gastric cancer risk. The finding in the study of Alakus *et al* that patients with the TIMP-2 303CC genotype more often have lymphatic and distant metastases seems to contradict with the findings of Kubben *et al*, who showed a significantly better tumor-related survival in patients with the CC genotype. This discrepancy

could possibly be due to the low number of patients with a CT or TT genotype in both studies.

To conclude, an increased gastric cancer susceptibility seems to be present in Asian (but not in Caucasian) G-allele carriers of the MMP-7 -181 A/G polymorphism, an association also seen with ESCC. While some studies reported an association between genotype and clinicopathological parameters or prognosis, these results were not confirmed by others and are thus not consistent, except for the finding that MMP-7-181 AG or GG genotype patients in the Asian population seem to have a more advanced tumor stage than patients with the AA genotype[63,64].

SMALL INTESTINAL CANCER

Tumors of the duodenum, jejunum and ileum are rare and there are in fact no data on the effect of functional polymorphisms of matrix metalloproteinases in these tumors. Only one paper describes MMP-2, -7, -9, -11 and -13 protein levels in 25 patients with a carcinoid tumor localized in the ileum. Except for MMP-2, none of the MMP protein levels were associated with survival[67]. Surprisingly, low MMP-2 expression in the primary carcinoid tumor is correlated with an unfavorable outcome of the disease. This finding, which contrasts with observations made in many other gastrointestinal tumors, might indicate that these neuro-endocrine tumors have a different proteolytic phenotype compared to the other tumors which are adenocarcinomas or (in case of proximal or mid-esophageal cancers) squamous cell carcinomas.

PANCREATIC CANCER

Over-expression of MMP-1, MMP-2, MMP-7 and MMP-9 protein in pancreatic cancer is associated with more advanced tumor stage and poor prognosis[68-74], whereas high glandular TIMP-2 expression is associated with better survival in pancreatic ductal adenocarcinoma[75]. However, until now, there are no reports on the functional polymorphisms of MMPs and TIMPs in malignant tumors of the pancreas.

CHOLANGIOCARCINOMA

Bile duct tumors are rare in the general population. Patients with primary sclerosing cholangitis (PSC) have an increased risk of developing cholangiocarcinoma. Wiencke *et al*[76] investigated the association of MMP-1 and MMP-3 promoter polymorphisms in 165 PSC patients. Fifteen of these patients developed cholangiocarcinoma; all of these were 1G-allele carriers of the MMP-1 -1607 1G/2G polymorphism, compared to 72% of the whole PSC population. This finding is somewhat surprising since the 2G allele of this SNP is associated with a higher level of transcription and in most cancers, as for example esophageal adenocarcinomas, associated with increased cancer risk or worse prognosis.

The number of PSC patients in this study was too small to draw definite conclusions about the role of this promoter-SNP in PSC-associated cholangiocarcinoma.

HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the eighth in women worldwide[77]. The geographic distribution of this most common form of primary liver cancer follows the distribution of hepatitis B and C infection, as most of the patients have a background of liver cirrhosis or hepatitis B-infection. Several studies have looked into the effect of single nucleotide polymorphisms of MMPs on the incidence of HCC and its relation to survival of the patients (Table 4). None of the MMP gene polymorphisms that have been studied in HCC patients is correlated with cancer risk. In one paper, the number of 2G/2G homozygotes of the MMP-1 -1607 1G/2G polymorphism was slightly increased in HCC patients with a background of chronic hepatitis C virus (HCV)-related liver disease compared to patients with HCV-related chronic liver disease without HCC[78]. However, when the same patient group was compared with healthy controls, this relationship was no longer present[79]. In hepatitis B virus (HBV) patients with or without HCC, the genotype distribution of the MMP-1 -1607 polymorphism was similar[80]. No association was found between the MMP-2 -1306 C/T polymorphism and the risk of hepatocellular carcinoma[80], although patients with the CC genotype did experience an increase in HCC recurrence after liver transplantation compared to patients with the CT genotype (CT *vs* CC, OR = 0.42, 95% CI: 0.18-0.99, $P < 0.05$; there were no TT patients in the cohort). When compared with healthy controls, HCV-infected patients with HCC were more often 5A-allele carriers of the -1171 5A/6A polymorphism[79]. However, when compared to HCV infected patients without HCC, no difference in genotype distribution was found[78], which might suggest that the 5A-allele interferes with the development of the underlying disease instead of with the development of HCC. 5A allele carriers did have larger tumor diameters at the time of diagnosis and a poorer prognosis[78,79]. The SNPs MMP-2 -735 C/T, MMP-7 -181 A/G, MMP-8 -799C/T, MMP-9 -1562 C/T, MMP-12 -82 A/G, MMP-13 -77 A/G and MMP-21 C572T were found not to be associated with an increased risk of developing HCC[78-82]. One paper which describes the impact of the TIMP-2 -418 G/C polymorphism in a group of 92 HCC patients and 70 patients with chronic liver disease without signs of HCC found no association with HCC occurrence or prognosis[83].

In conclusion, polymorphisms of MMPs are not associated with HCC susceptibility. MMP-genotype may possibly influence the course of the disease in HCC patients, as HCV-infected HCC patients carrying the 5A allele of the MMP-3 -1171 5A/6A polymorphism appear to have worse survival rates[78,79]. In addition, it has been reported that HCC recurrence after liver transplantation is increased in MMP-2 -1306 CC genotype carriers when compared to CT patients[82].

Table 4. Polymorphisms of matrix metalloproteinases in hepatocellular carcinoma

Gene	SNP	Ref.	Ethnicity	Case/control	Parameter	Results	Parameter OR	OR	95% CI	*P* value
MMP-1	-1607 1G/2G	Zhai[80]	Chinese	434/480	Cancer risk	No difference in cancer risk				
		Okamoto[78]	Japanese	95/83	Cancer risk	Increased risk of HCC in 2G/2G				0.002
					Clinicopath. par.	No correlation with any clinicopath. par.				
					Survival	No difference in survival				
		Okamoto[79]	Japanese	92/170	Cancer risk	No difference in cancer risk				
					Survival/ clinicopath. par.	No difference in survival/ clinicopath. par.				
MMP-2	-735 C/T	Zhai[80]	Chinese	434/480	Cancer risk	No difference in cancer risk				
					Cancer risk	No difference in cancer risk				
MMP-2	-1306 C/T	Zhai[80]	Chinese	434/480	Cancer risk	No difference in cancer risk				
		Wu[82]	Chinese	93/0	HCC recurrence after LTx	More CC in recurrence group	CT *vs* CC	0.42	0.18-0.99	< 0.05
MMP-3	-1171 5A/6A	Zhai[80]	Chinese	434/480	Cancer risk	No difference in cancer risk				
		Okamoto[78]	Japanese	95/83	Cancer risk	No difference in cancer risk				
					HCC diameter at diagnosis	Larger diameter in 5A allele carriers				
					Survival	Decreased survival in 5A allele carriers				
		Okamoto[79]	Japanese	92/170	Cancer risk	Increased cancer risk in 5A allele carriers				
					Survival/ clinicopath. par.	Decreased survival in 5A allele carriers				0.035
MMP-7	-181 A/G	Qiu[81]	Chinese	434/480	Cancer risk	No difference in cancer risk				
MMP-8	-799 C/T	Qiu[81]	Chinese	434/480	Cancer risk	No difference in cancer risk				
MMP-9	-1562 C/T	Zhai[80]	Chinese	434/480	Cancer risk	No difference in cancer risk				
		Wu[82]	Chinese	93/0	HCC recurrence after LTx	No difference in HCC recurrence				
		Okamoto[78]	Japanese	95/83	Cancer risk	No difference in cancer risk				
					Differentiation grade	Differentiation worse in T allele carriers		0.03		
					Survival	No difference in survival				
		Okamoto[79]	Japanese	92/170	Cancer risk	No difference in cancer risk				
					Survival/ clinicopath. par.	No difference in survival/ clinicopath. par.				
MMP-12	-82 A/G	Zhai[80]	Chinese	434/480	Cancer risk	No difference in cancer risk				
MMP-13	-77 A/G	Zhai[80]	Chinese	434/480	Cancer risk	No difference in cancer risk				
MMP-21	C572T	Qiu[81]	Chinese	434/480	Cancer risk	No difference in cancer risk				
TIMP-2	-418 G/C	Okamoto[83]]	Japanese	92/70	Cancer risk HCC	No difference in cancer risk				
					Survival	No difference in survival				

SNP: Single nucleotide polymorphism; LN: Lymph node; Clinicopath. par.: Clinicopathological parameters; OR: Odds ratio; 95% CI: 95% confidence interval; HCC: Hepatocellular carcinoma; LTx: Liver transplantation.

COLORECTAL CANCER

Worldwide, colorectal cancer (CRC) is the fourth most common cancer in men and the third most common cancer in women[84]. Continents with a high incidence of colorectal cancer include Europe and North America. The lowest incidence is found in Asia, Africa and South America. In Eastern Europe and Japan CRC incidence has increased over recent years, probably due to a “Westernization” of lifestyle[84]. The effect of MMP polymorphisms on lung, breast and colorectal cancer has been reviewed previously by Decock *et al*[85]. The studies that are included in the present review are shown in table 5.

Table 5. Polymorphisms of matrix metalloproteinases in colorectal cancer

Gene	SNP	Ref.	Ethnicity	Case/control	Parameter	Results	Parameter OR	OR	95% CI	*P* value
MMP-1	-1607 1G/2G	Ghilardi[92]	Caucasian	60/164	Cancer risk	Increased cancer risk in 2G/2G	2G/2G *vs* 1G/1G + 1G/2G	2.21	1.17-4.16	0.014
					Distant metastases	Increased risk of metastases in 2G/2G	2G/2G *vs* 1G/1G + 1G/2G	4.73	1.46-15.26	0.008
		Zinzindohoue[99]	Caucasian	201/0	Survival	Overall survival worse in 2G/2G	2G/2G *vs* 1G/1G	5.4	2.0-14.7	0.001
		Hettiaratchi[96]	Australian	503/471	Cancer risk	No difference in cancer risk				
					Survival	Increased survival in 2G/2G	2G/2G *vs* 1G/2G + 1G/1G	0.43	0.19-0.96	0.040
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Woo[89]	Korean	185/304	Cancer risk	Increased cancer risk in 2G/2G and G-allele	2G/2G in patients *vs* controls	1.8	1.23-2.64	0.044
					LN metastases	More often >10 LN in 2G/2G				
		Fang[94]	Chinese	237/252	Cancer risk	No difference in cancer risk				
		Xu[97]	Chinese	126/126	Cancer risk	No difference in cancer risk				
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Przybylowska[98]	Caucasian	33/52	Cancer risk	No difference in cancer risk				
		Hinoda[91]	Japanese	101/127	Cancer risk	Increased cancer risk in 2G/2G	2G/2G *vs* 1G/1G + 1G/2G	2.08	1.22-3.53	0.007
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Biondi[93]	Caucasian	63/164	Cancer risk	More 2G allele in cancer patients				< 0.08
		de Lima[95]	Brasilian	130/130	Cancer risk	No difference in cancer risk				
					Distant metastases	Increased risk of metastases in 1G allele (trend)				
					LN metastases	No difference in LN metastases				
		Elander[90]	Caucasian	127/208	Cancer risk	Increased cancer risk in 2G allele carriers	2G allele *vs* 1G allele	1.41	1.02-1.96	0.037
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Kouhkan[88]	Iranian	150/100	Cancer risk	Increased cancer risk in 2G/2G and G-allele				
					Distant metastases	Earlier metastases in 2G/2G				
MMP-2	-1306 C/T	Xu[102]	Chinese	126/126	Cancer risk	Increased cancer risk in CC	CC *vs* CT+TT	1.96	1.06-3.64	< 0.05
					Infiltration depth	More serosa/ adventitia involvement in CC	CC *vs* CT+TT			0.042
		Hettiaratchi[96]	Australian	503/471	Cancer risk	No difference in cancer risk				
					Survival	No difference in survival				
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Langers[105]	Caucasian	215/0	Survival	10 year survival worse in TT	CC/CT *vs* TT	1.4	1.02-1.91	0.038

Table 5. Polymorphisms of matrix metalloproteinases in colorectal cancer (continued)

		Ohtani[103]	Japanese	47/67	Cancer risk	No difference in cancer risk				
		Elander[90]	Caucasian	127/208	Cancer risk	No difference in cancer risk				
					Clinicopath. par.	No correlation with any clinicopath. par.				
MMP-2	-790 T/G	Xu[104]	Chinese	126/126	Cancer risk	No difference in cancer risk				
					Infiltration depth	No difference in infiltration depth				
MMP-2	-955 A/C	Xu[104]	Chinese	126/126	Cancer risk	No difference in cancer risk				
					Infiltration depth	No difference in infiltration depth				
MMP-2	-1575 G/A	Xu[104]	Chinese	126/126	Cancer risk	Increased cancer risk in GG and G allele	GG *vs* GA+AA	1.96	1.06-3.64	0.04
					Infiltration depth	More serosa/ adventitia infiltration in GG	GG *vs* GA+AA			< 0.05
MMP-3	-1171 5A/6A	Hinoda[91]	Japanese	101/127	Cancer risk	Increased cancer risk in 6A/6A	6A/6A *vs* 5A/5A + 5A/6A	2.11	1.17-3.82	0.01
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Biondi[93]	Caucasian	63/164	Cancer risk	No difference in cancer risk				
		Ghilardi[92]	Caucasian	60/164	Cancer risk	No difference in cancer risk				
					Distant metastases	No difference in metastases				
		Woo[89]	Korean	185/304	Cancer risk	No difference in cancer risk				
					LN metastases	No difference in LN metastases				
		Ohtani[103]	Japanese	47/67	Cancer risk	No difference in cancer risk				
		Elander[90]	Caucasian	127/208	Cancer risk	No difference in cancer risk				
					Clinicopath var.	No correlation with any clinicopath. par.				
		Zinzindohoue[99]	Caucasian	201/0	Survival	No difference in overall survival				
		Hettiaratchi[96]	Australian	503/471	Cancer risk	No difference in cancer risk				
					Survival	No difference in survival				
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Xu[97]	Chinese	126/126	Cancer risk	No difference in cancer risk				
					Clinicopath. par.	No correlation with any clinicopath. par.				
MMP-7	-153 C/T	Ghilardi[111]	Caucasian	58/111	Cancer risk	Increased cancer risk in T allele carriers	T allele in patients *vs* controls	2.2	0.89-5.48	0.05
					Clinicopath. par.	No correlation with any clinicopath. par.				
		Langers[110]	Caucasian	174/0	Survival	Better survival in CC patients	CC *vs* CT+TT (LR)	14		0.001
MMP-7	-181 A/G	Woo[89]	Korean	185/304	Cancer risk	No difference in cancer risk				
					LN metastases	No difference in LN metastases				
		Fang[94]	Chinese	237/252	Cancer risk	No difference in cancer risk				

Table 5. Polymorphisms of matrix metalloproteinases in colorectal cancer (continued)

		Ghilardi[111]	Caucasian	58/111	Cancer risk	Increased cancer risk in GG	GG in patients *vs* controls	2.41	0.98-5.89	0.03
					Distant metastases	GG more often distant metastases	G in M+ *vs* M-	7.5	2.07-27.19	0.001
					LN metastases	GG more frequent LN metastases				
		Ohtani[103]	Japanese	47/67	Cancer risk	No difference in cancer risk				
		Langers[110]	Caucasian	174/0	Survival	No difference in survival				
		de Lima[95]	Brasilian	130/130	Cancer risk	No difference in cancer risk				
					Distant metastases	No difference in metastases				
					LN metastases	No difference in LN metastases				
MMP-9	R279Q	Woo[89]	Korean	185/304	Cancer risk	No difference in cancer risk				
					LN metastases	No difference in LN metastases				
		Xing[106]	Chinese	137/199	Cancer risk	No difference in cancer risk				
					LN metastases	No difference in LN metastases				
		Fang[94]	Chinese	237/252	Cancer incidence	Increased cancer risk in RR	RR *vs* QQ	2.21	1.25-3.93	0.006
MMP-9	-90(CA)n	Woo[89]	Korean	185/304	Cancer risk	No difference in cancer risk				
					LN metastases	No difference in LN metastases				
MMP-9	-1562 C/T	Xu[107]	Chinese	126/126	Cancer risk	No difference in cancer risk				
					Infiltration depth	No difference in infiltration depth				
		Woo[89]	Korean	185/304	Cancer risk	Increased cancer risk in CC patients	GG in patients *vs* controls	1.7	1.04-2.66	0.03
					LN metastases	No difference in LN metastases				
		Xing[106]	Chinese	137/199	Cancer risk	No difference in cancer risk				
					LN metastases	Increased risk of LN metastases in CT+TT	CT+TT *vs* CC			0.02
		Langers[105]	Caucasian	215/0	Survival	No difference in survival				
		Ohtani[103]	Japanese	47/67	Cancer risk	No difference in cancer risk				
		Elander[90]	Caucasian	127/208	Cancer risk	No difference in cancer risk				
					Clinicopath. par.	No relationschip with clinicopath. par.				
MMP-12	-82A/G	Woo[89]	Korean	185/304	Cancer risk	No difference in cancer risk				
					LN metastases	No difference in LN metastases				

SNP: Single nucleotide polymorphism; LN: Lymph node; Clinicopath. par.: Clinicopathological parameters; OR: Odds ratio; aOR: Adjusted odds ratio; 95% CI: 95% confidence interval.

MMP-1, an interstitial collagenase, degrades fibrillar collagens type I, II, III, V, IX, and X, that form the most abundant class of extracellular matrix proteins in the interstitium[86]. The MMP-1 gene is located on chromosome 11q22. Insertion of an extra guanine (G) at the -1607 promoter position creates an Ets-1 transcription factor binding site (5'-GGA-3') leading to a significant increase in transcription activity in normal fibroblasts[18]. Both alleles are common in the general population; the allele frequency of the 2G allele is 64% in the Asian population and 52% in the European population[87]. In the Caucasian population, the frequency of the homozygote -1607 2G/2G polymorphism is about 30%. Several papers reported an increased colorectal cancer susceptibility in either 2G/2G homozygotes or 2G-allele carriers of the MMP-1 -1607 polymorphism[88-93]. However, a number of other studies found no association between cancer risk and MMP-1 genotype[94-98]. With exception of the studies of Fang *et al* and Hettiaratchi *et al*, all studies included a relatively small number of patients, which could explain the differences in results. Hettiaratchi *et al* included the largest cohort (503 Australian CRC patients, 471 controls) of all studies so far[96]. Besides the lack of association between the genotype and CRC susceptibility, in this cohort the 5-year survival was increased in 2G/2G homozygotes. All other studies, which have either looked at survival or correlation with clinicopathological parameters, showed that the 2G/2G genotype is either associated with worse survival[99], with unfavourable clinicopathological parameters, like increased risk of metastases at time of diagnosis[92], a higher number of affected lymph nodes[89], or with earlier distant metastases[88]. Patient selection could possibly account for this discrepancy, since Hettiaratchi *et al* only included patients who did not have synchronous metastases at the time of diagnosis, and the influence of MMP-1 on the cancer process may change during different stages of cancer progression. De Lima *et al* reported a higher risk of lymph node metastases in patients carrying a 1G-allele, although this association was not significant with a *P* value of 0.09[95]. In all the other abovementioned papers, no association of MMP-1 gene polymorphisms and clinicopathological parameters was found. In two meta-analyses, 2G-allele carriers showed a significantly increased risk of developing colorectal cancer when compared with homozygous 1G allele carriers[87,100]. However, the large cohort of Hettiaratchi *et al*[96] was not included in these meta-analyses and inclusion of this study might lead to loss of significance.

Lièvre *et al*[101] studied the influence of genetic polymorphisms in the MMP-1 (-1607 1G/2G) gene in 295 patients with large adenomas and 302 patients with small adenomas, the premalignant condition to colorectal cancer, and in 568 polyp-free controls. No difference was found in the genotype distribution between patients with large adenomas and patients with small adenomas or healthy controls.

In a population of 126 CRC patients and 126 healthy controls, Xu *et al*[102] found an increase in CRC susceptibility in patients with the CC genotype of the MMP-2 -1306 C/T polymorphism. These findings were not supported by Hettiaratchi *et al* [96], Elander *et al*[90] and Ohtani *et al*[103], who found no influence of the MMP-2 genotype on the colorectal cancer

risk (Figure 2). Difference in ethnicity (Australian *vs* European *vs* Japanese) or sample size might be the underlying cause of this discrepancy. Two metaanalyses, both including the study of Xu *et al*, showed no association between the MMP-2 -1306 C/T polymorphism and colorectal cancer susceptibility[87,100]. Xu *et al*[104] also reported that patients with the CC genotype had more frequent serosa/adventitia involvement, while none of the other studies described any correlation with clinicopathological parameters or survival, except for the study of Langers *et al*, where the TT genotype was shown to be an indicator of poor 10-year survival[89-93,96,97,99,103,105]. In the Xu *et al*[104] cohort of 126 CRC patients and 126 control patients, two other polymorphisms of MMP-2 (-790 T/G, -955 A/C) were not associated with cancer susceptibility or infiltration depth, while GG genotype carriers of the MMP-2 -1575 G/A polymorphism had an increased risk of developing CRC and more frequent serosa or adventitia invasion compared to the other genotypes, similar to that with the -1306 CC genotype[102]. The similarity in these observations is probably because the MMP-2 -1575 G/A, -1306 C/T, -790 G/T and -735 C/T polymorphisms have been found to be in almost complete pair-wise linkage (dis)equilibrium[28].

The most frequently studied MMP-9 polymorphism is the C to T substitution at position -1562 of the promoter region, which increases transcriptional activity. In a population of 185 Korean colorectal cancer patients and 304 controls, individuals with the CC genotype had an increased risk for developing CRC (OR = 1.7, 95% CI: 1.04-2.66, P = 0.033)[89]. None of the other studies found similar results[90,103-107]. A meta-analysis that included the studies of Elander *et al*[90], Xu *et al*[104,107], Woo *et al*[89] and Xing *et al*[106] showed no significant association of the -1562 C/T MMP-9 polymorphism and colorectal cancer[100]. The same conclusion was reached in a second meta-analysis[87] . Xing *et al*[106] reported a decrease of lymph node metastases in 137 Chinese CRC patients with the CC genotype of the MMP-9 -1562 SNP, whereas the other studies did not find an association with lymph node metastases, survival, infiltration depth or any other clinicopathological variable. The mechanism of action of MMP-9 in cancer is intriguing and not as straightforward as some of the other MMPs. In colorectal cancer, both very high and very low levels of MMP-9 in tumor tissue seem to be associated with poor prognosis compared to intermediate MMP-levels[105]. Similarly, in ovarian cancer, the presence of MMP-9 within the ovarian cells is associated with better survival, whereas higher stromal expression is a marker of worse prognosis[108]. The -90$(CA)_{14-27}$ polymorphism, in which the number of CA repeats influences expression of MMP-9, is not associated with cancer risk or the risk of lymph node metastases[89]. Thus, no consistent relation emerges between MMP-9 genotypes and CRC expression. In a single study of 185 Taiwanese colorectal cancer patients, no association of the MMP-12 -82A/G polymorphism and colorectal cancer risk of development or lymph node metastases was found[89].

Insertion of an extra Adenosine (A) at position -1171 of the MMP-3 promoter generates a 6A allele with lower promoter activity compared to the 5A allele[19]. This polymorphism has

been studied quite extensively in colorectal cancer, and in all but one paper, no contribution to cancer risk, clinicopathological parameters or survival was demonstrated[89-93,96,97,99,103]. Only Hinoda *et al* found a two-fold increase in CRC risk in the 6A/6A homozygotes (OR = 2.11, 95% CI: 1.16-3.82, *P* = 0.013) in the previously mentioned study of a Japanese cohort of 101 CRC patients and 127 controls. In 302 patients with small adenomas and 568 polyp-free controls, the 6A/6A genotype of the -1171 MMP-3 polymorphism was associated with a significant risk of small adenomas (OR = 1.50, 95%CI: 0.99-2.28, *P* = 0.008) and this association was even stronger in individuals with the combined genotype MMP-3 -1171 6A/6A + MMP-1 -1607 2G/2G (OR =1.88, 95%CI: 1.08-3.28, *P* = 0.001)[101]. When the MMP-3 genotype of 295 patients of that study with large adenomas was compared to either the patients with small adenomas or the polyp-free controls, no difference in genotype distribution was found. These findings suggest that this MMP-3 5A/6A polymorphism (and the -1607 1G/2G polymorphism) might be of importance early in the process of adenoma formation. The 6A/6A genotype of the MMP-3 -1171 5A/6A polymorphism has a lower transcriptional activity and higher plasma levels of MMP-3 were measured in 5A/5A homozygote patients with acute coronary syndrome compared to 6A/6A homozygotes[109]. Apparently, the association between this polymorphism and increased susceptibility for developing early colorectal adenomas does not provide an insight into the functional activity of the protein.

No clear association between MMP-7 -181 A/G polymorphism and colorectal cancer incidence, lymph node metastases or survival was found in most of the publications[89,94,95,103,110]. The only exception is by Ghilardi *et al*[111] who showed that the GG genotype increases the colorectal cancer risk. Furthermore, in the 58 patients with colorectal cancer included in this study, the CC genotype predisposed for lymph node metastases and distant metastases at the time of diagnosis[111]. Ghilardi *et al*[111] also studied the C/T polymorphism at position -153 of the MMP-7 promoter and found an increase in colorectal cancer risk in T allele carriers, but no association with any of the clinicopathological variables. In a study of 174 colorectal cancer patients, Langers *et al*[110] reported that patients with the CC genotype had a better 10-year survival than the patients with the CT or TT genotype (CC *vs* CT+TT: Log Rank 14.0, *P* = 0.0009). The study of Ghilardi *et al*[111] included 58 patients, a relatively small number for studying the influence of gene polymorphisms on cancer susceptibility and prognosis. This may explain the discordant results between the different studies and illustrates the need for larger sample sizes. Peng *et al* tried to solve this problem by performing meta-analyses of case control studies investigating the role of gene polymorphisms of MMP-1, -2, -3, -7 and -9 on cancer susceptibility in lung, head and neck, esophageal, gastric, colorectal, hepatocellular, breast, renal, bladder, cervical, ovarian, endometrial, prostate and skin cancer[38,87]. In these meta-analyses, a consistent positive association with colorectal cancer risk was observed for the

MMP-1 -1607 1G/2G polymorphism, but not for MMP-2 -735C/T, MMP-2 -1306 C/T, MMP-7 -181A/G and MMP-9 -1562 C/T.

In summary, although data are still emerging, there appears to be evidence for associations between the MMP-1 -1607 1G/2G, MMP-2 -1306 C/T, MMP-7 -181 A/G and MMP-9 -1562 C/T polymorphisms and CRC susceptibility. In affected individuals, an association of the MMP polymorphism with the course of the disease or prognostic parameters was reported in some studies as shown in Table 3, although these results await further confirmation.

DISCUSSION

The three major regulatory mechanisms that eventually determine the function of MMPs are transcription, activation of latent MMPs and inhibition by specific inhibitors. Along with local activation and inhibition, regulation of transcription seems to be of major importance for the function of MMPs[112]. Most of the promoter polymorphisms that are described in this review have been shown to influence promoter activity and to increase or decrease transcription *in vitro*, as shown in table 1. Some SNPs are associated with gastrointestinal cancer susceptibility and in some cases, a correlation with clinicopathological parameters and outcome of the disease was observed. Surprisingly, only a few studies have actually looked at the correlation between the promoter polymorphism of MMPs and the corresponding tumor protein levels. Two studies reported no association between the different genotypes and MMP protein expression in the tumor[56,105]. It would be interesting to correlate the values in normal tissues from these patients with their genotypes to further elucidate their contribution to the phenotypic expression of the MMPs in cancer patients. Besides the regulation of expression by transcription, the presence of MMPs in the (tumor) microenvironment depends on the inactivation/clearing, which is regulated by the inhibitors. High clearance could lead to low protein levels despite high levels of expression. Furthermore, a specific genotype can have different (and even opposite) effects in different cell types. Wang *et al* showed that the -799T/-381G/+17G haplotype of MMP-8 increased promoter activity in cells resembling chorion cytotrophoblasts, but the same haplotype decreased promoter activity in a leukocyte cell line and had no effect on promoter activity in a macrophage cell line[23]. Cell-specific functional effects of SNPs have been described for several cancer-associated proteins[113,114]. This phenomenon makes the translation of the effect of a promoter polymorphism on gene transcription to the *in vivo* situation even more complex, especially as many different stromal cell types as well as tumor cells are involved in the production of MMPs (Figure 1). A correlation between a particular polymorphism and cancer susceptibility does not necessarily demonstrate the implication of the corresponding gene in the process of cancer development or progression. It could also be the result of a

linkage (dis)equilibrium between the examined (potentially functionally neutral) SNP and another (potentially functionally important) SNP[85].

Although for some MMP-polymorphisms the results between different studies are unanimous, there is often a discrepancy between the results of different studies on the same polymorphism. However, some trends can be observed. An increased incidence of esophageal cancer in CC carriers of the MMP-2 -1306 C/T polymorphism was reported in 2 studies[15,39] and this association was corroborated in a meta-analysis[38]. In the Asian population, G-allele carriers of the MMP-7 -181 A/G polymorphism have an increased risk of developing both esophageal cancer and gastric cancer[45,63,64]. In hepatocellular cancer, no association was found between any of the MMP SNPs and cancer risk, although 5A-allele carriers of the MMP-3 5A/6A polymorphism might have a worse prognosis. Although some studies concerning CRC report a correlation between cancer incidence and the MMP-1 1G/2G, MMP-2 -1306 C/T, MMP-7 -181A/G and MMP-9 -1562 C/T polymorphism, the only association that was found to be significant in a meta-analysis was a higher cancer risk in 2G allele carriers of the MMP-1 1G/2G polymorphism[87]. Sometimes, as for the MMP-7 -181 A/G polymorphism in gastric cancer, the variability in results between the different studies is likely to be explained by ethnic differences between the study groups. Different genotype distributions of MMP-2 and MMP-9 SNPs have been reported in Caucasians and African-Americans, which seem to be associated with differences in prevalence of cancer and cardiovascular disease[115]. The diverse results in the publications described in this review emphasize the need for studies on larger numbers of patients before definite associations between genetic polymorphism and susceptibility to cancer or with the course of the disease in affected individuals can be established. There is a need for large cohorts of patients who are genotyped, and information about disease progression, lymph node metastases, distant metastases and prognosis needs gathered. In the meantime meta-analyses rather than single studies are the best indicators of the practical value of single SNPs. The recent meta-analysis of Zhou *et al*[116] including almost 3000 breast carcinoma patients, suggested MMP-2 -1306 C/T as a potential indicator, whereas the SNPs of MMP-1, MMP-3 and MMP-9 were not indicative.

Genome-wide association studies (GWAS) may further highlight the genes that are important in identifying people at high risk for the development of cancer or patients who are likely to have an unfavorable outcome of their disease. To date, fourteen loci identified by GWAS analysis have been shown to influence the risk of developing colorectal cancer[117]. None of them is located in any of the MMP genes. However, in an extensive mutation analysis of the human genome in which 13.023 genes were involved, Sjöblom *et al*[118] identified MMP-2, ADAM29 and three ADAMTS family members among the 69 CAN genes that are often mutated in colorectal cancer.

CONCLUSION

To predict the cancer risk in a population and the outcome of the disease in affected individuals, a genomic profile including functional SNPs of several genes would probably be a better tool than the use of a single SNP. Being key players in the process of cancer development and progression, SNPs of selected MMPs or TIMPs could be included in such a profile to predict disease susceptibility and/or the course of a disease. Because of the heterogeneity of previous studies that have included a relatively small number of patients, further research on large cohorts of cancer patients and healthy controls is needed before a definite conclusion can be drawn about the impact of these genes on gastro-intestinal cancer risk and prognosis.

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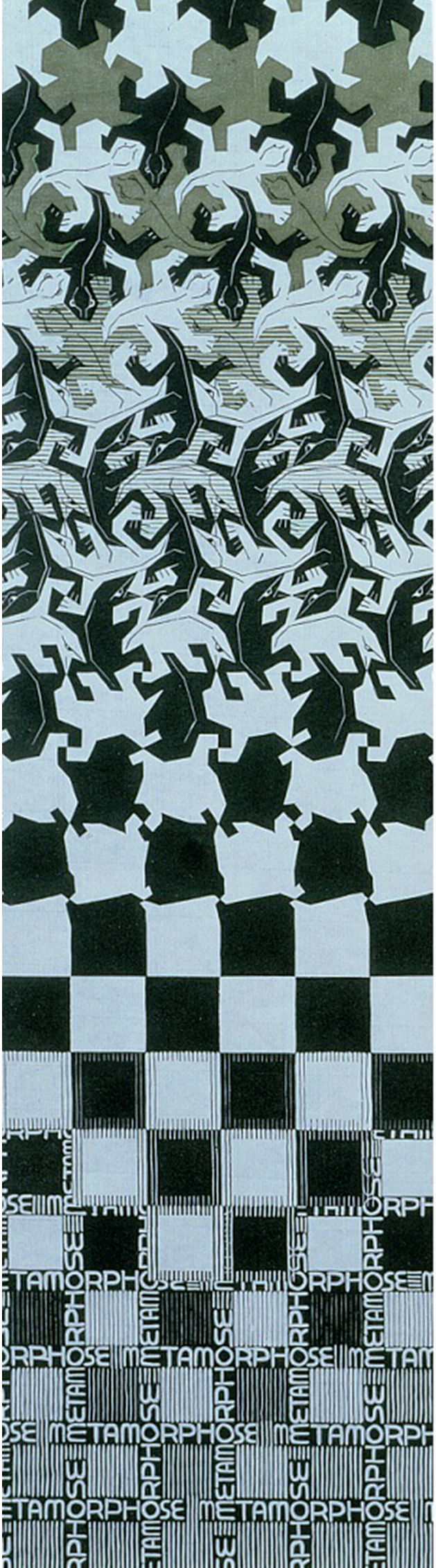

CHAPTER 5

MMP-2 geno-phenotype is prognostic for colorectal cancer survival, whereas MMP-9 is not

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ABSTRACT

The prognostic significance of single-nucleotide polymorphisms (SNPs) and tumour protein levels of MMP-2 and MMP-9 was evaluated in 215 colorectal cancer patients. Single- nucleotide polymorphism MMP-2 -1306 C/T and high MMP-2 levels were significantly associated with worse survival. Extreme tumour MMP-9 levels were associated with poor prognosis but SNP MMP-9 -1562 C/T was not. Tumour MMP levels were not determined by their SNP genotypes.

INTRODUCTION

Enhanced tumour matrix metalloproteinase (MMP) levels have been correlated to decreased patients' survival in various types of cancer[1-5]. Next to sheer degraders of extracellular matrix, MMPs are presently regarded as general molecular switches in the microenvironment[6]. For instance, MMPs function as major regulators of tumour growth by catalysing the release or activation of growth factors, activation or shedding of membrane receptors, or cleavage of matrix/membrane-bound substrates involved in cell proliferation[7]. A subgroup of MMPs, the gelatinases (MMP-2 and MMP-9), have been particularly implicated in progression, angiogenesis and metastasis of various cancer types[8]. In the present study, we evaluated the association between, and the prognostic relevance of tissue protein levels of MMP-2 and MMP-9 and their gene promoter single-nucleotide polymorphisms (SNPs) in a cohort of 215 Dutch colorectal cancer patients.

MATERIALS AND METHODS

Patients and study design

Representative, nonnecrotic samples of cancer tissue were collected from 215 patients with colorectal cancer, operated in the Leiden University Medical Centre (December 1983 - September 1991). Tissues were snap-frozen and stored at -70°C. Clinical data and follow-up were available for at least 10 years. Macroscopic and microscopic parameters were obtained from the pathology reports. The study was performed according to the instructions and guidelines of the LUMC Medical Ethics Committee.

Tissue preparation and protein concentration

Tissues were homogenised in 0.1 M Tris-HCL (pH 7.5) with 0.1% (v/v) Tween 80 buffer and centrifuged twice all at 4°C, as described before[9], the protein concentrations were determined[10] and the supernatants were aliquoted and stored at -70°C. Storage-induced degradation of MMP-2 and MMP-9 was checked by western blots and gelatin zymography prior to ELISA measurements[11].

Determination of MMP-2 and MMP-9 in tissue homogenates

The MMP-2 and MMP-9 levels were determined by previously described ELISAs[12]. Polyclonal anti-MMP-2 or monoclonal anti-MMP-9 antibodies were used as catching antibody, incubated with appropriately diluted samples (o/n, 4°C), and polyclonal anti-MMP-2/biotin-labelled goat anti-rabbit-IgG and biotin-labelled polyclonal anti-MMP-9 antibodies for immunedetection combined with avidin-peroxidase and the 3,3´,5,5´-tetramethyl

benzidine/H_2O_2 substrate solution, and the absorption was read at 450 nm. Sample MMP concentrations were calculated from standard curves and expressed in ng per mg protein.

Single-nucleotide polymorphism analysis

Tissue DNA was isolated using the salting out method[13]. Single-nucleotide polymorphism (SNP) analysis for MMP-2 -1306 C/T and MMP-9 -1562 C/T was performed by tetraprimer ARMS PCR, involving four oligonucleotide primers but no restriction enzymes, or RFLP-PCR, as described earlier[14, 15].

Statistical analysis

Statistical analyses were performed using SPSS 12.0 Statistical Package (2004, SPSS Inc., Chicago, IL, USA). Expression differences between groups were calculated using the Mann-Whitney's *U*-test. Log rank statistics was used for optimal cutoff point analysis. Hardy-Weinberg's analysis was performed using χ^2 or Fisher's exact test to examine differences in the distribution of alleles and genotypes. Correlations between parameters were according to Pearson or Spearman, where appropriate. Overall survival curves were according to Kaplan and Meier. Univariate and multivariate survival analyses were performed using the Cox's proportional hazards method. *P* values ≤ 0.05 were considered significant.

RESULTS

The genotype distributions of the SNPs for MMP-2 and MMP-9 in 215 colorectal cancer patients are shown in Table 1. The distribution of the polymorphisms in the patients was according to the predicted Hardy-Weinberg's distribution. Both SNPs showed a weak but significant association with TNM stage (Table 1) and MMP-2 -1306 C/T also with survival (Figure 1A). All other clinicopathological parameters did not show an association with either SNP MMP-2 -1306 C/T or MMP-9 -1562 C/T, and the latter was also not associated with survival (Figure 1B).

Table 1. Genotype distribution of single-nucleotide polymorphisms (SNPs) for MMP-2 and MMP-9 in 215 colorectal carcinoma patients compared with the expected Hardy–Weinberg distribution (H-W).

SNP		All	TNM stage*			
Genotype	H-W	Patients	1	2	3	4
MMP-2 -1306 C/T	(%)	% (N)	% (N)	% (N)	% (N)	% (N)
CC	55.4	54.4 (117)	69.4 (25)	57.1 (48)	52.2 (35)	32.1 (9)
CT	38.1	40.0 (86)	27.8 (10)	40.5 (34)	41.8 (28)	50.0 (14)
TT	6.6	5.6 (12)	2.8 (1)	2.4 (2)	6.0 (4)	17.9 (5)
MMP-9 -1562 C/T	(%)	% (N)	% (N)	% (N)	% (N)	% (N)
CC	69.7	71.2 (153)	83.3 (30)	70.2 (59)	73.1 (49)	53.6 (15)
CT	27.6	24.7 (53)	11.1 (4)	28.6 (24)	23.9 (16)	32.1 (9)
TT	2.7	4.1 (9)	5.6 (2)	1.2 (1)	3.0 (2)	14.3 (4)

MMP = matrix metalloproteinase *χ^2 values for TNM stage distribution of MMP-2 and MMP-9 were, respectively, 15.9 (P = 0.01) and 14.9 (P = 0.02).

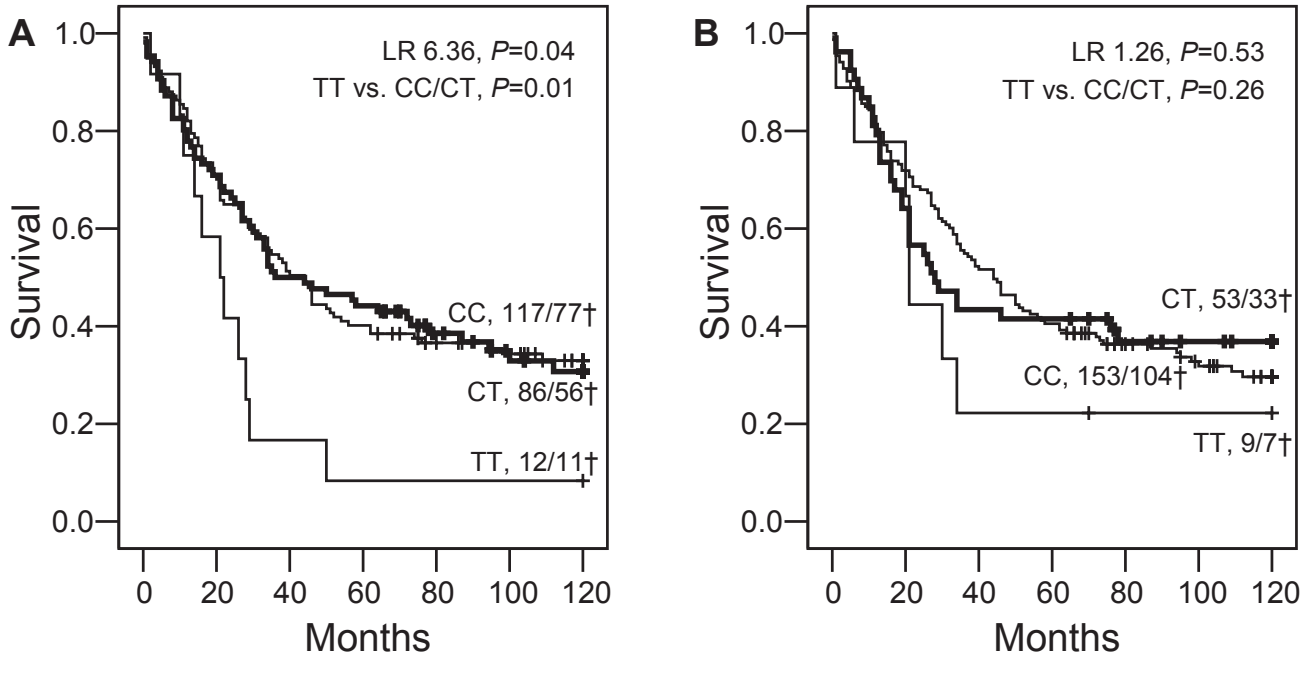

Figure 1: Kaplan-Meier's 10-year survival curves of 215 colorectal cancer patients grouped by their genotype for SNP MMP-2 -1306 C/T **(A)** and MMP-9 -1562 C/T **(B)**. The total number of patients and the deceased patients (†) are indicated per subgroup.

The protein levels of MMP-2 (median 10.6; range: 0.0-76.6 ng per mg protein) and MMP-9 (median 37.3; range: 0.5-201.9 ng per mg protein) in the colorectal carcinomas were found not to be related to any of the clinicopathological parameters, although for MMP-2 a stepwise increase with TNM stage was discernable (not shown). For the survival analysis, an optimised cutoff point value was identified for the tumour MMP-2 level (18.5 ng per mg protein, LR 5.07, *P* = 0.024, Figure 2A). The same approach for MMP-9 resulted in two differently oriented cutoff points; a low MMP-9 value (11.2 ng per mg protein, LR 9.18, *P*=0.010) and a high value (125.0 ng per mg protein, LR 5.31, *P* = 0.021), both associated with poor prognosis (Figure 2B).

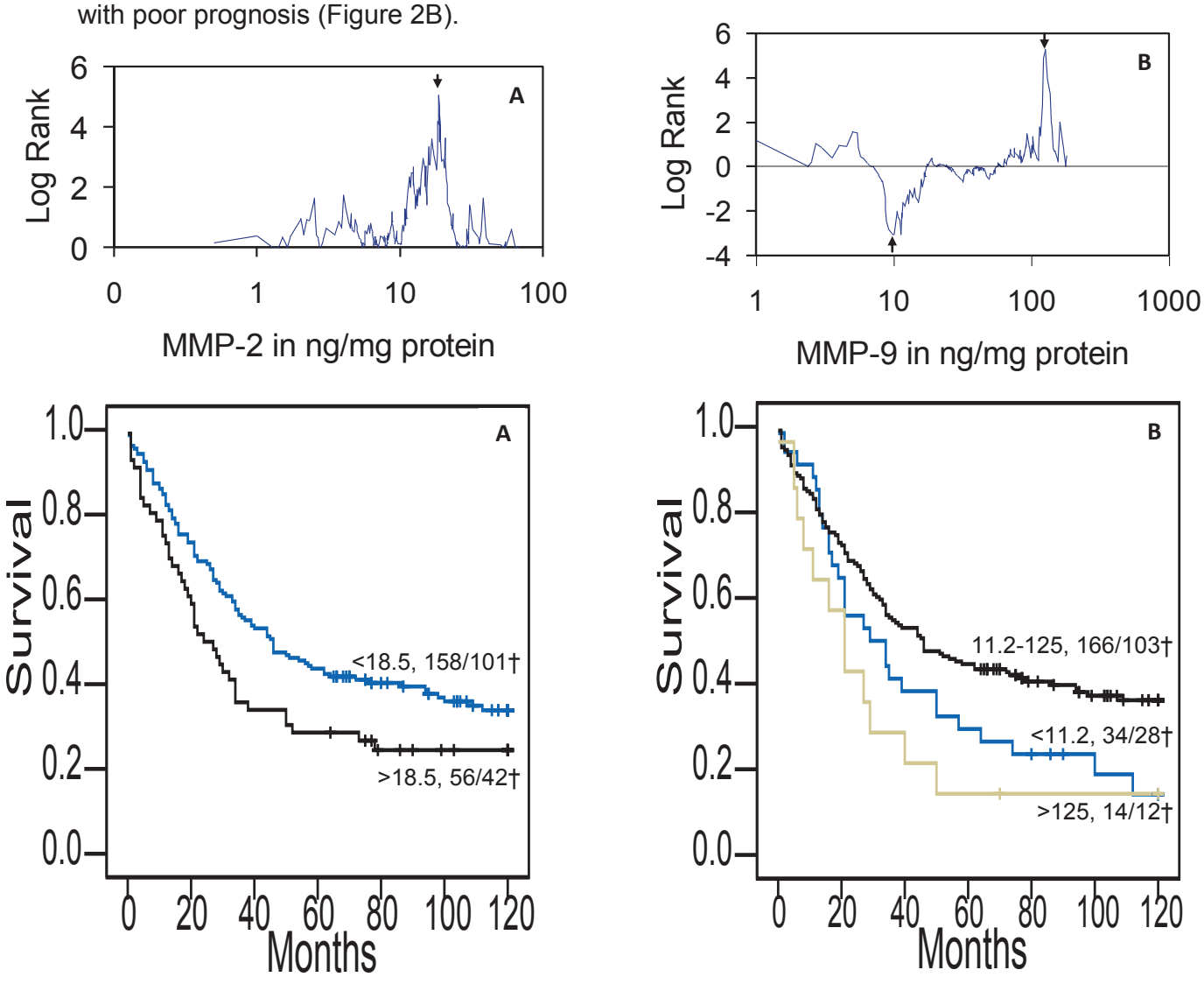

Figure 2: Optimal cutoff point analysis and corresponding Kaplan-Meier's 10-year survival curves for MMP-2 **(A)** and MMP-**9 (B)** in tumour tissue homogenates from 215 colorectal cancer patients. The optimal cutoff points are indicated with arrows. In the survival curves the total number of patients and the deceased patients (†) are indicated per subgroup.

Table 2: Univariate and multivariate Cox's proportional hazard 10-year survival analysis of 215 colorectal cancer patients

			Univariate			Multivariate		
		n	HR	CI 95%	*P*	HR	CI 95%	*P*
Parameter								
Gender	F *vs* M	91-124	1.363	0.973-1.908	0.071	1.362	0.973-1.908	0.072
Age	< 65 years >	73-142	2.123	1.448-3.113	**0.000**	2.390	1.625-3.516	**0.000**
TNM	1/2 *vs* 3/4	120-95	2.316	1.662-3.228	**0.000**	2.548	1.822-3.564	**0.000**
Localization	Right *vs* rest	75-140	1.023	0.726-1.442	0.896		Not included	
Diameter	<5 cm >	92-123	1.310	0.936-1.834	0.116		Not included	
Differentiation	Good *vs* rest	38-174	1.129	0.789-1.614	0.506		Not included	
MMP Proteins								
MMP-2	<18.5ng/mg>	158-56	1.504	1.048-2.158	**0.027**	1.417	0.982-2.043	**0.062**
MMP-9	11.2-125	166	1.000					
	<11.2	34	1.526	1.004-2.319	**0.048**	0.787	0.516-1.201	0.266
	>125	14	2.135	1.171-3.895	**0.013**	1.109	0.561-2.189	0.766
MMP SNPs								
MMP-2	CC/CT *vs* TT	203-12	1.471	1.079-2.006	**0.015**	1.395	1.019-1.911	**0.038**
MMP-9	CC/CT *vs* TT	206-9	1.239	0.847-1.813	0.270	1.114	0.757-1.638	0.584

CI = confidence interval; HR = hazard ratio; MMP = matrix metalloproteinase. Multivariate analysis was performed by adding every single MMP-related parameter to the dichotomised, prognosis-associated clinicopathological parameters gender, age and TNM stage. Entries in bold indicate significant, or in case of MMP-2 almost significant *P*-values.

The MMP-2 and MMP-9 protein levels in the colorectal cancer homogenates did not correlate with their respective SNP genotypes. The median values for MMP-2 -1306 C/T were 10.9, 9.5, and 11.6 ng mg^{-1} for genotypes CC, CT and TT respectively. Even an apparent enhancement of MMP-9 protein associated with the TT genotype of SNP MMP-9 -1562 C/T in tumours did not reach significance (CC/CT *vs* TT, medians 36.5/26.3 *vs* 44.4 ng mg^{-1}, $P = 0.28$).

Univariate Cox's survival analyses confirmed the association of the MMP-2 SNP and the protein levels of MMP-2 and MMP-9 with survival (Table 2). Multivariate analysis against the prognosis-associated parameters gender, age and TNM classification showed that the MMP-2 SNP was independently associated with survival, whereas the tumour protein levels of MMP-2 just lost and MMP-9 completely lost their significance.

DISCUSSION

MMP-2 and MMP-9 are proteinases implicated in cancer progression. We showed previously that high tumour levels of MMP-2 in gastric carcinomas were consistently associated with a worse survival[3, 11]. For enhanced MMP-9 levels the relation with survival was more ambivalent[3, 14]. Also in our present cohort of colorectal carcinoma patients, we found that MMP-2 and MMP-9 levels within the tumours are of significance to survival. The cutoff point analysis showed a broad range of MMP-2 levels with a significant and unidirectional relation with survival outcome: high tumour MMP-2 levels are unfavourable for the patients' prognosis. Our ELISA-derived MMP-2 data correspond very well with a recent immunohistochemical study in a group of 351 colorectal cancer patients, showing that high expression of MMP-2 in malignant epithelium as well as in the surrounding stroma was associated with reduced survival[2]. Similar analysis on our tumour MMP-9 data revealed that not only patients with the highest, but also those with the lowest levels had a worse survival than patients with intermediate levels. Duality of tumour MMP-9 levels with respect to survival has been recognised before in immunohistochemistry-based studies. For example, extensive MMP-9 staining in ovarian cancer cells was associated with a longer survival, as opposed to a shorter survival with a higher stromal expression of MMP-9[16]. Earlier morphometric studies indicated that the degree of MMP-9 expression in tumour-associated lymphocytes, macrophages and neutrophils was inversely associated with invasion and metastasis in colorectal cancer[17]. These observations are relevant to our findings because a low level of MMP-9 in the tumour homogenates might indicate a lack of infiltration of the tumours with MMP-9 containing leukocytes, known to exert anticancer effects[18, 19], leading to an adverse prognosis. In addition, MMP-9- mediated cleavage of extra-cellular matrix components is also known to generate antiangiogenesis inhibitors like angiostatin[20]. Low MMP-9 expression in the tumour, leading to insufficient production of antiangiogenic factors, could also contribute to the worse prognosis of these patients[21]. However, MMP-9 has a wide range of substrates, including various growth factors and several types of collagen, which after cleavage contribute to the process of invasion, angiogenesis, and metastasis of tumours, explaining why high tumour levels of MMP-9, in general, are associated with a poor prognosis.

Differences in expression of metalloproteinases might, in part, be explained by genotypic variation. Because the investigated SNPs of MMP-2 and MMP-9 are located in the promoter region of the gene, a correlation between polymorphism and protein expression might be expected. The C→T transition at the -1306 position of the MMP-2 gene promoter prevents binding of the stimulating Sp1 transcription factor whereas a change at the -1562 position of the MMP-9 gene decreases binding of a repression factor[22, 23]. *In vitro* MMP-2 expression levels by colon cancer cell lines containing the CC genotype were indeed higher compared with cells with the CT genotype[24]. In our cohort of patients, however, we found no

relationship between MMP-2 and MMP-9 polymorphisms and the tumour protein levels, likely caused by the complicated regulatory posttranslational mechanisms for proteinases in multicellular tumour tissues. Nevertheless, we did find significant associations between MMP-2 -1306 C/T and TNM stage and survival, that is, worse prognosis in patients with the TT genotype. This association with the outcome of the patients support the recent confirmation of MMP-2 as one of the candidate cancer genes (CAN-genes) by the number and nature of mutations and pathways in colorectal cancer[25]. Our results for SNP MMP-9 -1562 C/T correspond well with data from Asiatic patients[26], indicating that this polymorphism is not directly involved in the process of colorectal carcinogenesis.

Summarising, we showed that MMP-9, mechanistically probably the most interesting of the gelatinases, is not a likely candidate as a simple prognostic indicator. Despite the absence of a correlation between promoter-located SNP -1306 C/T in the MMP-2 gene with tumour MMP-2 levels, both parameters were significantly associated with survival, indicating MMP-2 as a consistent independent prognostic factor in colorectal cancer.

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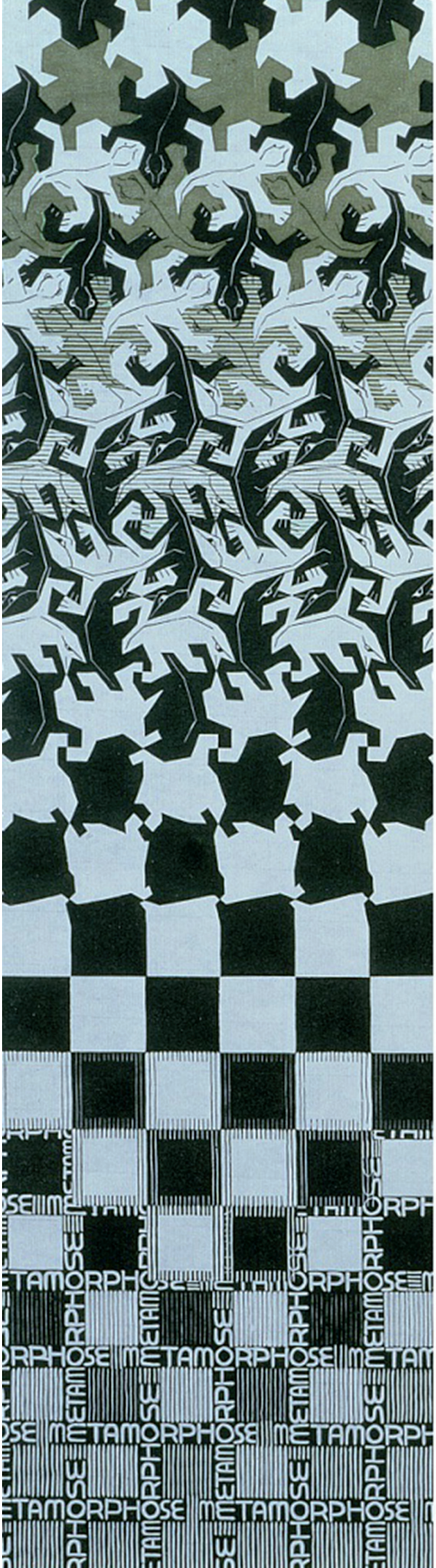

CHAPTER 6

MMP-2 and MMP-9 in normal mucosa are independently associated with outcome of colorectal cancer patients

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ABSTRACT

Background: Upregulation of the matrix metalloproteinases MMP-2 and MMP-9 in various cancers has been associated with worse survival of the patients.

Methods: We assessed MMP-2 and MMP-9 levels in normal colorectal mucosa from colorectal cancer patients in relation to the course of the disease.

Results: A high protein expression of MMP-2 as well as MMP-9 in normal mucosa was found to be correlated with worse 5-year survival. The combination of both parameters was an even stronger prognostic factor. These protein levels were found not to be related to the corresponding single-nucleotide polymorphisms of MMP-2 (-1306C>T) and MMP-9 (-1562C>T). Multivariate analyses indicated that the MMP-2 and MMP-9 levels in normal mucosa are prognostic for survival, independent of TNM classification.

Conclusion: MMP-2 and MMP-9 levels in normal mucosa are indicative of the course of disease in colorectal cancer patients.

INTRODUCTION

The gelatinases MMP-2 and MMP-9 are implicated in the process of colorectal cancer progression, angiogenesis and metastasis[1]. We previously reported that MMP-2 genotype and high levels of tumour MMP-2 are associated with a poor survival in colorectal cancer patients. For MMP-9 we found a bivalent correlation between MMP-9 expression and survival, that is, patients with either very low or very high MMP-9 tumour protein levels have worse survival compared with patients with intermediate MMP-9 expression[2]. In contrast, little is known about the impact of MMP expression in normal mucosa of cancer patients. We hypothesised that the expression of MMP-2 and MMP-9 in normal mucosa of colorectal cancer patients could be relevant as well to the outcome in colorectal cancer. The aim of the present study was to evaluate the relation of the genotype and phenotype of MMP-2 and MMP-9 in normal appearing mucosa with outcome of patients with colorectal cancer.

MATERIALS AND METHODS

Patients and study design

Tumour tissue and normal appearing mucosa at a distance of 5-10 cm from the tumour was collected from 198 consecutive patients (85 female and 113 male) who underwent surgery for colorectal cancer between 1983 and 1991 at Leiden University Medical Centre. All patients from whom tissue was obtained and data collection was complete were included in this retrospective study of prospectively collected tissues. None of the patients received (neo-) adjuvant chemo- and/or radiotherapy. The surgical procedure consisted of removal of the tumour with *en bloc* resection of the lymph nodes. Tissue samples were snap frozen and stored at -70°C until use. Macroscopic and microscopic parameters were obtained from the pathological reports, including TNM classification. Clinical data and follow-up information was available for a period of at least 5 years. The primary end point was survival at 5 years after surgery. Patient characteristics were as follows: 67% of patients was >65 years of age, TNM classification was stage 1 in 17%, stage 2 in 40%, stage 3 in 29% and stage 4 in 14% of the patients. The tumour was localised in the right hemicolon in 36% of the patients, 42% had left-sided cancer and in 22% the tumour was localised in the rectum. In 42% of the patients the tumour was smaller than 5 cm in diameter and in 23% there was a mucinous component in the histology. Histological differentiation grade was poor in 13% of the patients, moderate in 70% and good in 17% of the patients. Parameters considered for inclusion in the multivariate analysis were: MMP-2 and MMP-9 in normal mucosa, tumour stage, age and gender. The study was performed according to the instructions and guidelines of the LUMC Medical Ethics Committee and in accordance with the Helsinki Declaration.

Tissue preparation and protein concentration

Tissue homogenates were prepared in 0.1 M Tris-HCL (pH 7.5) with 0.1 % (v/v) Tween 80 extraction buffer as previously described[3]. The protein concentration was determined[4].

Determination of MMP-2 and MMP-9 in tissue homogenates

Levels of MMP-2 and MMP-9 were determined in homogenates by previously described ELISAs[5]. In short, polyclonal anti-MMP-2 or monoclonal anti-MMP-9 antibodies were used as catching antibody and appropriately diluted samples were incubated overnight at 4°C. Immune-detection was performed using polyclonal rabbit anti-MMP-2 followed by biotin-labelled goat anti-rabbit-IgG for MMP-2 and biotin-labelled polyclonal anti-MMP-9 antibodies for MMP-9. After incubation with avidinperoxidase, the chromogenic substrate 3,3´,5,5´-tetramethyl benzidine was added in the presence of hydrogen peroxide. The reaction was stopped with H_2S0_4 and the absorption was measured at 450 nm. The amount of MMP was calculated from parallel incubated standard curves of MMP-2 or MMP-9 and expressed in ng per mg protein of the homogenate.

Single-nucleotide polymorphism (SNP) analysis

Genomic DNA was isolated from the tissues using the salting out method[6]. The SNP analysis for MMP-$2_{-1306C>T}$ and MMP-$9_{-1562C>T}$ was performed by restriction fragment length polymorphism – polymerase chain reaction as described earlier[7, 8] or by tetra primer ARMS PCR, involving four oligonucleotide primers but no restriction enzymes[7]. Genotype frequencies for MMP-2-1306C>T were CC: n=108: CT: n=79 and TT: n=11, and for MMP-9-1562C>T CC: n=139; CT: n=50 and TT: n=9.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 Statistical Package (SPSS Inc., Chicago, IL, USA). Expression differences between groups were calculated using the Mann-Whitney U-test. Log Rank statistics (LR) was used for optimal cut point analysis[9]. Hardy-Weinberg analysis was performed using chi-square or Fisher's exact test to examine differences in the distribution of alleles and genotypes. Multivariate analyses were performed by adding every single MMP-related parameter to the dichotomised, prognosis-associated clinico-pathological parameters gender, age and TNM stage. Correlations between parameters were calculated according to Pearson's correlation test. *P*-values smaller than 0.05 were considered significant.

RESULTS

The median protein level of MMP-2 and MMP-9 in the normal mucosa of the 198 colorectal cancer patients was 4.8 ng mg^{-1} protein (range 0 - 30.5) for MMP-2 and 3.2 ng mg^{-1} protein (range 0.2-164.7) for MMP-9. As expected, MMP expression in normal mucosa was lower than in carcinoma tissue: 2-fold lower for MMP-2 (Figure 1A, median carcinomas 10.6 ng mg^{-1} protein) and 12-fold lower for MMP-9 (median carcinomas 36.7 ng mg^{-1} protein). The MMP-2 levels in the normal mucosa correlated significantly with the levels in cancer tissue (R = 0.489, $P \leq 0.0001$ for all genotypes together; results per genotype are shown in figure 1A), whereas the MMP-9 levels did not (R = 0.034, $P = 0.637$). The highest levels of MMP-2 and MMP-9 were found in mucosa from TNM stage IV patients, but the differences with and between the other stages were not statistically significant (data not shown). Optimal cutoff point analyses of mucosal MMP-2 and MMP-9 protein levels divided the patients in subgroups with significant differences in survival. High levels of MMP-2 (cutoff 8.7 ng mg^{-1} protein, LR 12.82, $P < 0.001$, Figure 1B) or MMP-9 (cutoff 1.6 ng mg^{-1} protein, LR 10.41, $P = 0.001$, Figure 1C) were associated with poorer 5-year survival. The combination of both mucosal MMP-based parameters appeared a highly significant discriminator (LR 20.30 $P < 0.0001$) for subdivision of patients into good, intermediate, and poor survivors as shown in Figure 1D.

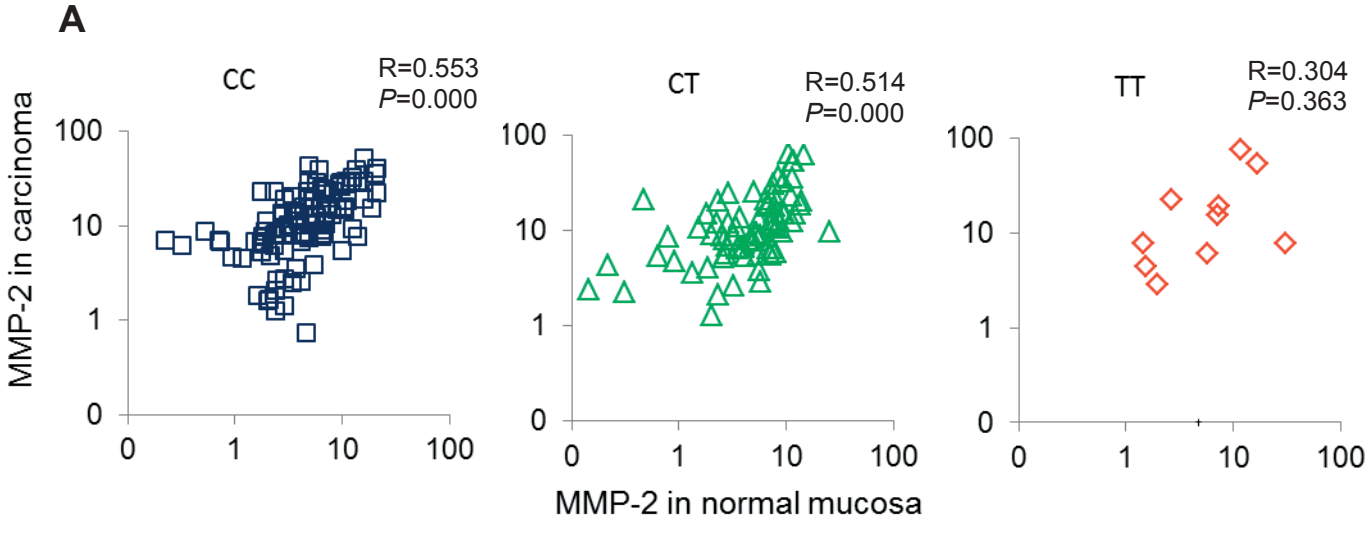

Figure 1. (A) Correlation between the tissue MMP-2 levels (in ng mg^{-1} protein) in normal mucosa and corresponding carcinoma tissue obtained from 198 colorectal cancer patients. The left panel shows the correlation in patients with a CC genotype, the middle panel in patients with a CT genotype and the right panel shows the correlation in patients with a TT genotype of the -1306 CT polymorphism of MMP-2. Indicated are the Pearson's correlation coefficients for MMP-2 levels in normal mucosa and corresponding tumour and their corresponding *P*-value.

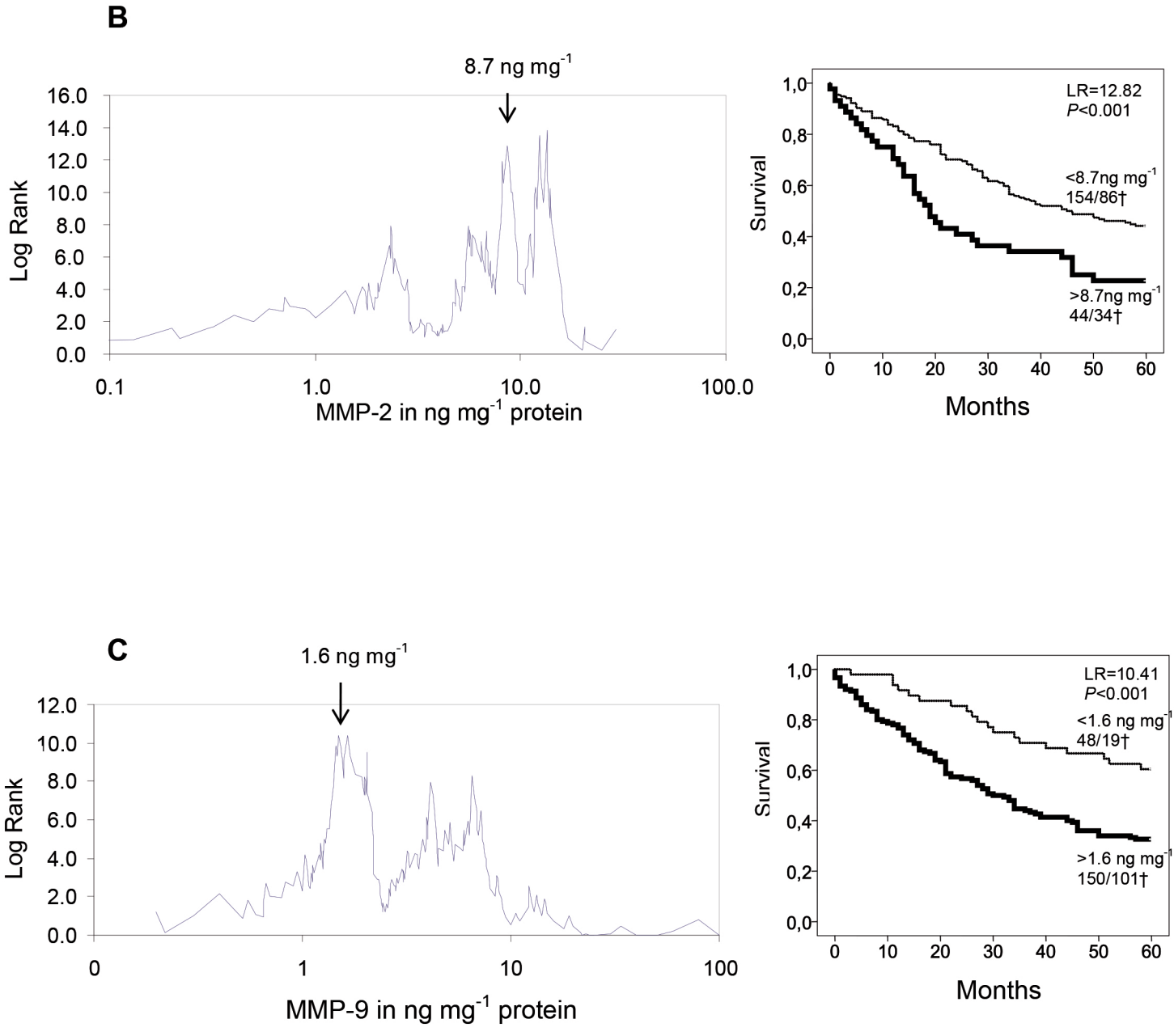

Figure 1. (**B** and **C**): Optimal cutoff point analysis and corresponding Kaplan-Meier 5-year survival curves for dichotomized (high/low) levels of MMP-2 (**B**) and MMP-9 (**C**) in normal mucosa. The optimal cutoff points are indicated with arrows. In the survival curves, the total number of patients and the deceased patients (†) are indicated per subgroup.

D

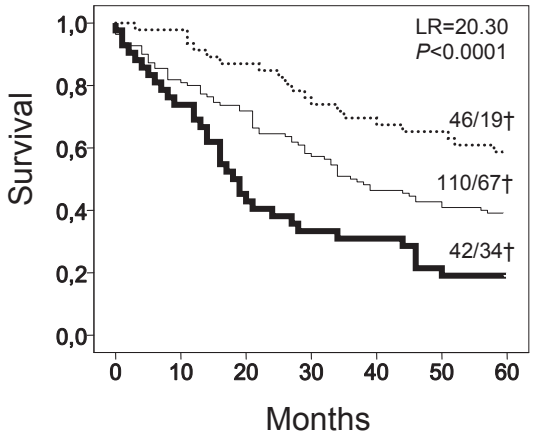

Figure 1. (**D**) Kaplan Meier 5-year survival curves for combination of MMP-2 and MMP-9 in normal mucosa (both MMP-2 and MMP-9 high (lower, thick line), both MMP-2 and MMP-9 low (upper, dashed line), either MMP-2 high and MMP-9 low or MMP-2 low and MMP-9 high (intermediate, thin line); for cutoff points see **B** and **C**). The total number of patients and the deceased patients (†) are indicated per subgroup.

The genotypes of the MMP-2-1306C>T and MMP-9-1562C>T polymorphism were distributed according to the Hardy-Weinberg equilibrium (MMP-2 χ^2=0.49, *P* = 0.48; MMP-9 χ^2=2.5, *P* = 0.11) and there was no association between the different genotypes and the levels of MMP-2 and -9 in normal mucosa (data not shown).

Univariate Cox analysis confirmed the association of the tissue levels of MMP-2 and MMP-9 with survival (Table 1). Multivariate Cox analysis, including the clinical parameters gender, age and TNM stage of the tumour, showed both mucosal MMPs to be independent indicators of prognosis (hazard ratios >1.7, *P* = 0.009). A separate multivariate analysis of the MMP-2/MMP-9 combination indicated a highly significant hazard ratio for patients with high mucosal levels of both MMPs.

Table 1. Univariate and multivariate Cox proportional hazard analysis for 5-year survival in normal appearing mucosa from 198 patients with colorectal cancer

			Univariate			Multivariate		
		n	HR	CI 95%	*P*	HR	CI 95%	***P***
Parameter								
Gender	F-M	85-113	1.195	0.830-1.719	0.339	1.164	0.808-1.677	0.414
Age	<65yrs>	66-132	1.861	1.230-2.815	**0.003**	2.003	1.322-3.035	**0.005**
TNM	I+II-III+IV	113-85	2.972	2.056-4.297	**0.000**	3.083	2.129-4.465	**0.000**
MMP mucosal								
MMP-2	<8.7>	154-44	1.898	1.273-2.828	**0.002**	1.718	1.145-2.578	**0.009**
MMP-9	<1.6>	48-150	2.323	1.421-3.796	**0.001**	1.948	1.184-3.204	**0.009**
MMP-2/MMP-9 combination[a]	<,<	46	Ref	-	-	Ref	-	-
	rest	110	1.839	1.105-3.063	**0.019**	1.582	0.946-2.645	0.080
	>,>	42	3.310	1.882-5.821	**0.000**	2.638	1.486-4.683	**0.001**

CI = confidence interval; F-M = female-male; HR = hazard ratio; MMP = matrix metalloproteinase; Ref = reference group. [a]In the multivariate analysis for the MMP-2/MMP-9 combination, the separate variables MMP-2 and MMP-9 protein were not included. <,< = MMP-2<8.7 ng mg $^{-1}$ protein and MMP-9<1.6 ng mg $^{-1}$ protein; >,> = MMP-2>8.7 ng mg $^{-1}$ protein and MMP-9>1.6 ng mg $^{-1}$ protein. Multivariate analysis was performed by adding every single MMP-related parameter to the dichotomised, prognosis-associated clinico-pathological parameters gender, age and TNM stage. Statistically significant values are given bold.

DISCUSSION

In the present study we found high protein levels of MMP-2 and MMP-9 in mucosa adjacent to colorectal cancer tissue to be indicative for the course of disease, that is, independently associated with a worse survival of the patients. Mucosa *immediately* adjacent to colon cancer (within 2 cm) shares histochemical, ultrastructural and biological features with the corresponding tumour and is referred to as transitional mucosa[10]. But even

at longer distances from the tumour enhanced calcium levels and increased numbers of aberrant crypts are detected, indicating differences with mucosa from healthy controls[11, 12]. Some of these changes are clearly related to changes in the endothelium. 'Normal' mucosa adjacent to larger, more invasive tumours showed enhanced microvessel densities compared with less invasive tumours, which was associated with increased levels of the angiogenic factors vascular endothelial growth factor (VEGF), interleukin-8, and factor VIII-related antigen[13]. These differences in the normal mucosa were not related to the survival of the patients. We have previously shown that low levels of the endothelium-derived serine proteinase tissue-type plasminogen activator (tPA) in normal appearing mucosa of colorectal and gastric cancer patients is also associated with poor prognosis[14, 15]. The correlation of high protein levels of MMP-2 and MMP-9 in mucosa adjacent to colorectal cancer tissue with worse survival, as we found, is probably also more, but not exclusively, related to endothelial cells in combination with epithelial cells and/or leukocytes. Previously we showed that MMP-2 in the intestinal mucosa of colorectal cancer patients is predominantly expressed in the submucosal extracellular matrix and MMP-9 in the mucosal macrophages and neutrophils[5]. We hypothesised previously that the poor prognosis found for patients with very low tumour MMP-9 levels is possibly due to the lack of infiltrating leukocytes, which may possess anti-cancer effects[2]. Diffuse staining of a subset of microvessels in tumour-adjacent mucosa of the oesophagus by CD105/endoglin, a neovascularisation marker, has been found to correlate statistically with worse overall survival of the patients[16]. Interestingly, we previously observed that MMP-9 is involved in the VEGF-mediated neoangiogenesis in colorectal cancer and that MMP-mediated endoglin mobilisation is involved in the regulation of the angiogenic potential of endothelial cells in colorectal cancer[17, 18]. Angiogenesis in the adjacent mucosa might very well enhance the development of multi-focal tumours or regional metastasis, which could explain the correlation with survival.

We have previously shown that there is no association between SNPs of MMP-2 and MMP-9 and the amount of corresponding protein in colorectal carcinomas[2]. But in a tumour environment the final concentration of MMP-2 and -9 would depend on many different cell types, mechanisms and interactions. Therefore, one could speculate that an association between the SNP and the amount of MMP protein in normal mucosa would be more likely. T allele carriers of the MMP-2$_{-1306C>T}$ polymorphism have lower promoter activity due to disruption of an Sp-1-binding site[19], while T allele carriers of the MMP-9$_{-1562C>T}$ polymorphism have increased transcriptional activity due to preferential binding of a transcription suppressor protein to the C allele[20]. Although normal mucosa MMP-2 levels correlated significantly with their corresponding tumour levels, this was not restricted to or within a specific genotype. The tissue levels of MMP-9, either from normal mucosa or tumour did not correlate and were also independent from the genotypic background. In a recent review about SNPs of MMPs and gastrointestinal cancer we already emphasised

that due to the complicated regulatory processes after transcription, SNPs are not very strong effectors of overall synthesis/presence of the corresponding MMP *in vivo*[21].

In conclusion, this study shows that increased MMP-2 and MMP-9 protein expression in normal mucosa at some distance of colorectal tumours is strongly related to the course of disease, that is, independently associated with a poor prognosis of colorectal cancer patients. The difference in mucosal expression of the gelatinases cannot be attributed to genotypic variations.

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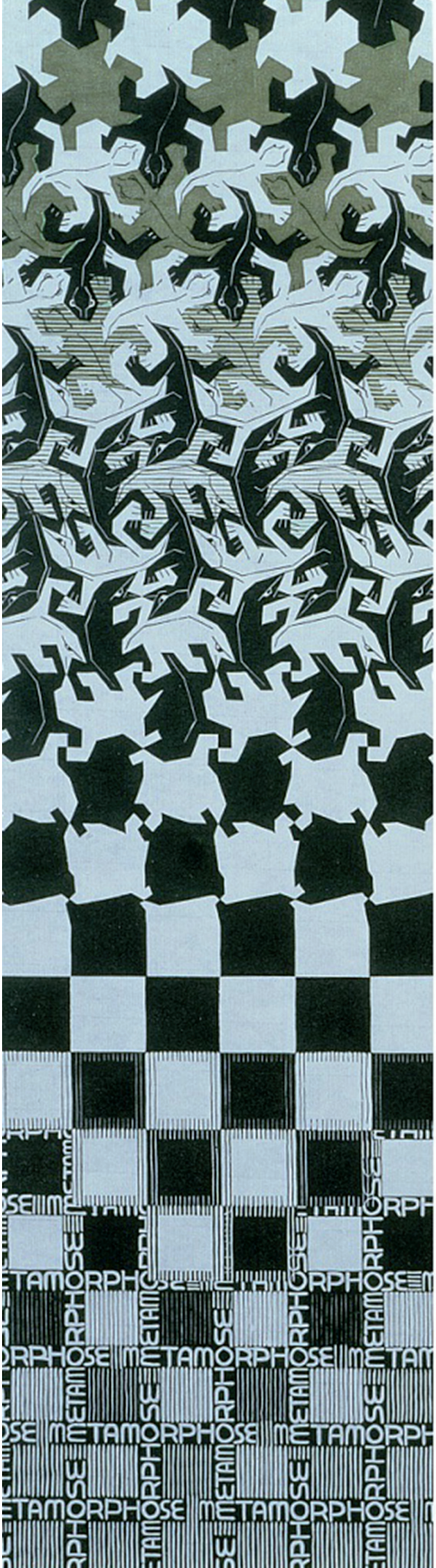

CHAPTER 7

Functional and clinical impact of matrilysin in human colorectal carcinogenesis; level, activity and gene polymorphisms in the adenoma-carcinoma sequence

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ABSTRACT

Background and aim of the study: Matrilysin (or MMP-7) is a metalloproteinase (MMP) abundantly produced by (pre)malignant epithelial cells and associated with invasion and metastasis. The aim of the present study was to investigate whether the levels of total and active matrilysin in the adenoma-carcinoma sequence were altered and to study the relationship between the MMP-7 genotype, matrilysin level and activity with the outcome of colorectal cancer patients.

Methods: The tissue level and activity state of matrilysin was evaluated in 58 colorectal adenomas and in normal mucosa and tumour tissue from 174 patients with colorectal cancer. Matrilysin protein levels and the MMP-7 SNPs -153 C/T and -181 A/G were determined, respectively, by a specific activity assay detecting total and active matrilysin and RFLP-PCR-based techniques.

Results: Total matrilysin level was found to increase gradually in the normal tissue – adenoma – carcinoma sequence, and in the adenomas in association with dysplasia. Matrilysin activity, however, was only enhanced in the carcinomas. SNP -153 C/T correlated with active matrilysin in cancer tissue, but not with the total amount of MMP-7. Matrilysin activity and the SNP -153 C/T were found to be independently associated with the overall survival of the cancer patients.

Conclusion: Matrilysin up-regulation was associated with dysplasia in adenomas and therefore seems to be an early event in colorectal carcinogenesis. Enhanced MMP-7 activity, however, was only present in the more advanced stages of colorectal cancer development. A high level of either total or active MMP-7 in the carcinoma is associated with worse 10-year survival, but only active MMP-7 was independently associated with the outcome of the patients. MMP-7 SNP -153 C/T was not accompanied by enhanced tumour matrilysin levels, but was associated with a higher matrilysin activity and a worse patient's survival.

INTRODUCTION

Matrix metalloproteinases (MMPs) consist of a family of proteinases implicated in (patho)physiological processes like cell migration and neo-angiogenesis. The smallest family member, MMP-7 or matrilysin, is considered the most promising candidate for cancer-associated diagnostic purposes, because of a number of specific properties: 1] Matrilysin is able to degrade a variety of tumour-associated extracellular matrix components, like elastin, collagen type IV, fibronectin, vitronectin, aggrecan and proteoglycans[1]. 2] Through active cleavage and via activation of other matrix metalloproteinases, matrilysin is involved in the shedding of a range of trans-membrane proteins like E-cadherin, FasL and TNF-alpha precursor[1]. 3] Unlike other MMPs, matrilysin is mainly produced by epithelial cells, implicating an active role of matrilysin at the level of the invading malignant cells[2]. Matrilysin over-expression is associated with mutational activation of the Apc/Wnt signalling pathway and is therefore considered an early event in carcinogenesis[3, 4]. The transformation from normal colon mucosa to cancer is a multi-step process, where consequent mutations in oncogenes and tumour suppressor genes lead to the development of aberrant crypt foci, adenomatous polyps, carcinoma and eventually colorectal metastasis[5]. Enhanced levels of matrilysin have previously been found in adenomas as well as carcinomas, mainly in immunohistochemical studies[6-9]. Furthermore, matrilysin has been associated with a poor prognosis of cancer patients[6, 7, 10-12].

In the present study we assessed the association of matrilysin with the multi-step model for colorectal cancer by including adenomas and by measuring not only the total level of matrilysin, but focusing especially on the activity state of matrilysin in the successive stages. Furthermore, the association of two promoter-located gene polymorphisms (MMP-7 SNPs) with matrilysin tissue levels (total/active) was determined. The MMP-7 SNP variants and carcinoma matrilysin levels were also evaluated for their prognostic value for the overall survival of the patients.

PATIENTS AND METHODS

Patients

Tissue was collected from 174 patients with colorectal cancer, operated in the Leiden University Medical Center between December 1983 and September 1991. Samples from the non-necrotic mid-central part of the tumour and normal mucosa at a distance of 5 to 10 cm were obtained from the surgical specimen immediately after resection. In the same period, tissue samples from 58 endoscopically removed colonic adenomas were collected. In these adenomas, the degree of dysplasia was classified as mild in 19, moderate in 23, severe in 10 and unknown in 6 adenomas. All tissues were frozen and stored at -70°C until

use. Clinical data and follow-up was available for a period of at least 10 years. Macroscopic and microscopic data were obtained from pathology reports, including localization, diameter, histological type, grade of dysplasia and differentiation according to the WHO classification, and classification of the carcinomas according to the TNM classification. All human samples were collected according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Center and in accordance with the Helsinki declaration.

Tissue preparation and protein concentration

Homogenates were prepared by adding 1 ml of ice-cold 0.1 M Tris-HCL (pH 7.5) with 0.1 % (vol/vol) Tween 80 extraction buffer per 60 mg tissue sample. The tissues were homogenized on ice for two minutes in a Potter S (B Braun). Homogenates were centrifuged twice at 8000 x *g* for 2.5 minutes at 4°C and the supernatants were stored at -70°C (15). The protein concentrations in the homogenates were determined according to the method of Lowry *et al*[13].

Matrix metalloproteinase-7 activity assay

An immunocapture bioactivity assay (BIA) for MMP-7 was performed as recently described by Hawinkels[14]. Briefly, MaxiSorp 96-well plates (Nunc) were coated with matrilysin-specific antibodies for 2 hours at 37°C, blocked and washed (3x) with PBS/0.05% Tween-20. Appropriately diluted samples or human recombinant matrilysin (R&D Systems Europe, Abingdon, UK) were incubated overnight at 4°C in assay buffer. After washing (4x), samples were incubated with 1mM APMA or assay buffer for determination of total or endogenously active MMP-7 levels, respectively. MMP-7 activity was detected using modified MMP-sensitive pro-urokinase in combination with peptide substrate S-2444. Colour development was detected by kinetic OD_{450} readings during 7 hours. MMP-7 concentrations were calculated from the standard curves and expressed in ng MMP-7/mg protein.

MMP-7 SNP analysis

Tissue DNA was isolated using the salting out method[15]. Analyses for matrilysin SNP -181 A/G and -153 C/T were performed by Restriction Fragment Length Polymorphism – Polymerase Chain Reaction as described earlier[16, 17]. For SNP -181A/G the following primers were used: forward primer TGGTACCATAATGTCCTGAATG, reverse primer TCGTTATTGGCAGGAAGCACACAATGAATT. The primers for SNP -153 C/T were: forward primer ACGAATACATTGTGTGCTTCCTGCCAATCA and reverse primer TTTATATAGCTTCTCAGCCTCG (reverse). Deliberate mismatches in primers are underlined. PCR conditions were: annealing at 55°C, cycle number 35, BP 150, Restriction enzyme *Eco*RI for MMP-7 -181 A/G and annealing at 55°C, cycle number 30, BP 158, Restriction enzyme *Nla*III for MMP-7 -153 C/T.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 Statistical Package (SPSS Inc., Chicago, IL, USA). Hardy-Weinberg analysis was performed using chi-square (χ^2) or Fisher's exact test to examine differences in the distribution of alleles and genotypes. Differences between groups and group means were calculated using the chi-square (χ^2) or Fisher's exact test, Mann-Whitney U-test or Wilcoxon's signed rank test for related samples (N/T pairs). Correlations between parameters were calculated with Pearson's rank test (Rho). Uni- and multivariate analyses were performed according to the Cox proportional hazard model.

RESULTS

Patient characteristics

The clinicopathological characteristics of the patients are presented in table 1, also showing the correlation of these parameters with the 10-year overall survival. The majority of the tumours were of TNM stage 2 or 3. TNM stage and older age were significantly associated with survival, whereas tumour localization, size, differentiation grade and histology were not.

Table 1. Clinicopathological variables from 174 patients with colorectal cancer and their relation with the 10-year overall survival.

Variable	Category	n	%	% 10-year survival	*P*-value
Gender	Female	71	40.8	33.8	
	Male	103	59.2	29.1	0.508
Age	<65 yrs	52	29.9	49.1	
	≥65 yrs	122	70.1	23.1	**0.002**
TNM classification	1	31	17.8	64.5	
	2	71	40.8	32.4	
	3	47	27.4	21.3	
	4	25	14.4	0	**0.000**
Tumour localization	Right	62	35.6	29.0	
	Left	71	40.8	26.4	
	Rectum	41	23.6	40.0	0.137
Tumour diameter	<5 cm	75	43	38.7	
	≥5 cm	99	57	25.3	0.202
Differentiation	Good	31	18.0	38.7	
	Moderate	116	67.4	28.4	
	Poor	25	14.5	36.0	0.542
Mucinous	No	127	73.8	27.6	
	Yes	45	26.2	37.8	0.423

* data for 2 patients not available. Statistical significant *P*-values are bold.

Matrilysin protein and activity levels

Matrilysin total protein level and activity in colorectal carcinomas and their corresponding normal mucosa and in adenomas is shown in figure 1. The total group of carcinomas contained significantly more matrilysin protein (Figure 1A) and activity (Figure 1B) than normal mucosa (medians respectively 3.37 versus 1.19 and 1.59 versus 0.59 ng/mg protein, n=174, both $P \leq 0.001$). The adenomas (n=58) contained significantly more matrilysin than normal mucosa (2.97 ng/mg, $P \leq 0.001$) but similar levels to the carcinomas ($P = 0.161$), whereas the matrilysin activity level of the adenomas (0.35 ng/mg, n=58) was similar to that in the normal mucosa but significantly lower compared to the carcinomas ($P \leq 0.001$). In the adenomas, a trend towards higher levels of both total and active matrilysin in adenomas with moderate or severe dysplasia compared to the adenomas with mild dysplasia was observed (Figure 1C). The correlation between total versus active matrilysin in all tissue samples was high (Rho = 0.609, $P < 0.001$, n=414).

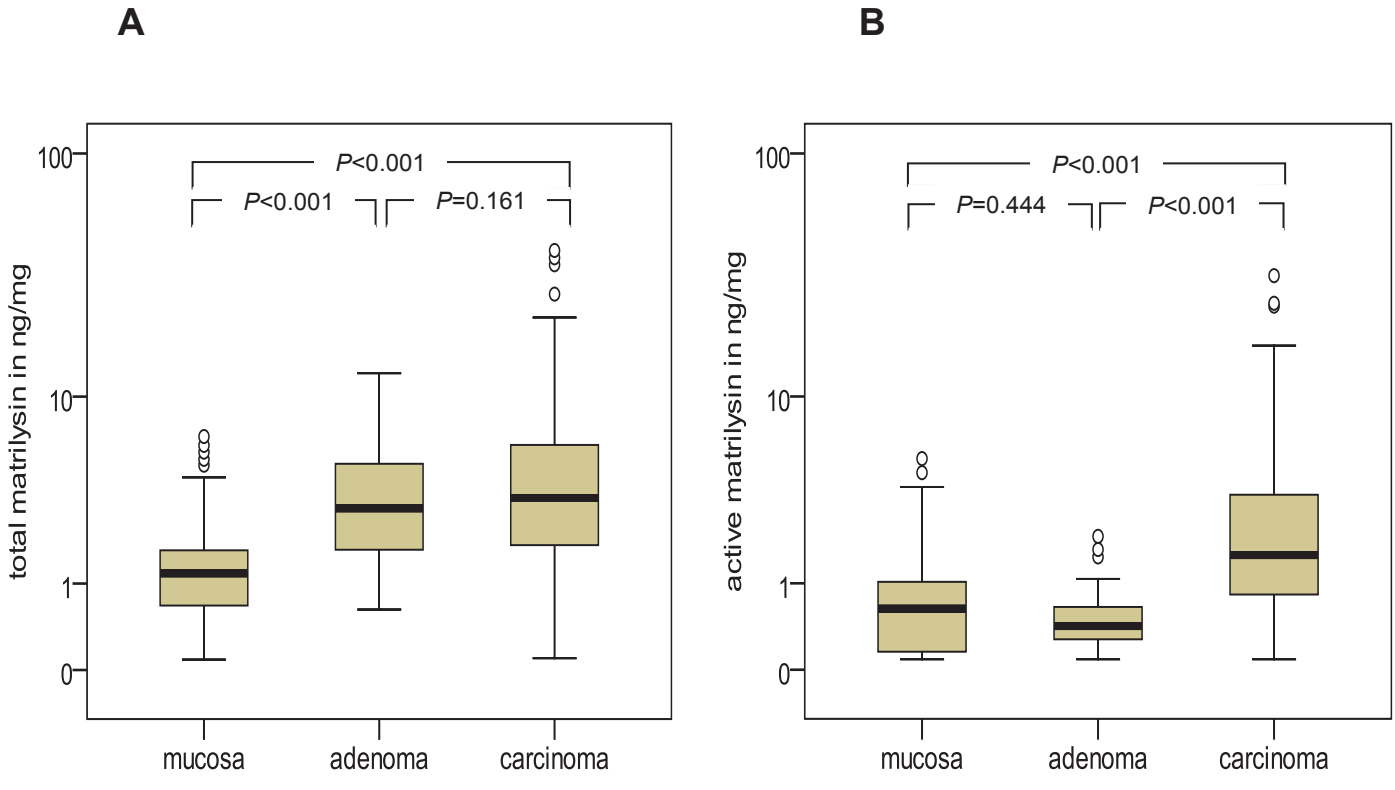

Figure 1. Matrilysin levels in normal appearing mucosa at 5 to 10 cm from the carcinomas, adenomatous polyps and colorectal carcinomas. A) total matrilysin in ng/mg protein and B) active matrilysin in ng/mg protein. The box-plots represent median, quartiles and extreme values (o). Significant differences between groups are indicated.

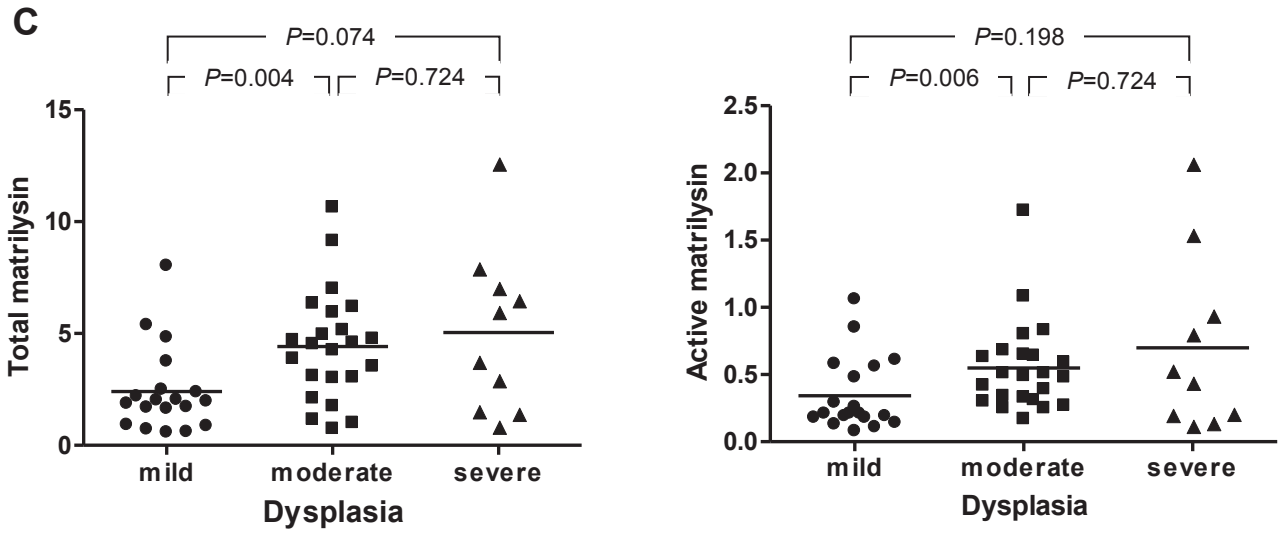

Figure 1C) total (left panel) and active (right panel) matrilysin levels in colorectal adenomas, subdivided by grade of dysplasia. Significant differences between groups are indicated.

Single-nucleotide polymorphisms for matrilysin

The genotype distributions of the promoter-located SNPs MMP-7 -181 A/G (AA: n=54, AG: n=85 and GG: n=35) and MMP-7 -153 C/T (CC: n=159, CT: n=14 and T: n=1) were both according to the Hardy-Weinberg prediction. There was no relation between these matrilysin SNPs and the TNM stage, diameter, localization and differentiation grade of the tumour (data not shown). Evaluation of both matrilysin genotypes with the total and active protein levels in homogenates from the corresponding cancer tissue and adjacent normal mucosa revealed a weak but statistically significant correlation between SNP -153 C/T and matrilysin activity in cancer tissue (χ^2 = 7.0, P = 0.03). Because the MMP-7 -153T/-181G haplotype was reported to be associated with increased MMP-7 expression *in vitro*, we analyzed the MMP-7 levels in this group of patients (n=15) compared to the rest of the patients (n=159), but did not find any difference in MMP-7 expression in the colorectal carcinomas or corresponding normal mucosa between the two groups (data not shown).

Survival analyses

Figure 2A shows that SNP -153 C/T was significantly associated with 10-year survival: better survival of patients with the CC genotype compared to those with a CT or TT genotype (LR 14.0, P < 0.001), and that the SNP -181 A/G was not related to survival in these patients (Figure 2B).

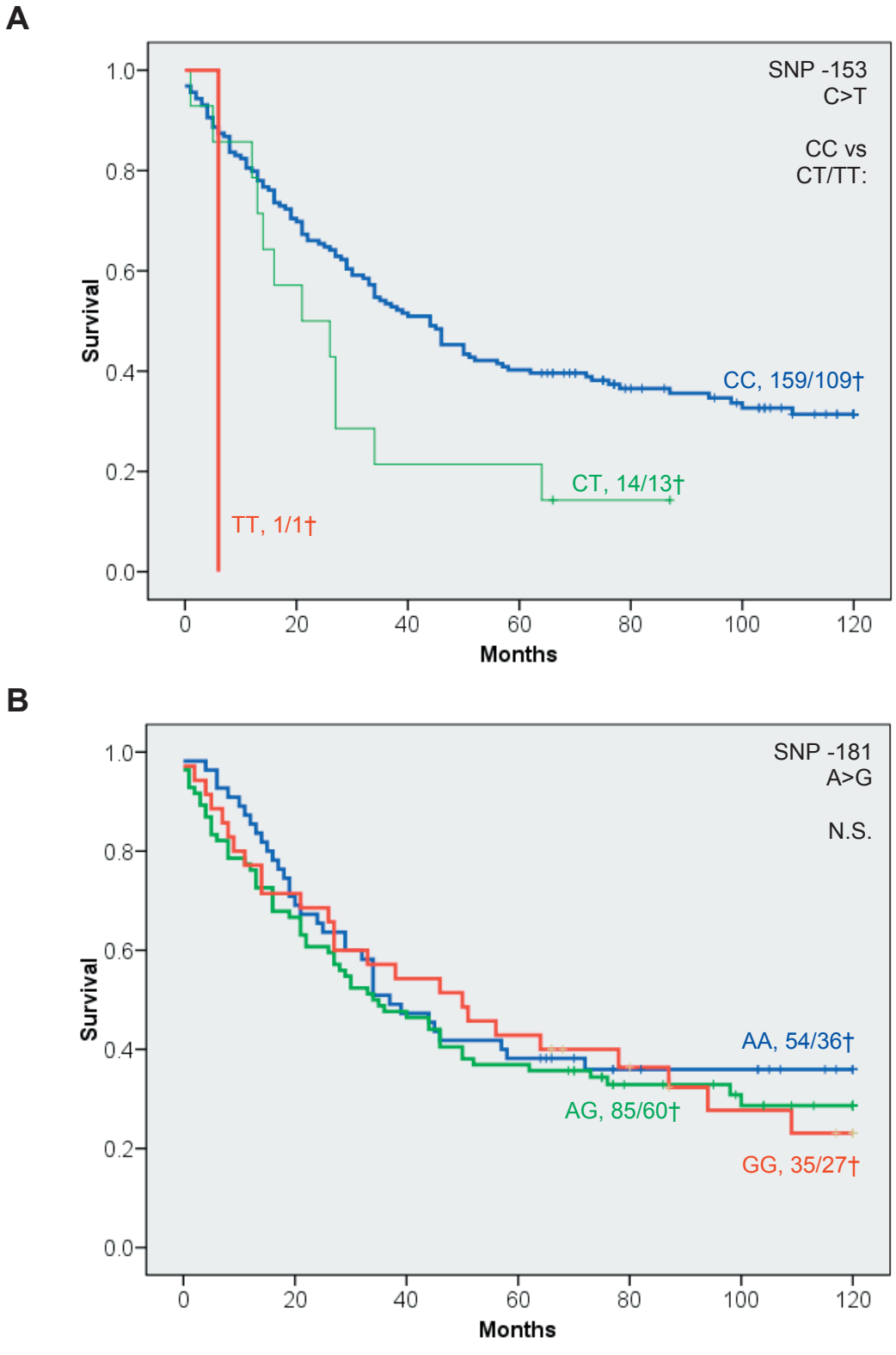

Figure 2. Kaplan-Meier 10-year overall survival curves of 174 colorectal cancer patients subdivided by A) MMP-7 SNP -153 C/T and B) MMP-7 SNP -181 A/G.

Optimized cut off stratification, dividing the patients in 2 groups, revealed a carcinoma cut-off point of 2.25 ng/mg for total matrilysin (Figure 3A). The Kaplan-Meier curve shows that patients with a low total matrilysin level had a significantly better survival than patients with higher levels (LR 9.23, *P* = 0.002). The same procedure for active matrilysin revealed a cut-off point of 3.72 ng/mg (LR 7.73, *P* = 0.005, Figure 3B).

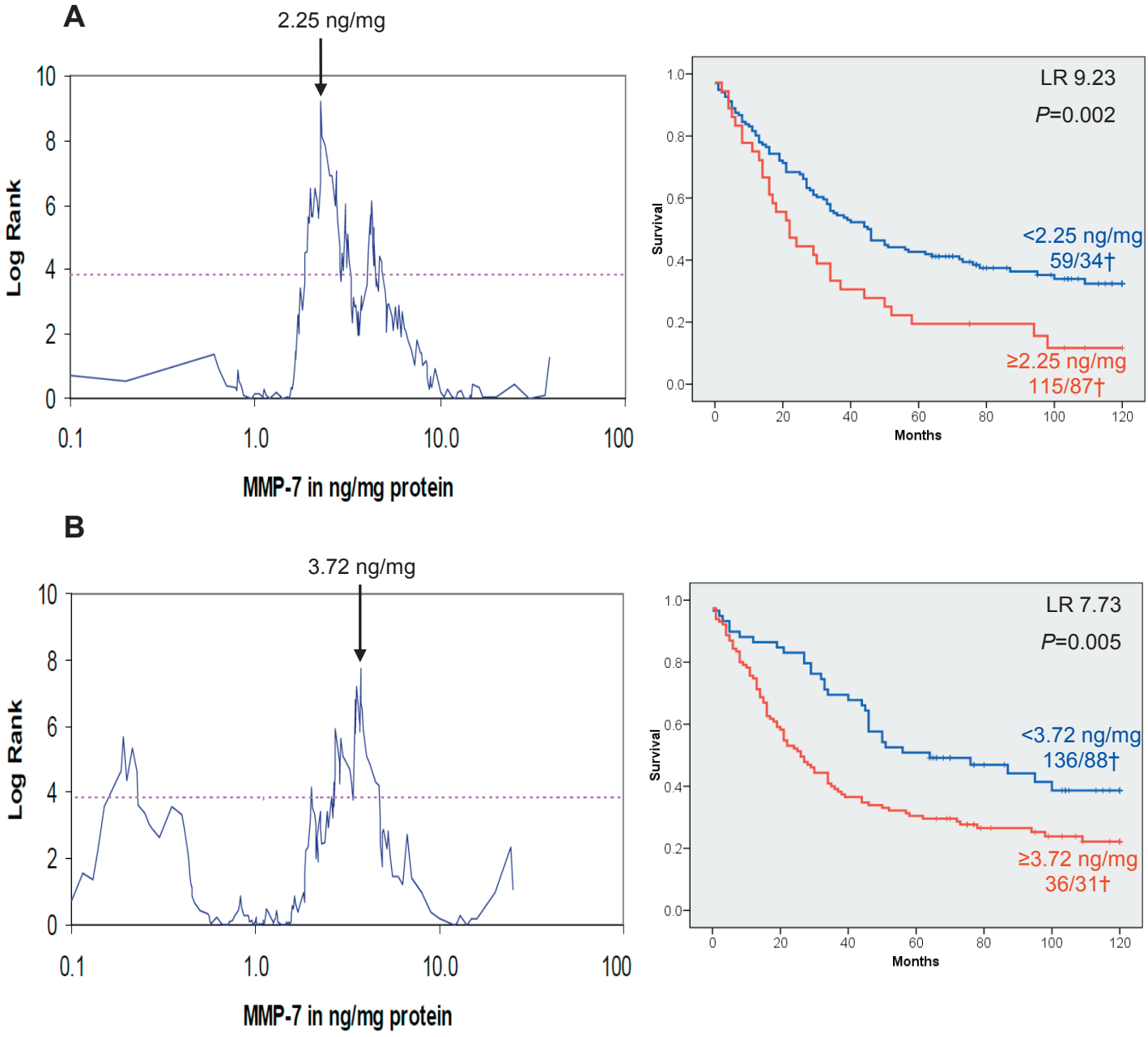

Figure 3. Optimal cut-off point analysis and corresponding Kaplan-Meier 10-year survival curves for dichotomized (high/low) levels of total matrilysin (A) and active matrilysin (B) in carcinoma. The optimal cut-off points are indicated with arrows. In the survival curves, the total number of patients and the number of deceased patients(†) are indicated per subgroup.

The matrilysin-derived variables were evaluated for their association with 10-year survival using univariate and multivariate Cox analyses (Table 2). In the univariate analysis, patients with a CT or TT genotype of the -153 C/T polymorphism, or those with high levels of matrilysin protein or matrilysin activity showed a significantly worse 10-year overall survival. From these parameters only matrilysin activity (*P* = 0.004) and the MMP-7 -153 C/T SNP (*P* = 0.05) proved to be independent prognostic markers in the multivariate analyses, next to TNM stage of the carcinomas and age of the patients, whereas the level of total MMP-7 just lost its prognostic significance (*P* = 0.051).

Table 2. Univariate and multivariate Cox's proportional hazard 10-year survival analyses of 174 colorectal cancer patients. Statistical significant *P*-values are bold, * indicates missing data.

			Univariate			Multivariate		
Parameter	Cat.	n	HR	CI 95	*P*	HR	CI 95	*P*
Gender	F *vs* M	71/103	1.131	0.784-1.631	0.512	1.133	0.779-1.649	0.513
Age	< 65 yr ≥	53/121	1.952	1.270-2.999	**0.002**	2.228	1.431-3.469	**0.000**
TNM	1,2 *vs* 3,4	102/72	2.620	1.817-3.777	**0.000**	2.763	1.880-4.060	**0.000**
Localization	Right *vs* rest	62/112	0.958	0.661-1.389	0.820	1.171	0.788-1.739	0.435
Diameter	< 5 cm ≥	75/99	1.268	0.877-1.832	0.207	1.097	0.741-1.622	0.644
Differentiation	Good *vs* rest	31/141*	1.305	0.798-2.133	0.298	1.063	0.639-1.767	0.815
Matrilysin SNPs								
SNP -153 C/T	CC *vs* CT,TT	159/15	2.487	1.412-4.381	**0.002**	1.798	1.000-3.232	**0.050**
SNP -181 A/G	AA *vs* AG,GG	54/120	1.258	0.845-1.871	0.258	1.263	0.841-1.896	0.261
Matrilysin proteins								
Total protein	<2.25≥	59/115	1.886	1.261-2.821	**0.002**	1.519	0.998-2.312	0.051
Activity	<3.72≥	136/36*	1.786	1.183-2.698	**0.006**	1.857	1.216-2.834	**0.004**

DISCUSSION

Tumour development from normal mucosa to colorectal cancer is a multistep process[5]. Our results show that matrilysin (or MMP-7), which is normally only present in small amounts in paneth cells of normal colonic mucosa, is already strongly up-regulated in colorectal adenomas. The total levels of matrilysin hardly increased further in more advanced stages of the adenoma-carcinoma sequence. Although the mechanism is not entirely cleared, early mutations in WNT/Apc, K-ras and E1AF are reported to be involved in matrilysin up-regulation[4, 18, 19]. Besides by mutations in regulating pathways of (pre)-malignant cells, the up-regulation of matrilysin could also originate from SNP variations in the promoter part of the MMP-7 gene. Although SNP -153 C/T was associated with patients' survival in this study, we could not establish a clear correlation with up-regulated levels of matrilysin, neither in the carcinomas nor in the normal mucosa. Also patients with the MMP-7 -153T/-181G haplotype, which has been shown to increase MMP-7 expression *in vitro*[16], did not have elevated MMP-7 levels compared with the rest of the patients. In contrast to the overall level, the activity of matrilysin was weakly but significantly correlated with MMP-7 SNP -153 C/T, which cannot easily be explained. Being genotypic variations, MMP-7 SNP derived up-regulation would not be restricted to (pre)malignant epithelial cells and therefore the activity could as well originate from matrilysin expressing stromal cells within the tumour in the individuals carrying the SNP variants. Other studies that reported association of MMP-7 SNPs with invasive properties of colon or other gastrointestinal cancers or with worse survival of the patients did not establish a clear relation with matrilysin levels either[20-25].

Adenomas greater than 1 cm in diameter and/or with villous components and/or with severe dysplasia are regarded as advanced adenomas, because they are at increased risk for transforming into an invasive carcinoma[26, 27]. An immunohistochemical study has demonstrated an increase in MMP-7 expression with a more advanced histology stage of colorectal adenomas, that is, tubular adenomas had the lowest level of MMP-7 expression, villous adenomas the highest and tubulovillous adenomas were in between the other two groups[7]. In the present study we have looked into the correlation between the degree of dysplasia (mild/moderate/severe) and levels of (total and active) matrilysin. We noticed a trend towards increased MMP-7 levels in the adenomas with more advanced (moderate, severe) grades of dysplasia compared to the adenomas with mild dysplasia. A similar trend was observed for MMP-7 activity, although the absolute levels of active MMP-7 were within the same range as in the normal mucosa.

The association between up-regulation of MMP-7 in the colorectal cancer sequence and poor outcome of colorectal cancer patients has been described before in immunohistochemical and tissue microarray studies[10, 28]. Whereas Koskensalo *et al.* observed a difference in survival between patients with high and low immunohistochemical

staining for MMP-7 after 5 but not after 10 years, the difference in survival in our cohort was still present 10 years after the surgical intervention for the cancer. The technique that was used in the present study, an MMP-7 activity assay, provides insight in the impact of MMP-7 activity during colorectal carcinogenesis and prognosis. We have demonstrated that not only the total amount of MMP-7 within a tumour, but also the activity state of MMP-7, is an important prognostic parameter. Others have demonstrated that MMP-7 is present in the circulation of colorectal cancer patients and that patients with higher serum MMP-7 levels are more likely to relapse compared to patients with lower levels[29, 30]. Even in patients with advanced colorectal cancer who have been treated with anti-EGFR therapy as a third line therapy, those patients who had the highest serum MMP-7 levels had the poorest overall and progression-free survival[31]. These findings suggest that MMP-7 might be used as a (serological) prognostic marker and may even serve as a target for therapy.

Our data clearly show that despite the overall enhanced protein levels of matrilysin in all stages of adenomas and carcinomas, the activity is only enhanced in carcinomas. Apparently, next to over-expression, also an up-regulated activation mechanism is essential for biological activity. This phenomenon has been shown before for other proteinases and tumour promoting growth factors[32-34]. Besides the fact that immunohistochemical staining of matrilysin is found mainly in the cytoplasm and therefore presumably represents inactive pro-matrilysin, activation of pro-matrilysin outside the cell is a complex mechanism depending on the presence of pro-matrilysin activators and MMP inhibitors such as TIMP-1 and TIMP-2[35]. Also local binding of released (pro-)matrilysin to the surface of the cells might play a substantial role. Soluble pro-matrilysin is susceptible to auto-cleavage, resulting in uncontrolled activation[35]. Docking of pro-matrilysin to CD151, a cell-surface trans-membrane protein present on epithelial cells[36], prevents auto-activation of MMP-7[37]. Down-regulation of CD151 expression by hypoxic conditions in colorectal cancer was reported to promote migration of malignant epithelial cells[38]. Hypoxia and the subsequent loss of CD151, in combination with the gain of other alternative sites for (pro)-matrilysin protection against TIMPs[39], may therefore be factors stimulating uncontrolled auto-activation around malignant cells. These observations might provide an explanation why high amounts of pro-matrilysin but concomitant low activity levels are present in adenomas and high levels of activated MMP-7 are present in the more hypoxic carcinomas. Further studies are needed to investigate whether this hypothesis is correct.

In conclusion, we found enhanced matrilysin levels in most neoplastic colorectal tissues. Especially the active form of MMP-7 correlated with more advanced stages of the disease. MMP-7 activity as well as the MMP-7 -153 C/T SNP were independently associated with 10-year survival of the carcinoma patients, whereas the total amount of MMP-7 just lost its prognostic significance. There was a weak correlation between matrilysin activity and the MMP-7 SNP -153 C/T genotype in the carcinomas. In conjunction with earlier mRNA studies reporting that matrilysin levels were increase with enhancing Dukes stage[40], related

to the presence of distant metastases[41], and were also measurable in the circulation[29, 30, 42-45] our data suggest a role for matrilysin as diagnostic or prognostic tool in colorectal cancer patients.

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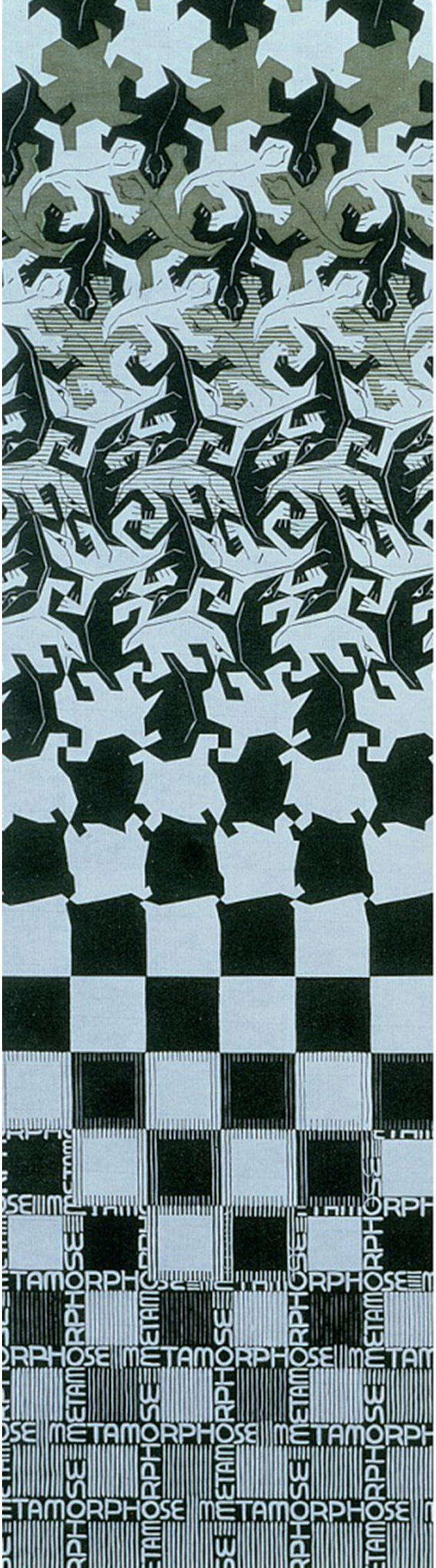

CHAPTER 8

Summarizing discussion

Colorectal cancer is the second leading cause of cancer death in the Netherlands and the fourth worldwide. Understanding the underlying mechanisms in cancer development and cancer progression is crucial for the development of more effective treatment strategies, especially in those patients who have an increased risk of developing cancer or who already developed either lymph node or distant metastasis. In this thesis, the focus is on the clinical impact of matrix metalloproteinases (MMPs) in the different stages of colorectal cancer development and metastasis.

Matrix metalloproteinases in the colorectal cancer sequence

To better understand the potential involvement of MMPs in the different phases of colorectal cancer development, the degree of expression of MMP-2, -8, -9 and neutrophil granulocyte-associated lipocalin (NGAL) was studied in the different phases of colorectal carcinogenesis. NGAL is a 25 kDA glycolylated protein that prevents inactivation of MMP-9 by the formation of heterodimers with the 92 kDa gelatinase and thereby preventing its degradation[1]. **Chapter 2** describes the results of enzyme-linked immunosorbent assay (ELISA) and bio-immunoassay (BIA) measurements of these proteins in tissues from various stages of colorectal cancer progression, that is, normal colorectal mucosa, adenomatous polyps, adenocarcinomas and liver metastases. Samples of normal liver tissue were included to serve as a standard for the liver metastases. MMP-8 (2-fold increase in adenoma, 8-fold increase in cancer), and NGAL (4-fold increase in adenoma, 8-fold increase in cancer) showed a significant stepwise up-regulation in the development from normal mucosa to adenoma and subsequent colorectal cancer development, a trend that was also observed for MMP-9. In carcinomas and liver metastases, these results were persistent after correction for the degree of inflammation, whereas in adenomas the increase of MMP-8, -9 and NGAL could be ascribed entirely to this increase in inflammation. MMP-2 up-regulation was observed only in the later phases of colorectal carcinogenesis, once cancer had developed. These data provided insight in the expression of MMPs throughout the process of colorectal cancer development, which needs to be taken into account when developing and choosing MMP-inhibitors as a therapy to intervene in this process, as will be discussed later in this chapter.

MMP-2 and MMP-9 as biomarkers for colorectal cancer

The follow-up of colorectal cancer patients has become more important now that more advanced treatment options are available. To be able to evaluate the possibility of the use of MMP-2 and MMP-9 as biomarkers, the presence of these MMPs in easily accessible biofluids like urine and plasma needs to be demonstrated. This had so far not been done for MMP-2 and MMP-9 in the urine of colorectal cancer patients. In a cohort of 49 patients who were operated because of colorectal cancer, MMP-2 and MMP-9 levels were measured in plasma, urine and in tissue and correlated with the clinical and surgical outcome (**chapter**

3). Different techniques (zymography, ELISA and BIA) were used to measure the MMPs in the different specimens. The presence of measurable amounts of MMP-2 and MMP-9 in the urine of colorectal cancer patients was demonstrated for the first time. Plasma and urine MMP-9 levels showed a peak value shortly after surgery, most probably reflecting the process of wound healing after major surgery. Especially urinary MMP-2 levels, when measured by zymography, were associated with a poor clinical and surgical outcome. High preoperative plasma MMP-9 levels were also predictive of poor clinical outcome, but this correlation only appeared to be modest.

Currently, carcinoembryonic antigen (CEA) is used as a serum marker in the follow-up of patients who have undergone surgery for colorectal cancer. The sensitivity for this marker to predict recurrent disease after curative resection varies from 58 to 89%[2, 3]. However, thirty to forty percent of colorectal cancers do not produce CEA and in those patients who consequently did not have preoperatively elevated CEA levels, the sensitivity of CEA for the detection of recurrent disease is only about 40%[2, 4]. The treatment options for recurrent and even metastatic colorectal cancer have increased over the last decades[5]. Because early detection of limited liver metastases creates the possibility of surgical metastasectomy and increases long-term disease-free survival, it would be useful to have disposition of an additional biomarker to predict recurrent disease. Circulating MMP-2 levels are unsuitable tools for CRC follow-up, as several studies have demonstrated that those levels are lower in colorectal cancer patients compared to healthy controls[6, 7]. Plasma MMP-9 levels have been shown to be increased in CRC patients compared to healthy individuals[8-14] and might therefore be more useful in the follow-up of these patients. However, in one study of 61 postoperative CRC patients in whom plasma MMP-9 levels were measured three-monthly over a two year follow-up period, MMP-9 levels were not able to predict recurrent disease.

As both MMP-2 and MMP-9 are detectable in the urine of colorectal cancer patients, whereas they are hardly detectable in the urine of healthy controls, urine levels of the gelatinases might be more useful as biomarkers in the follow-up. A problem that occurs in this context is the aspecificity of MMP-elevation in urine, as elevated urine levels of the gelatinases can also be found in a number of other malignant (e.g. bladder cancer, prostate cancer, brain tumours) as well as benign (e.g. kidney disease, endometriosis) diseases[15-21]. Still, in patients without significant comorbidity, it might be worth investigating whether urinary MMPs could be of additional value in the follow-up after surgery for colorectal cancer.

Single-nucleotide polymorphisms of matrix metalloproteinases in gastrointestinal cancer

The most common type of genetic variation is single-nucleotide polymorphism (SNP). The replacement of one single nucleotide by another can occur in either the coding or non-coding region of the gene. To be considered a SNP, the gene frequency of the minor allele needs to be at least 1%. SNPs can be associated with, for example, disease susceptibility

(i.e., a particular genotype is more or less prevalent in diseased than in healthy controls), the course of a disease or the response of an individual to drug treatment. One or more of the known SNPs of MMP-1, -2, -3, -7, -8, -9, -12, -13 and -21 have been studied in oesophageal cancer (both adenocarcinoma and squamous cell cancer), gastric cancer, hepatocellular carcinoma and colorectal cancer. **Chapter 4** gives an overview of these studies. Some of the SNPs in the MMP-genes are associated with cancer susceptibility, and in some cases there was a correlation with clinicopathological parameters and/or the outcome of disease in patients with gastrointestinal (GI) cancer. Probably due to differences in ethnicity, sample size or environmental factors, there was quite some heterogeneity between the results of the different studies that were included in this overview. The same diversity in results has been reported in lung and breast cancer[22]. Some of the most consistent results are the association between the -1306 C/T MMP-2 polymorphism and oesophageal cancer (increased cancer risk in CC carriers) and between the MMP-7 -181 A/G polymorphism and gastric as well as oesophageal cancer (increased cancer risk in G-allele carriers, in the Asian population only). Some studies report an association between colorectal cancer susceptibility and the MMP-1 -1607 1G/2G, MMP-2 -1306 C/T, MMP-7 -181 A/G and MMP-9 -1562 C/T polymorphism; however, in two large meta-analyses only the correlation between 2G allele carriers of the MMP-1 1G/2G polymorphism and an increased risk for development of colorectal cancer remained significant[23, 24]. Studies that actually looked at the correlation between SNPs and protein levels of MMPs are scarce and the few studies that did investigate this correlation yielded negative results[25, 26].

Almost all known functional SNPs of MMPs are located in the promoter region of the gene and have been demonstrated to influence gene expression *in vitro*. For example, C to T substitution at the -1306 position of the MMP-2 gene leads to disruption of a binding site for the Sp1 transcription factor and as a result a decreased promoter activity *in vitro*[27], whereas the activity of the MMP-9 promoter is increased in T-allele carriers of the -1562 C/T polymorphism as a result of decreased binding of a repression factor[28]. For MMP-7, two functional SNPs have been identified in the promoter of the gene, namely the -181 A/G and -153 C/T polymorphisms[29]. Nuclear proteins bind with higher affinity to the G-allele than to the A-allele at the -181 position of the MMP-7 gene. The T-allele at the -153 position binds additional nuclear proteins compared to the C-allele, but those proteins that bind to both alleles have a higher affinity for the C-allele than for the T-allele. The overall effect is a two- to three-fold increase in *in vitro* transcriptional activity in carriers of both minor alleles (-181G and-153T) compared to carriers of at least one of the major alleles (i.e. -181A and/or -153C carriers)[29]. The (cor)relation of the MMP-2, -7 and -9 polymorphisms with protein levels and outcome of colorectal cancer patients will be discussed in the next two paragraphs.

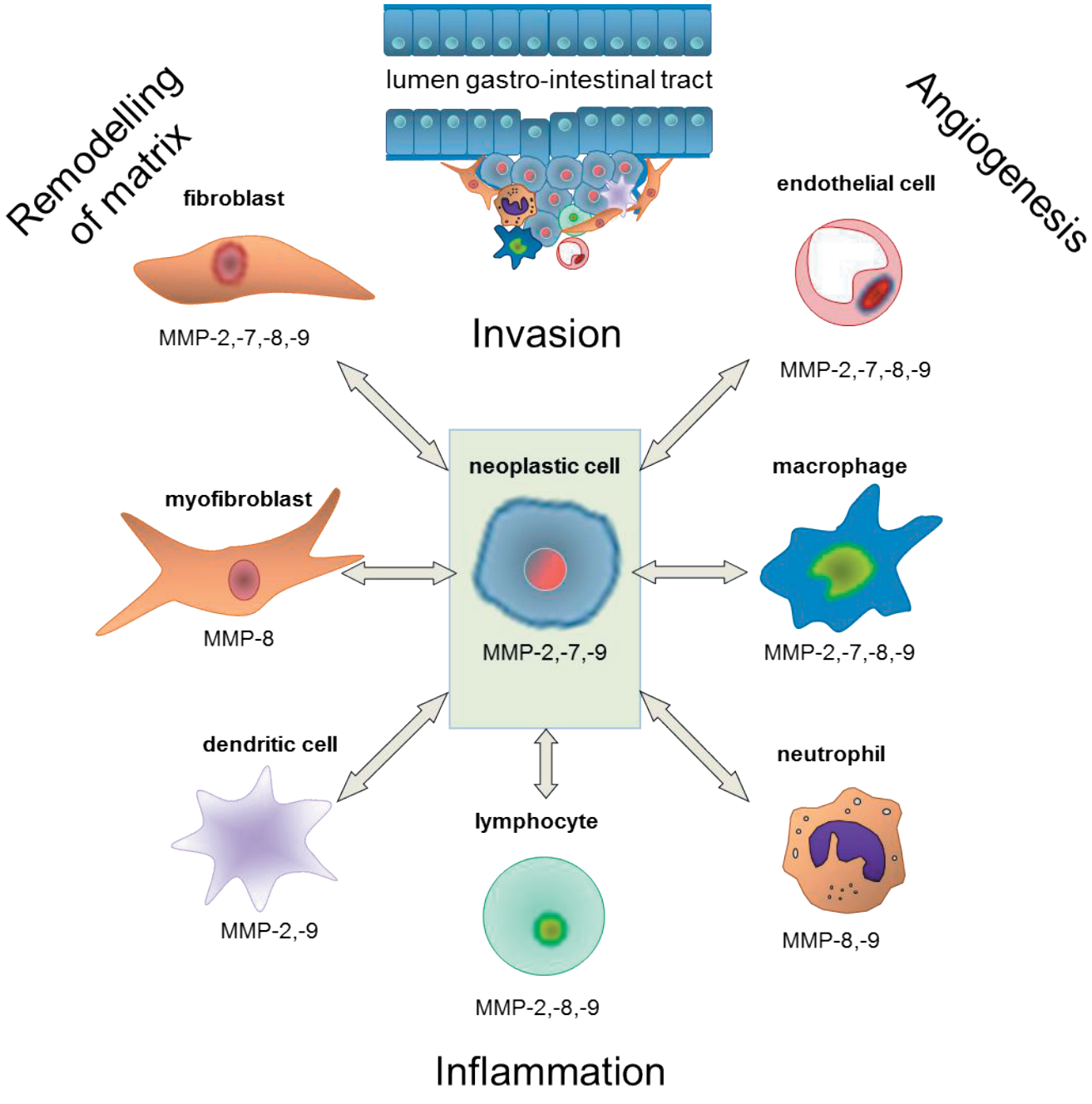

Figure 1. The matrix metalloproteinases MMP-2, -7, -8 and -9 that have been studied in this thesis are expressed by different cell types present in the tumour microenvironment. Adapted from Kessenbrock *et al* (2010)[30] and Langers *et al* (2011; chapter 4).

Gelatinases in colorectal cancer

Because of their involvement in angiogenesis, regulation of inflammation, metastatic niche formation, tissue invasion and cell growth[30], MMP-2 and MMP-9 have received particular interest in cancer research. Both gelatinases have indeed been shown to be implicated in the progression and metastasis of various solid tumours[31]. In the studies described in **chapter 5**, the questions were addressed whether functional SNPs of MMP-2 and MMP-9 and/or their protein levels in peroperatively obtained carcinoma tissue are associated with survival in a cohort of 215 colorectal cancer patients, and whether there is a correlation between the functional polymorphisms and tissue levels of MMP-2 and MMP-9. For the MMP-2 -1306 C/T and the MMP-9 -1562 C/T polymorphisms, no correlation between SNP and tumour level of MMP-2 or MMP-9 was found. The absence of this correlation could be explained by the fact that protein levels not only depend on the transcription levels of the MMP-gene, but also on producing cell type, activation and clearing of the protein. Some of the cell types expressing the metalloproteinases that have been studied in this thesis are shown in figure 1. Especially in a tumour microenvironment, many different cell types, mechanisms and interactions determine the final concentration of MMPs. Therefore, it would be more rational to look at the association between genotype and protein levels of MMPs in normal colorectal mucosa, which was actually done in the study described in **chapter 6**. In 198 of the 215 colorectal cancer patients described in chapter 5, tissue derived from normal colon at a distance of 5-10 centimetres from the tumour site was available for analysis. MMP-2 and MMP-9 levels in normal mucosa were determined by ELISA and compared to the different genotypes of MMP-2 (-1306 C/T polymorphism) and MMP-9 (-1562 C/T polymorphism). Also in normal mucosa no association between the protein levels of the gelatinases and their promoter genotypes was found. In spite of the lack of a correlation between the SNPs and protein levels, patients with a TT genotype of either the MMP-2 -1306 C/T polymorphism or the MMP-9 -1562 C/T polymorphism were found to have a more advanced TNM stage than C-allele carriers. Furthermore, a multivariate Cox's analysis demonstrated that the MMP-2 -1306 C/T SNP (but not the MMP-9 -1562 C/T SNP) was independently associated with survival of the patients.

At the protein level, there was a significant association between MMP-2 levels in carcinoma tissue and survival. Patients with a tumour MMP-2 level of higher than 18.5 ng/mg protein had a worse 10 year survival that those patients with MMP-2 levels below this cut-off point. Interestingly, patients with either very low (<11.2 ng/mg protein) or very high (>125 ng/mg protein) tumour levels of MMP-9 had a worse survival compared to those patients with intermediate tumour MMP-9 levels. These somewhat surprising results could be explained by putative pro- and anti-tumour effects of MMP-9, especially concerning its ambivalent role in angiogenesis. The pro-angiogenic effects of MMP-9 are well established, as is illustrated by the MMP-9 induced increase of Vascular Endothelial Growth Factor

(VEGF) and basic Fibroblast Growth Factor (FGF-2) bioavailability [32, 33]. On the other hand, MMP-9 generates anti-angiogenic factors like angiostatin, endostatin and tumstatin, which inhibit angiogenesis[34-37]. Furthermore, very low levels of MMP-9 within the tumour might reflect a lack of influx of MMP-9 generating cells (like neutrophilic leukocytes) into the tumour, which has been associated with decreased survival and a higher rate of local recurrence[38-42].

In normal mucosa, gelatinase expression is lower than in carcinoma tissue. This difference is more pronounced for MMP-9 (12-fold lower protein levels in normal mucosa compared to carcinomas) than for MMP-2 (2-fold lower levels). Survival of patients with low MMP-2 (<8.7 ng/mg) or low MMP-9 (<1.6 ng/mg) levels in normal mucosa was better than in patients with higher levels, and the combination of both mucosal gelatinases provided an even stronger instrument to classify patients as good, intermediate and poor survivors. These results were confirmed by a uni- and multivariate analysis, which identified both mucosal MMPs as independent prognostic variables and their combination as the strongest independent prognostic parameter. The fact that MMP-2 and MMP-9 in normal colorectal tissue corresponded even better with patient outcome than their levels in tumour tissue, is an interesting phenomenon that cannot easily be explained. Because the samples of normal tissue were obtained at a distance of 5-10 cm from the primary tumour, they should be regarded as distant mucosa and should therefore not display the changes that might be expected in mucosa immediately adjacent to the tumour[43]. However, also in mucosa not immediately adjacent to the tumour mucosal changes have been observed, like for example aberrant crypts and enhanced calcium levels[44, 45]. The strong correlation with higher gelatinase-levels and worse outcome suggests that this is either a response to the presence of a tumour elsewhere in the colon, or there might be some kind of “field defect” in which these changes in MMP profile occur even before cancer develops. To be able to better answer this question, it would be interesting to look at MMP-levels in normal colorectum of patients who are at an increased risk of CRC development or who have developed dysplasia in the context of an inflammatory bowel disease. Processes like neo-angiogenesis and breakdown of the basement membranes are limited to the site of malignancy and do not take place at distance of the tumour. Still, some microvascular changes have been observed in normal mucosa adjacent to large invasive colorectal tumours, like enhanced microvessel density associated with increased levels of vascular endothelial growth factor (VEGF) and interleukin 8[46]. The changes that were observed in the normal mucosa could reflect the creation of a tumour-friendly environment (even at distant sites from the tumour) that promotes local spread of the tumour and, once the tumour reaches this area, could facilitate dissemination and enhance the development of multifocal tumours, which could subsequently explain the worse prognosis in patients with high mucosal MMP-levels.

Matrilysin in colorectal cancer

Because of its ability to induce angiogenesis, increase cell survival, create resistance to apoptosis, degrade ECM components and to activate other MMPs, MMP-7 (or matrilysin) is probably one of the most important MMPs in colorectal carcinogenesis[47]. **Chapter 7** focuses on the genotype and phenotype of matrilysin in relation to outcome of CRC patients. Furthermore, MMP-7 expression and activity were examined throughout the colorectal carcinoma sequence. No correlation between either of the two promoter-located functional SNPs of MMP-7 (–181A/G and -153C/T) and protein levels of MMP-7 was found. However, there was a small but significant association between the -153 C/T SNP and MMP-7 activity, and this polymorphism also correlated with the survival of the patients. Matrilysin levels were increased in colorectal adenomas, especially in those with moderate or severe dysplasia, as well as in colorectal carcinomas. This suggests that MMP-7 is already involved in the early stages of colorectal carcinogenesis, which is in line with the finding that MMP-7 overexpression is associated with mutational activation of the APC/Wnt signalling pathway which is also involved in the early stages of colorectal cancer development.[48, 49]. Activation of MMP-7 was particularly seen in colorectal carcinomas and thus seems to take place later in the colorectal carcinogenesis process. High tumour levels of either total matrilysin or matrilysin activity were associated with poor 10-year survival, but only matrilysin activity proved to be an independent prognostic parameter. These data provide further insight in the implication of MMP-7 throughout the colorectal cancer sequence and suggest that it might be useful as a prognostic tool and maybe even as a target for therapy, as will be discussed in the next paragraph.

Therapeutical options for matrix metalloproteinase inhibitors

The fact that MMP expression is increased in various types of cancer[50], combined with the fact that increased MMP expression is often associated with poor outcome of these patients, provided the rationale for the use of MMP inhibitors as therapeutics in cancer[51]. In the $APC^{Min/+}$ mouse, which is a model of intestinal cancer development, treatment with broad spectrum MMP inhibitors led to a more than 50% decrease in polyp count[52, 53]. However, the results of MMP-inhibitors (MMPIs) in humans have been disappointing. One of the reasons for this lack of success is the occurrence of side effects (in particular musculoskeletal complaints)[51]. Furthermore, the first generation of MMPIs that have been used in patients were all broad spectrum MMPIs. The original concept that all MMPs promote tumour progression has proven to be incorrect and what makes things even more complicated is that one particular MMP can be harmful in one situation, but beneficial under different circumstances. Even in one particular disease, the role of MMPs can be positive or negative depending on the stage of progression, as has been shown for MMP-2 and MMP-9 in a mouse model of the Alport syndrome[54]. These observations illustrate that to design MMP inhibiting therapies, not only knowledge is required about the (either beneficial or

detrimental) role of a particular MMP in the progression of a certain tumour, but also the stage of disease should be taken into account when determining the optimal profile for MMP inhibition as anti-cancer therapy. Furthermore, concomitant therapy, like surgery, for colorectal cancer will be followed by a period of wound healing, a process in which MMPs are involved under physiological circumstances and where MMP inhibition may be harmful. Increased knowledge of the role of individual MMPs in the different stages of cancer development, combined with the development of antibodies against specific MMPs, might open the road to directed anti-MMP therapy in the future[55]. If MMPIs would be considered as chemoprotective agents to prevent adenoma formation and/or progression in individuals at high-risk for colorectal cancer development, the need to understand and overcome the side-effects becomes even more important.

Conclusions and future perspectives

The studies described in this thesis provide further evidence for the role of MMPs in colorectal cancer development and identifies MMPs (both at a SNP and protein level) as predictors of outcome in patients with colorectal carcinoma. MMP-2 and -9 levels in normal mucosa of CRC patients are an even stronger predictor of survival than their levels in tumour tissue. Whereas the MMP-2 -1306 C/T SNP, the MMP-7 -153 C/T SNP, high tumour MMP-2 and MMP-7 levels and tumour MMP-7 activity are associated with poor survival, MMP-9 shows a bidirectional association, with poor 10 year survival in patients with either very low of very high tumour levels of MMP-9. For the first time ever, measurable levels of MMP-2 and MMP-9 have been shown to be present in urine of colorectal cancer patients and, in case of MMP-2, also to correlate with clinical and surgical outcome of the patients. MMP-7, MMP-8 and probably also MMP-9 are involved in the early stages of carcinogenesis, that is, in colorectal adenomas, whereas MMP-2 levels and MMP-7 activity are only enhanced once cancer has developed.

The development of genome-wide association studies (GWAS) and whole genome sequencing allows to search a large number of genetic variations throughout the genome or even to sequence the entire genome of an individual. As these constantly improving technologies become available at lower costs, they will probably replace the individual SNP determinations, causing a shift from hypothesis-driven research towards a non-hypothesis based collection of a large number of genetic data. These data then need to be linked to information about disease susceptibility and/or disease outcome, with the intrinsic and problematic ethical implications, in order to be able to draw conclusions about the presence or absence of a genotype-phenotype association. So far, the GWAS studies did not locate any of the colorectal cancer susceptibility loci in one of the MMP genes[56]. However, the MMP-2 gene was identified as one of the CAN genes that are often mutated in colorectal cancer[57]. Whether the results of the whole genome sequencing will identify the MMP-genes as pivotal genes in colorectal cancer susceptibility and/or progression, remains to be seen.

Since the role of MMPs in cancer progression has been well established, this would not be very surprising.

Personalized medicine is evolving, and MMP inhibiting therapies could be part of such an individualized treatment approach, especially in those patients with elevated tissue levels of MMP-2, MMP-7 or other MMPs that have shown to have detrimental effects on tumour progression and/or are associated with a poor outcome. MMP-9 inhibition would be less obvious as a target for therapy, because of the negative effect on survival of very low tumour MMP-9 levels. The major drawbacks of MMP directed therapy have been described before, but with detailed knowledge of the role of the different MMPs in the various stages of cancer progression and the possibility to aim directly at targeted MMPs, could reopen the way to MMP-inhibiting therapy in colorectal cancer.

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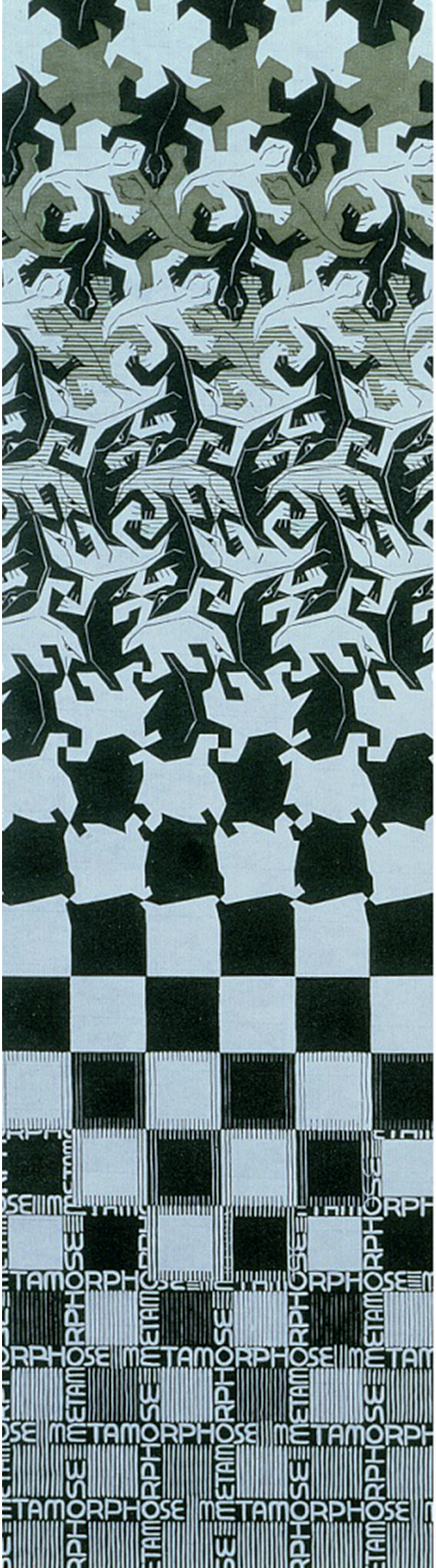
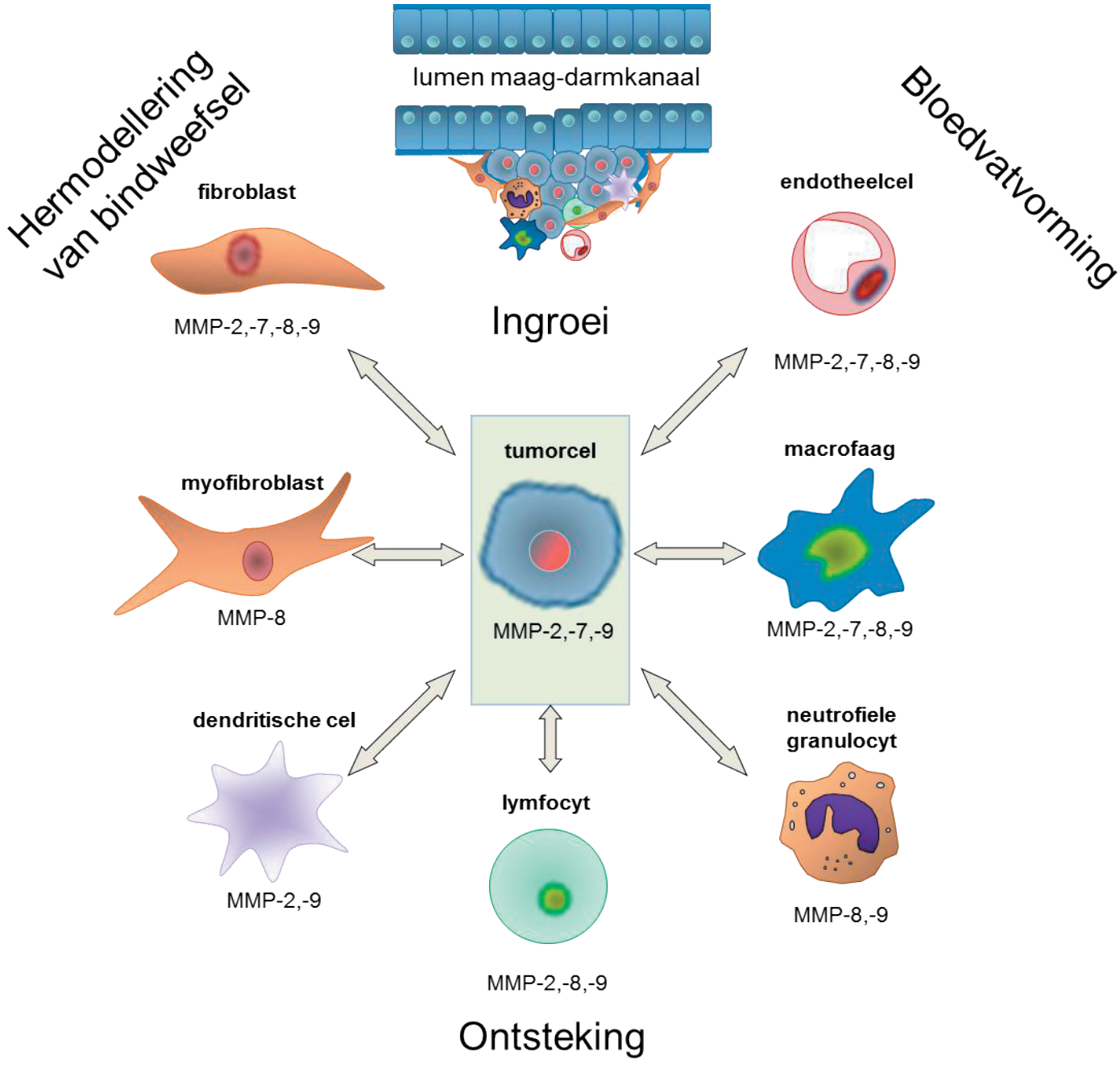


CHAPTER 9

Samenvatting

INTRODUCTIE

In Nederland wordt jaarlijks bij meer dan 12.000 personen de diagnose darmkanker gesteld. Onder darmkanker, ook wel colorectaal carcinoom (Engels: colorectal cancer, afgekort CRC) genoemd, wordt in de praktijk een kwaadaardige (maligne) tumor van de dikke darm en/of de endeldarm verstaan. Dikke darmkanker ontstaat bijna altijd uit een darmpoliep; dit is een goedaardige afwijking die, indien tijdig opgespoord, verwijderd kan worden door middel van een endoscopie. Bijna de helft van de darmkankerpatiënten zal uiteindelijk aan de ziekte overlijden, meestal ten gevolge van de aanwezigheid van uitzaaiingen (metastasen). Bij het ontstaan van uitzaaiingen worden de natuurlijke barrières, zoals de basaalmembraan van het darmslijmvlies, door de tumorcellen doorbroken. Dit leidt tot doorgroei in de diepere lagen van de darmwand (invasie), waardoor het enerzijds mogelijk wordt dat de tumorcellen doorgroeien in de lymfevaten en via het lymfevat-stelsel kunnen uitzaaien (lymfogene metastasering); anderzijds kan doorgroei tot in de bloedvaten leiden tot uitzaaiingen in andere organen (hematogene metastasering). Andere belangrijke factoren in het proces van metastasering zijn de vorming van bloedvaten (angiogenese) door en naar de tumor en het “voorbereiden” van de organen waarin de uitzaaiingen kunnen ontstaan door het creëren van een zogenaamde “metastatische niche”. Dit laatste houdt in dat er in het orgaan waarin de uitzaaiingen kunnen gaan optreden, meestal in de lever, veranderingen plaatsvinden die het mogelijk maken dat tumorcellen die zich via de bloedbaan hebben verplaatst, zich kunnen gaan nestelen en verder uitgroeien. Matrix metalloproteïnasen (MMPs) zijn eiwitsplitsende enzymen (proteasen) die een belangrijke rol spelen bij de progressie van tumoren. Zij zijn in staat verschillende componenten van de basaalmembraan af te breken, zodat de tumorcellen door de basaalmembraan heen kunnen breken en kunnen doorgroeien in de diepere lagen van de darmwand. Ook zijn zij van belang bij andere processen die meespelen in de ontwikkeling van kanker en vervolgens de metastasering, zoals later in dit hoofdstuk wordt beschreven. MMPs worden geproduceerd in een inactieve pro-vorm en moeten geactiveerd worden alvorens ze hun eiwitsplitsende werking kunnen uitoefenen. De MMPs die in dit proefschrift aan bod komen zijn MMP-2 en MMP-9, samen ook wel de gelatinases genoemd, en tevens MMP-7 en MMP-8. In figuur 1 wordt weergegeven door welke celtypen deze MMPs worden geproduceerd; tevens zijn enkele belangrijke processen aangegeven in de progressie van dikke darmkanker, waarbij de bestudeerde MMPs betrokken zijn.



Figuur 1. De matrix metalloproteïnasen die zijn bestudeerd in dit proefschrift, te weten MMP-2, MMP-7, MMP-8 en MMP-9, worden geproduceerd door verschillende celtypen die aanwezig zijn in het micromilieu van de tumor. Ze zijn betrokken bij diverse processen die te maken hebben met de ontwikkeling en progressie van darmkanker.

Rol van matrix metalloproteïnasen in het ontstaan van darmkanker

Darmkanker ontstaat in het merendeel van de gevallen uit een darmpoliep (adenoom). Via een stapsgewijs proces, waarbij er in elke opeenvolgende stap (van vroege poliep, naar verder gevorderde poliep, naar kanker en vervolgens uitzaaiingen) bepaalde genetische veranderingen optreden, ontwikkelt zich uiteindelijk kanker. Dit model voor darmkanker-ontwikkeling wordt ook wel het Vogelstein-model genoemd. De in dit proefschrift beschreven studies tonen aan dat sommige MMPs al vroeg in het proces betrokken zijn. Zo zijn MMP-7, MMP-8 en MMP-9 al in het poliepstadium in verhoogde mate aanwezig. Hetzelfde geldt voor neutrofiel gelatinase-geassocieerd lipocaline (NGAL). Lipocaline verhindert de afbraak van MMP-9 door de vorming van complexen met MMP-9. MMP-7 blijkt al in het poliep-stadium verhoogd te zijn; activatie van MMP-7 treedt echter pas later op, op het moment dat zich kanker heeft ontwikkeld. Toename van MMP-2 wordt pas gezien op het moment dat zich reeds darmkanker heeft ontwikkeld.

MMP-2 en MMP-9 in urine en plasma van patiënten met darmkanker

De behandelingsmogelijkheden voor darmkanker zijn de afgelopen decennia fors toegenomen. Belangrijkste behandeling blijft natuurlijk het operatief verwijderen van de darmtumor, waar ook nieuwe technieken zijn ingevoerd. Terwijl er in het verleden weinig tot geen opties waren voor patiënten bij wie zich uitzaaiingen hadden ontwikkeld, zijn er nu voor deze patiëntengroep diverse behandelopties zoals nieuwe vormen van chemotherapie, maar ook bijvoorbeeld resectie van de levermetastasen. De follow-up van darmkanker patiënten wordt hiermee steeds belangrijker, want hoe eerder je de uitzaaiingen opspoort, hoe meer opties er zijn voor behandeling. Technieken die toegepast kunnen worden om metastasen in een vroeg stadium op te sporen zijn de echografie, de CT scan en het bepalen van de CEA (carcino-embryonaal antigeen) waarde in het bloed. Een stijging van de CEA waarde na een operatie voor darmkanker kan duiden op terugkeer van de ziekte. Bij 40% van de tumoren is het CEA op het moment van stellen van de diagnose niet verhoogd. Bij deze 40% van de patiënten is de waarde van het vervolgen van CEA dan ook beperkt. In dit proefschrift staat beschreven dat MMP-2 en MMP-9 meetbaar blijken te zijn in de urine en in het plasma van patiënten met darmkanker. Indien deze waarden gemeten worden door middel van zymografie (een bepaling die MMPs kan aantonen op basis van hun enzymatische activiteit), blijken met name patiënten met hoge MMP-2 waarden in de urine een slechte prognose te hebben. Tot nu toe was nog niet eerder aangetoond dat MMP-2 en MMP-9 meetbaar zijn in de urine van patiënten met darmkanker. Deze bevinding, gecombineerd met het feit dat zowel MMP-2 als MMP-9 eveneens meetbaar zijn in het plasma van darmkankerpatiënten, maakt dat deze MMPs mogelijk gebruikt zouden kunnen worden in de follow-up na een operatie. Een probleem hierbij is wel dat beide waarden niet alleen specifiek bij darmkanker verhoogd zijn, maar ook bij een aantal andere aandoeningen.

Single-nucleotide polymorfismen (SNPs) van matrix metalloproteïnasen in kanker van het maagdarmkanaal

De meest voorkomende vorm van genetische variatie is het single-nucleotide polymorfisme (SNP), waarbij één enkel nucleotide van een gen vervangen wordt door een ander nucleotide. SNPs als genetische kenmerken kunnen geassocieerd zijn met de vatbaarheid voor bepaalde aandoeningen, maar ook met het beloop van een ziekte of de respons op therapie. Veel van de SNPs die zijn onderzocht bij maligniteiten van het maagdarmkanaal zijn gelokaliseerd in de zogenaamde promoter-regio van het gen, waar onder andere wordt bepaald in welke mate een gen tot expressie komt. Zij hebben daarom mogelijk effect op de translatie (vertaling) van het gen naar eiwit en dus ook op de hoeveelheid en soms ook de vorm waarin een bepaald eiwit geproduceerd wordt. Bij slokdarmkanker, maagkanker, darmkanker en levercelkanker is onderzocht in hoeverre er een associatie is tussen bepaalde SNPs en het ontstaan van de genoemde tumoren. Hierbij worden de meest consistente resultaten gevonden bij het -1306 C/T polymorfisme van MMP-2 en het -181 A/G polymorfisme van MMP-7, welke geassocieerd zijn met de kans op de ontwikkeling van slokdarmkanker (MMP-2) danwel slokdarm- en maagkanker (MMP-7). Hierbij dient opgemerkt te worden dat voor de SNPs in het MMP-7 gen de correlatie alleen aanwezig is in de Aziatische populatie. Met betrekking tot het ontstaan van darmkanker is de correlatie tussen de aanwezigheid van een dubbel 2G-allel op de -1607 positie van het MMP-1 gen en de verhoogde kans op het ontwikkelen van darmkanker het meest consistent. De associatie tussen SNPs in het MMP-2, MMP-7 en MMP-9 gen en darmkanker-risico wordt wisselend gerapporteerd. Soms zijn deze wisselende uitkomsten toe te schrijven aan een verschil in etniciteit van de onderzochte studiepopulatie.

Slechts een beperkt aantal studies heeft de relatie tussen SNPs en eiwitniveaus onderzocht. Van een aantal SNPs is bekend dat ze *in vitro* leiden tot een toegenomen transcriptie van het gen (d.w.z. omzetting van DNA naar messenger RNA), doordat er een toegenomen binding is van een transcriptiefactor. Dit is bijvoorbeeld het geval bij de MMP-7 -181A/G SNP. Het tegenovergestelde komt ook voor: de transcriptie-activiteit neemt dan juist af door verstoring van een bindingsplaats van een transcriptie-factor (zoals bijvoorbeeld bij de MMP-2 -1306 C/T SNP). De toe- of afgenomen activiteit van de transcriptie-factor *in vitro* laat zich echter lang niet altijd vertalen in toe- of afgenomen eiwitniveaus in de patiënten.

Gelatinases in darmkanker

MMP-2 en MMP-9 worden samen ook wel de gelatinases genoemd, vanwege het feit dat beide in staat zijn gelatine (gedenatureerd collageen) af te breken. Tevens zijn beiden in staat meerdere andere componenten van de basaalmembraan af te breken, zoals collageen type IV, elastine en laminine. Daarnaast zijn ze betrokken bij de activatie van groeifactoren, chemokinen en andere pro-MMPs. MMP-2 en MMP-9 spelen een rol in onder

andere angiogenese, regulatie van ontsteking en vorming van de metastatische niche. Hun betrokkenheid bij deze processen en het feit dat zowel MMP-2 als MMP-9 een rol speelt in de progressie van diverse tumoren was aanleiding voor het bestuderen van de relatie tussen de gelatinases en de progressie en prognose van dikke darmkanker, waarvan de resultaten zijn weergegeven in dit proefschrift. In een groep van 215 patiënten met dikke darmkanker is onderzocht of weefselniveaus van MMP-2 en MMP-9 correleren met de overleving. Tevens is onderzocht of de functionele SNPs van MMP-2 (-1306 C/T) en MMP-9 (-1562C/T) correleren met de desbetreffende eiwitniveaus. Dit laatste blijkt niet het geval te zijn.

Aangezien er zich in het micromilieu van een tumor diverse processen tegelijkertijd afspelen, wordt het uiteindelijke (activiteits-)niveau van MMPs in een tumoromgeving bepaald door meer factoren dan door de transcriptie alleen. Het ontbreken van een relatie tussen SNPs en eiwitniveaus in een tumor sluit daarom niet uit dat een dergelijke relatie wel degelijk aanwezig is in normaal darmweefsel. Dit is onderzocht door de eiwitniveaus van MMP-2 en MMP-9 te meten in normale colonmucosa (darmslijmvlies) en deze waarden te correleren met de SNPs. Ook in de normale mucosa blijkt er geen correlatie tussen eiwitniveau en gen-polymmorfisme te bestaan. Ondanks de afwezige correlatie tussen SNP en eiwitniveaus, blijken patiënten met een TT genotype van het MMP-2 -1306 C/T polymorfisme of het -1562 C/T polymorfisme een verder gevorderd tumorstadium te hebben dan dragers van het C-allel. Tevens blijkt de MMP-2 -1306 C/T SNP een onafhankelijke voorspeller te zijn van de overleving van de patiënten.

Patiënten met een laag MMP-2 eiwitniveau in het tumorweefsel hebben een significant betere 10-jaars overleving dan patiënten met een hoger eiwitniveau. Voor MMP-9 blijkt juist dat patiënten met ofwel een heel laag MMP-9 niveau in het tumorweefsel, ofwel een heel hoog niveau, een slechtere overleving hebben dan de groep patiënten die een gemiddeld MMP-9 eiwitniveau in de tumor heeft. Een mogelijke verklaring voor dit fenomeen is dat MMP-9 zowel pro- als anti-angiogenetische effecten heeft die potentieel afhangen van de concentratie waarin MMP-9 aanwezig is. Een andere verklaring kan worden gezocht in het feit dat zeer lage MMP-9 niveaus mogelijk veroorzaakt worden door een gebrek aan influx van (MMP-9 producerende) leukocyten in de tumor, hetgeen geassocieerd is met verminderde overleving en een hoger aantal lokale recidieven. Terwijl de eiwitexpressie van zowel MMP-2 als MMP-9 in normale mucosa van CRC-patiënten evident lager ligt dan in de tumoren, blijkt de correlatie tussen eiwitniveaus en overleving juist sterker aanwezig te zijn in de normale mucosa dan in de tumoren. In de normale mucosa is zowel voor MMP-2 als voor MMP-9 een afkappunt gevonden waarboven de patiënten een duidelijk slechtere prognose hebben dan de patiënten van wie de eiwitniveaus onder deze waarden liggen. De combinatie van de MMP-2 en MMP-9 waarden in normale mucosa is zelfs de sterkste voorspeller van de prognose van CRC-patiënten.

Matrilysine in darmkanker

MMP-7, ook wel matrilysine genoemd, is onder andere betrokken bij de vorming van nieuwe bloedvaten, de overleving van de cel, het minder gevoelig maken van de cel voor apoptose (geprogrammeerde celdood), de afbraak van componenten van de basaalmembraan en het activeren van andere proMMPs. De rol van MMP-7 in de ontwikkeling van darmkanker is onderzocht door MMP-7 spiegels en activiteit te meten in normale mucosa en tumoren van 174 darmkankerpatiënten en in 58 adenomen. Terwijl in adenomen reeds verhoogde MMP-7 spiegels worden gemeten in vergelijking met de normale mucosa, treedt activatie van MMP-7 pas op in de carcinomen. De MMP-7 activiteit in adenomen is gelijk aan de activiteit in de normale mucosa. Er is geen correlatie tussen een tweetal bekende functionele SNPs van MMP-7 (SNP -181A/G en SNP -153 C/T) en de MMP-7 eiwitniveaus (noch in de tumoren, noch in de normale mucosa). Wel correleert de -153 C/T SNP onafhankelijk met de 10-jaars overleving van de darmkankerpatiënten. Hoge MMP-7 spiegels en een hoge MMP-7 activiteit in de tumor correleren met een slechte 10-jaars overleving; de MMP-7 activiteit is hierbij een onafhankelijke voorspeller voor de 10-jaars prognose van darmkankerpatiënten.

Therapeutische opties voor matrix metalloproteïnase-remmers

Gebaseerd op de feiten dat MMPs betrokken zijn bij diverse processen die een rol spelen in de ontwikkeling van kanker én dat hoge MMP-spiegels meestal correleren met een slechte uitkomst, werd in het verleden het effect van diverse anti-MMP therapieën in studieverband onderzocht. De resultaten van deze studies waren helaas teleurstellend. Deels werden deze tegenvallende resultaten veroorzaakt door de ernstige bijwerkingen van de MMP-remmers, met name op het gebied van spier- en gewrichtsklachten, die het continueren van de therapie onmogelijk maakten. Een andere belangrijke verklaring ligt in het feit dat de meeste van de onderzochte MMP-remmers een breed spectrum aan MMPs remden. Terwijl er in het verleden vanuit werd gegaan dat alle MMPs het ziektebeloop negatief beïnvloeden, is inmiddels duidelijk is dat sommige MMPs (ook) anti-tumor effecten hebben en dat het remmen van deze MMPs juist niet wenselijk is. De meeste anti-MMP therapieën werden toegepast in vergevorderde stadia van de ziekte, terwijl niet alle MMPs in elk stadium van de ziekte evenzeer betrokken zijn. De studies waarvan de resultaten zijn terug te vinden in dit proefschrift dragen bij aan een toenemend inzicht in welke MMPs betrokken zijn in de diverse fasen van darmkankerontwikkeling en metastasering. Kennis hiervan is essentieel bij de ontwikkeling van nieuwe, specifiekere MMP-remmers en bij het bepalen in welk stadium van de ziekte deze nieuwe medicijnen ingezet zouden kunnen worden.

Conclusies en toekomstperspectief

De studies die zijn beschreven in dit proefschrift dragen bij aan een toenemende kennis van de rol van matrix metalloproteïnasen in de ontwikkeling van dikke darmkanker en tonen aan dat deze MMPs, zowel op genetisch (SNP) als op eiwitniveau, in belangrijke mate voorspellend zijn voor de uitkomst van patiënten met deze aandoening. MMP-2 en MMP-9 niveaus in de normale mucosa van dikke darmkankerpatiënten blijken zelfs een sterkere voorspeller van de overleving van deze patiënten te zijn dan MMP-2 en MMP-9 waarden in tumorweefsel. Terwijl de MMP-2 -1306 C/T SNP, de MMP-7 -153 C/T SNP, hoge tumor MMP-2 en MMP-7 spiegels en hoge tumor-MMP-7 activiteit geassocieerd zijn met een slechte overleving, toont het tumor-MMP-9 niveau geen eenduidige correlatie met de overleving en zijn zowel zeer hoge als zeer lage tumorniveaus van MMP-9 geassocieerd met een slechte 10-jaars overleving. Voor het eerst is aangetoond dat MMP-2 en MMP-9 in meetbare hoeveelheden aanwezig zijn in de urine van darmkankerpatiënten en dat er mogelijk een correlatie is tussen de zymografisch bepaalde urine-MMP-2 spiegels en de overleving van de patiënten. Pro-MMP-7, MMP-8 en waarschijnlijk ook MMP-9 zijn reeds betrokken in de vroege fases van de darmkanker-ontwikkeling, d.w.z. in het poliep-stadium, terwijl MMP-2 en MMP-7 activiteit pas in de latere stadia verhoogd zijn.

Met de komst van de “whole genome sequencing” wordt het mogelijk om in korte tijd het volledige menselijke genoom in kaart te brengen. De verwachting is dat deze nieuwe techniek de SNP-analyses op termijn zal vervangen. Op therapeutisch gebied is er een ontwikkeling gaande in de richting van geïndividualiseerde behandeling van patiënten (“personalized medicine”). Hierbij wordt er op basis van bepaalde tumor- en patiëntkenmerken de optimale therapie voor een patiënt bepaald. Gezien de betrokkenheid van de onderzochte MMPs in de ontwikkeling en progressie van dikke darmkanker, zouden MMPs in de toekomst mogelijk een rol kunnen spelen bij deze geïndividualiseerde therapie. Enerzijds kan deze rol bestaan uit het inschatten van de prognose van een patiënt en daarmee het bepalen van de noodzaak tot aanvullende behandeling; anderzijds kan gerichte anti-MMP therapie mogelijk als therapeuticum worden ingezet bij patiënten met darmkanker.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 17 november 1974 te Tilburg. In 1992 werd het Gymnasium-diploma behaald aan het Pauluslyceum te Tilburg en in datzelfde jaar werd gestart met de opleiding geneeskunde aan de Rijksuniversiteit te Leiden. In 1996 werd het doctoraalexamen behaald (cum laude). Tijdens het 3^{e} jaar van de studie werd een aanvang gemaakt met het verrichten van wetenschappelijk onderzoek op de afdeling nierziekten, wat uiteindelijk leidde tot een afstudeeronderzoek op het gebied van de tubulointerstitiële nierziekten onder leiding van prof. dr. L.A. van Es. In de periode 1996-1997 werkte zij gedurende 12 maanden als onderzoeker op het gebied van de nefrologie in Hôpital Tenon (INSERM Unité 64) in Parijs onder leiding van prof. dr. G.L. Striker en prof. dr. L.J. Striker. Hierna werd een aanvang gemaakt met de co-schappen, waarna in 1999 het artsexamen werd behaald. Tussen 2000 en 2002 vond het eerste deel van de opleiding tot internist plaats in het Rijnland ziekenhuis te Leiderdorp (opleider: dr. F.H.M. Cluitmans), gevolgd door vier jaar opleiding in het Leids Universitair Medisch Centrum (opleiders: prof. dr. A.E. Meinders en prof. dr. J.A. Romijn), leidend tot registratie als internist in 2006. Tijdens het laatste deel van deze opleiding werd tevens een begin gemaakt met de specialisatie tot maag-darm-leverarts in het Leids Universitair Medisch Centrum (opleiders: prof. dr. C.B.H.W. Lamers en prof. dr. D.W. Hommes) en registratie als maag-darm-leverarts volgde in september 2007. In de laatste fase van deze opleiding werd een aanvang gemaakt met onderzoek naar matrix metalloproteinasen bij het colorectaal carcinoom onder leiding van dr. ir. H.W. Verspaget en dr. C.F.M. Sier, waarvan de resultaten zijn beschreven in dit proefschrift. Sinds september 2007 is de auteur werkzaam als staflid op de afdeling maag-darm-leverziekten van het LUMC met als aandachtsgebied de gastrointestinale oncologie, in het bijzonder het (erfelijk) colorectaal carcinoom.

NAWOORD

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