Matrix metalloproteinases in gastric inflammation and cancer : clinical relevance and prognostic impact

Kubben, F.J.G.M.

CHAPTER 1

General introduction

Introduction

Helicobacter pylori infection of the stomach causes a chronic gastritis that is associated with the development of peptic ulcer disease and gastric cancer. Chronic inflammation and malignancy are diseases that are accompanied by excessive degradation of the extracellular matrix. Matrix metalloproteinases are zinc-dependent proteinases that are involved in these processes because of their capability of digesting various structural components of the extracellular matrix. The list of their known substrates has expanded over the years to a broad range of extracellular proteins including other proteinases, proteinase inhibitors, receptors, clotting factors, cytokines, growth factors and chemotactic molecules. Enhanced matrix metalloproteinase levels and activities have been described in *Helicobacter pylori*-induced gastritis and in gastric cancer. In this thesis several studies are described that assessed the putative role of matrix metalloproteinases in chronic *Helicobacter pylori*-induced gastritis and gastric cancer.

Extracellular proteolysis

Extracellular proteolysis plays an important role in cell-cell and cell-matrix interactions of physiological processes like mammary gland involution, ovulation, blastocyst implantation, cellular migration and angiogenesis, but also in pathological conditions like inflammation as well as invasion and metastasis of malignant tumours [1-8]. Whereas in physiological conditions this proteolysis is controlled and self-limiting, in inflammation and metastasis there appears to be an excessive or unbalanced production of proteolytic enzymes. These proteolytic enzymes play an important role in the remodelling and breakdown of the extracellular matrix (ECM). The ECM, consisting of basement membranes and interstitial stroma, is composed of a large number of components that interact with each other and with the different cell types present. Collagens are the most abundant ECM constituents besides adhesive glycoproteins like laminin, fibronectin, elastin and proteoglycans-glycosaminoglycans like hyaluronic acid and heparan sulphate [9-11]. Proteinases can be classified in four main groups: 1) Cysteine proteinases (e.g., cathepsin-B, -H, -L and -N); 2) Aspartyl proteinases (e.g., cathepsin-D); 3) Serine proteinases (e.g., cathepsin-G and -E, elastase, kallikrein, thrombin, trypsin, plasmin, plasminogen activators); and 4) Metalloendopeptidases. These metalloendopeptidases include the thermolysins, insulinases and metzincins (zinc-dependent proteinases). Metzincins can subsequently be subdivided into matrix metalloproteinases (MMPs, matrixins), adamalysins (ADAMs; a disintegrin and metalloproteinases, and ADAMTSs; a disintegrin and metalloproteinases with thrombospondin motifs), astacins and serralysins [12-14].

Matrix metalloproteinases

The matrix metalloproteinase (MMP) gene family consists of a group of proteolytic enzymes capable of degrading components of the ECM during physiological processes like pregnancy, parturition [15], development, growth and wound-healing [16], as well as in pathological conditions like rheumatoid arthritis [17], pulmonary emphysema [18], osteoarthritis [19], skin disorders [20] and malignancy [21]. MMPs share the following functional properties: 1) they contain a zinc ion at their active site and can be inhibited by chelating agents; 2) they are almost all secreted in a latent zymogen form that needs activation by partial proteolytic cleavage to become active; 3) they are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs); and 4) they share common amino acid sequences and cleave at least one component of the ECM [22]. Depending on their structure and substrate preference, the MMP family is divided into collagenases (MMP-1, -8, -13 and -18), stromelysins (MMP-3, -10, -11 and -28), matrilysins (MMP-7 and -26), gelatinases (MMP-2 and -9), elastases (MMP-12, -19 and -20) and membrane-type MT-MMPs (MMP-14, -15, -16, -17, -24 and -25). Some relevant characteristics of the specific MMPs and TIMPs studied as described in this thesis are summarized in Table 1 and discussed below.

Collagenases. The collagenases can degrade structural type I to III collagens only. Neutrophil collagenase or collagenase-2 (MMP-8) is one of the collagenases regarded as being synthesized exclusively by polymorphonuclear neutrophils before emigration from the bone marrow. In polymorphonuclear leucocytes it is stored in and released from secretory granules and its expression is stimulated by tumour necrosis factor-α. In addition, MMP-8 mRNA has been detected in mononuclear fibroblast-like cells in rheumatoid synovial fibroblasts and endothelial cells. Doxycycline has been shown to down-regulate MMP-8 induction, at both the mRNA and protein levels [17].

Matrilysins. Matrilysin (MMP-7) or putative matrix metalloproteinase-1 (PUMP-1) lacks a specific extracellular matrix-binding domain and is therefore the smallest of the MMP gene family, with a molecular weight in its inactive form of 28 kDa. The zymogen is activated by 4-aminophenylmercuric acetate, trypsin, plasmin and stromelysin-1 (MMP-3), but not by tissue collagenase (MMP-1), gelatinase-A (MMP-2) nor gelatinase-B (MMP-9). MMP-7 can activate pro-MMP-1 and pro-MMP-9 but not pro-MMP-2 nor pro-MMP-3. It has strong stromelysin-like activity and degrades insoluble elastin, type IV collagen, laminin-1, fibronectin, proteoglycan and gelatins [27].

Gelatinases. Gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are originally called gelatinases, enzymes which degrade denatured collagens (gelatin), although both gelatinases can degrade native collagens including type IV (basement membrane) and type V collagen and elastin as well. MMP-2 has been demonstrated in a variety of normal and malignant cells whereas MMP-9 is mainly expressed by alveolar mac-

Table 1 - Characteristics of the proteinases studied

Proteinase	MMPs				TIMPs		Lipocalins
Subgroup	Collagenase	Gelatinases		Matrilysins			
Nomenclature	MMP-8	MMP-2	MMP-9	MMP-7	TIMP-1	TIMP-2	Lipocalin-2
Common name	Neutrophil collagenase	Gelatinase-A	Gelatinase-B	Putative matrix metalloproteinase (PUMP-1); Matrilysin			Neutrophil gelatinase-associated lipocalin (NGAL)
Substrate specificity	Collagen I, II, III, VII, X	Gelatin, collagen type IV and V, elastin, laminin	Gelatin, collagen type IV and V, elastin, laminin	Gelatin, fibronectin, laminin, collagen type IV, procollagenase, TNF-α precursor	Pro-MMP-9	Pro-MMP-2	Bacterial formyl-peptide FMLP; MMP-9
Molecular mass (kDa)	75	72	92	28	28.5	21	25
Molecular mass of active species (kDa)	55	62; 64	67; 82	19			
Physiological activators	Serine proteases	MT-MMP; type 1 collagen	Serine proteases; MMP-2; MMP-7		EGF; IL-6; IL-1; IL-1β; Epo; TGF-β		
Native inhibitor	TIMP-1; TIMP-2	TIMP-2 > TIMP-1	TIMP-1 > TIMP-2	TIMP-1; TIMP-2			
Expression	Inducible	Constitutive	Inducible	Inducible	Inducible	Constitutive	Inducible
Localization	Neutrophils	Fibroblasts	Neutrophils	Epithelial cells; tumour cells	Fibroblasts; Epithelial and endothelial cells; tumour cells		Neutrophils
Reference	23	24	24	23	25		26

rophages, monocytes, keratinocytes, polymorphonuclear leucocytes and malignant cells [28, 29].

Tissue inhibitors of metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) are ubiquitous and naturally occurring inhibitors of MMPs that inhibit the MMP proteolytic activity by forming noncovalent 1:1 stoichiometric complexes resistant to heat denaturation and proteolytic degradation [for review see 25]. Up to now, four TIMPs have been described, TIMP-1, -2, -3 and -4, with molecular weights ranging from 21 to 28.5 kDa. TIMPs differ in solubility, interaction with proenzymes (pro-MMPs) and regulation of expression. Whereas TIMP-1, -2 and -4 are present in soluble forms, TIMP-3 is tightly bound to the matrix. Apart from binding to MMPs, TIMPs are also able to form complexes with the pro-MMPs influencing the MMP activation process. TIMP-2 is a constitutive protein, whereas TIMP-1 expression is influenced by external stimuli including growth factors, serum, phorbol esters, cytokines and erythropoietin. It has become apparent that apart from regulation of MMP activity, TIMPs are also involved in various other biological processes including cell-growth, apoptosis and angiogenesis. For example, TIMP-1 and -2 have been shown to potentiate the effect of erythropoietin on erythroid stem cell proliferation and differentiation. Moreover, TIMP-1 and -2 are able to induce the growth of normal and malignant cells and are associated with resistance against apoptosis in malignant cells. In addition, TIMP-1 has been shown to potentiate steroidogenesis. Moreover, TIMPs are also able to inhibit angiogenesis, partly by MMP inhibition, and are involved in embryogenesis by controlling the MMP-mediated remodelling of the extracellular matrix during blastocyst implantation. In tumours of various origins, downregulation of both TIMP-1 and -2 has been associated with increased invasiveness, while overexpression was associated with reduced tumour growth and metastasis.

Detection of matrix metalloproteinases

MMPs can be detected by various techniques including zymography, *in situ* zymography, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, mRNA *in situ* hybridization and quantitative RT-PCR analysis, Western blotting and quantitative activity assays, including radiolabelled collagen substrate degradation assays and bio immuno assays (BIAs). Gelatin zymography allows quantification of the active and the pro-enzyme form but is primarily suitable for measurement of gelatinases [30] and does not provide information at the cellular level. This disadvantage has been

overcome with the introduction of *in situ* zymography, that enables visualisation of activity of the gelatinases MMP-2 and -9 [31-34] and of MMP-7 [35] on frozen tissue sections. MMPs and their TIMPs can be localized by immunohistochemistry and mRNA *in situ* hybridization analysis without possibilities for quantification and detection of enzyme activity [36, 37]. In general, most ELISAs measure the grand total of pro-enzyme, active- and inhibitor-complexed forms of the respective MMP [38]. Enzymatic activities of MMPs can also be measured in blood samples and tissue homogenates using specific biochemical immunosorbent activity assays (BIA) [39, 40].

Regulation of matrix metalloproteinases

MMPs are tightly regulated at different levels, including gene expression, secretion, activity, and clearance [41].

1) Gene expression. The expression of most MMPs is regulated at the transcriptional level, by growth factors (e.g., epidermal growth factor and transforming growth factor-β), cytokines (e.g., tumour necrosis factor-α and interleukin-1), hormones [42-44], bacterial endotoxins, stress and oncogene activation [45, 46]. Apart from soluble factors, also cell-cell and cell-matrix interactions influence the expression of MMPs. For example, extracellular matrix metalloproteinase inducer (EMMPRIN), formerly called tumour cell-derived collagenase stimulatory factor, is a glycoprotein located on the outer surface of human tumour cells which interacts with fibroblasts to stimulate expression of several matrix metalloproteinases in fibroblasts [47, 48]. Various single nucleotide polymorphisms (SNPs) in gene promoter regions of MMPs have been shown to influence transcriptional activity resulting in altered protein levels. The -1306 C/T transition in the MMP-2 promoter sequence, for example, disrupts a Sp-1 binding site resulting in remarkably decreased promoter activity [49]. The -1562 C/T transition in the promoter region of the MMP-9 gene, on the other hand, results in higher promoter activity. This is due to preferential binding of a putative transcription repressor protein to the C allelic promoter [50].

2) Secretion in the latent form. MMPs are secreted in a latent proenzyme form, with the exception of MMP-11, MMP-28, and the membrane bound MT-MMPs. The other MMPs need to be activated in order to interact with the extracellular matrix. Several proteolytic activators, including the plasminogen activator/plasmin system, kallikreins, neutrophil elastase and trypsin, are involved in the activation of MMPs [51]. Unlike other MMPs, pro-MMP-2 is constitutively expressed by many cell types and activation occurs at the cell surface [52]. This activation appears to involve an interaction with MT1-MMP and TIMP-2 to form a membrane-bound complex that regulates the activation of pro-MMP-2 [53]. Pro-MMP-9 is not constitutively expressed

and its production and secretion can be induced and modulated by various factors. For example, pro-MMP-9 is synthesized by differentiating neutrophils in the bone marrow, stored in specific granules of circulating neutrophils, and released following neutrophil activation by inflammatory cytokines [54, 55].

3) Regulation of activity. TIMPs, the primary inhibitors of MMPs, form inhibitory complexes with most active MMPs [56, 57]. TIMP-1 also binds to pro-MMP-9, whereas TIMP-2 forms complexes with pro-MMP-2 [58]. TIMPs inhibit MMPs locally whereas alpha-2-macroglobulin, an abundant plasma protein, acts as a general non-specific endoproteinase inhibitor [59]. Recently, the transformation suppressor factor RECK, a membrane-anchored glycoprotein, was found to contain 3 protease inhibitor-like domains that negatively regulate MMP-2, -9 and MT1-MMP activity and inhibits tumour invasion and metastasis. Furthermore, down-regulation of RECK by oncogenic signalling leads to the excessive activation of MMPs thereby promoting malignant behaviour of cancer cells. In several types of tumours, a positive correlation between RECK expression and survival of the patients has been described [60-62].

4) MMP catabolism and clearance. Although MMPs are found in urine, the degradation and excretion pathways of MMPs and TIMPs in the body have not been fully elucidated [63]. Little is known about autoproteolysis of active MMPs, but certain cleavages clearly diminish MMP-activity [54]. Some MMPs are also found in association with accessory proteins, like lipocalins, which could serve as protection against autolysis.

Human neutrophil lipocalin

Lipocalins are a group of small extracellular proteins with great diversity at the sequence level. The lipocalins are member of an overall structural superfamily: the calycins. The other groups are the fatty-acid-binding proteins, a group of metalloprotease inhibitors, triabin and the avidins [64]. Lipocalins bind to a spectrum of small hydrophobic molecules and to specific cell-surface receptors and form complexes with soluble macromolecules. Lipocalins are supposed to function as transport proteins. They appear to be involved in biological processes like retinol transport, invertebrate cryptic coloration, olfaction, pheromone transport and prostaglandin synthesis. They also play a role in the regulation of cell homeostasis, the modulation of the immune response, and, as carrier proteins, act in the clearance of endogenous and exogenous substances [65]. Neutrophil gelatinase-associated lipocalin (NGAL), also named human neutrophil lipocalin (HNL), siderocalin or lipocalin-2 (lcn2), is a 25 kDa glycosylated protein constitutively expressed in myelocytes and stored in secondary granules of human neutrophils [66]. NGAL is present as a monomer, homodimer, or

as a heterodimer with neutrophil gelatinase [67]. It is highly induced in epithelial cells during inflammation or malignant conditions [68-74]. Because accumulation and infiltration by neutrophils is a prominent feature of the inflammatory process in ulcerative colitis, it has been suggested that NGAL may serve as a specific marker of intestinal neutrophil activation in ulcerative colitis [75]. In neutrophils, NGAL colocalizes with lactoferrin, whereas the gelatinases are localized in specific gelatinase granules [67, 76]. Interaction of NGAL with activation and enzymatic activity of gelatinase could initially not be shown [77]. The expression of NGAL in epithelial cells is totally dependent on NF-κB and also depends on a NF-κB-binding co-factor that is induced by interleukin-1β but not by tumour necrosis factor-α and is required for transcription of the NGAL gene [78]. It has been shown that NGAL can act as a bacteriostatic agent by sequestering siderophore bound iron and preventing its uptake by microorganisms [73, 79, 80]. Therefore, NGAL seems to play a role also in the innate immunity by reducing the availability of iron for microbial growth [78].

Helicobacter pylori infection and gastric neoplasia

Gastritis is inflammation of the stomach and is caused by infectious agents, drugs, and autoimmune and hypersensitivity reactions. Gastritis in childhood is very common in non-industrialized countries but uncommon in the Western world. The prevalence of gastritis increases with age, reaching a prevalence of approximately 60% in industrialized to 100% in non-industrialized countries at the age of 60 [81-83]. The most frequent cause of (chronic) gastritis is *Helicobacter pylori* (*H. pylori*), a Gram-negative bacterial species that preferentially colonizes human gastric mucosa resulting in a chronic gastritis [84, 85]. After eradication of *H. pylori,* the gastric mucosa is restored to normal [85]. In the majority of patients with peptic ulcer disease *H. pylori* is the causative factor [86-88] and eradication of *H. pylori* prevents recurrence of peptic ulcers [89]. *H. pylori* has also been associated with gastric carcinoma [90], gastric mucosa associated lymphoid tissue (MALT), lymphoma [91] and Ménétrier's disease [92]. *H. pylori* gastritis progresses gradually over the years from the non-atrophic form into the atrophic form. Atrophic gastritis and intestinal metaplasia are premalignant conditions for gastric cancer except for cancers of the gastrointestinal junction. Of gastric carcinomas, 80% are related to *H. pylori* gastritis. *H. pylori* infection is currently associated with an approximately two-fold increased risk of developing gastric cancer [93]. In addition to *H. pylori* gastritis, another 10% of gastric carcinomas are related to atrophic gastritis of the autoimmune type [94, 95]. A possible mechanism through which *H. pylori* infection could induce carcinogenesis is via the accompanying inflammatory response, which gives rise to the production of mutagenic substances like

nitric oxide [96]. Although *H. pylori* appears to be the most frequent cause of (chronic) gastritis [84, 85], only a minority of people who harbour this organism ever develop cancer. This process of gastric carcinogenesis seems to be influenced by differences in inflammatory response due to genetic diversity of both *H. pylori* isolates as well as host genes [97]. Four different genetic loci have been identified in the *H. pylori* genome with the potential to interact with host molecules that induce epithelial responses with carcinogenic potential: *CagA, VacA, BabA, and SabA*. *CagA*, derived from the cytotoxin associated gene, is injected in the host cells by the bacterium. *CagA*$^+$ strains enhance the risk for severe gastritis, atrophic gastritis, and distal gastric cancer compared with that incurred by *cag*$^-$ strains [98, 99]. One of the mechanisms involved is an enhanced NF-κB mediated interleukin-8 induction and inflammatory neutrophilic response in human gastric tissue by *H. pylori cagA*$^+$ strains compared to *cag*$^-$ strains [100]. *VacA* encodes a secreted bacterial cytotoxin that induces multiple structural and functional alterations in cells leading to gastric inflammation, haemorrhage, and ulcers [101, 102]. *H. pylori* strains with a type s1/m1 *vacA* allele are associated with enhanced gastric epithelial cell injury [103] and gastric cancer risk compared with *vacA* s2/m2 alleles [104]. *BabA2* encodes for an adhesin that binds the Lewis^b histo-blood-group antigen on gastric epithelial cells. The presence of *babA2* is associated with *cagA* and *vacA* s1 and *H. pylori* strains with all three genes induce the highest risk for gastric cancer [105]. Sialyl-Lewis^x antigen is a tumour antigen that is upregulated in gastric inflammation binding to gastric epithelial cells by the *H. pylori* adhesin *sabA* [106].

MMPs in gastrointestinal immunity, inflammation, infection and carcinogenesis

MMPs are tightly regulated and under normal conditions are involved in physiological tissue turnover and the host immune response. They play a role in matrix remodelling, the recruitment of inflammatory cells into the intestinal wall and other organs, cytokine and chemokine processing and defensin activation. MMP-9, for example, is secreted during neutrophil migration across the basement membrane, whereas TIMP-1 is able to inhibit this process [107]. MMP-3 is involved in the cellular immune response against intraluminal colonic pathogenic bacteria by facilitating the migration of T-helper lymphocytes into the intestinal lamina propria [108]. The gelatinases are also necessary for migration of dendritic cells out of the skin and of T-cells across the basement membrane [109]. Several MMPs, including the gelatinases, can release active tumour necrosis factor-α from the membrane-anchored precursor [110] and can both activate pro-interleukin-1β or inactivate active interleukin-1β [111, 112]. Defensins are MMP-7 activated antibiotic peptides that kill bacteria by membrane dis-

ruption. It has been shown that certain bacterial components can stimulate epithelial MMP-7 secretion, indicating its role in the early defence mechanism against infection [113].

Under pathological conditions, including gastrointestinal inflammation, infection and malignancy, enhanced levels and activities of MMPs have been described resulting in an imbalance in breakdown and remodelling of the extracellular matrix [114, 115]. In inflammatory bowel diseases, for example, enhanced expression of MMPs, either on the protein or mRNA level, or immunohistochemically, has been described to be associated with the severity of inflammation [116-118]. Protein and mRNA levels of MMP-2 and especially MMP-9 were markedly enhanced in inflammatory bowel tissues, with the highest concentrations in severely inflamed tissues. Polymorphonuclear leucocytes appeared to be the main source of MMP-9, whereas MMP-2 was predominantly located in the extracellular matrix [116]. Epithelial cells at the edge of gastrointestinal ulcers are strongly positive for matrilysin (MMP-7), probably because of their putative role in re-epithelization [119]. It has been demonstrated that activated lamina propria T-cells, for example elicited by luminal antigens, can cause a pathological chronic inflammatory response leading to intestinal damage by stimulating MMP secretion [120]. Enhanced MMP levels and activities have also been described in necrotising enterocolitis [121], celiac disease [122], collagenic colitis [123] and diverticulitis [124].

In infectious diseases, enhanced host MMP activity or decreased TIMP expression has been described in response to pathogens. In addition to inducing MMP secretion by host cells, it has been shown that bacterial pathogens are able to activate host pro-MMPs by secreting proteolytic enzymes themselves [125]. *H. pylori*, for example, produces several metalloproteases including one with MMP-3 like activity [126, 127].

In a number of human cancers enhanced expression of many MMPs, including MMP-1, -2, -3, -7, -9, -13 and -14, at the protein and mRNA levels or immunohistochemically, in both primary tumours and/or metastases has been associated with tumour progression and poor prognosis [128]. For instance, enhanced immunohistochemical expression of MMP-1, -7 and -13 has been associated with poor prognosis in colorectal cancer patients [129-131]. Enhanced expression of MMPs is found at the invasive front of tumours where malignant cells and stromal cells interact with each other and mutual induction of MMPs takes place. Several specific cellular mechanisms have been described that facilitate activation of local pro-MMPs and containment of MMP activity to the invasive front. These mechanisms include: 1) the expression of membrane-bound metalloproteinases; 2) the binding of soluble MMPs to membrane-bound docking factors; and 3) cell surface receptor-mediated activation of pro-MMPs. Pro-MMP-2, for example, can be activated at the cell membrane after forming a trimeric complex with TIMP-2 and MT1-MMP (MMP-14) [53].

Chronic inflammation is associated with enhanced cancer risk and both chronic inflammation and cancer are accompanied by enhanced MMP levels and activities [97]. Malignant cells, on their turn, secrete cytokines and MMPs, which stimulates influx of inflammatory cells to the tumour site and induces (neo)angiogenesis [132]. It has been shown, for instance, that MMP-9 is involved in tumour-induced angiogenesis by releasing Vascular Endothelial Growth Factor (VEGF) [133] with tumour-related inflammatory cells being the main source of MMP-9 [134].

MMPs in *H. pylori*-induced gastritis

Immunohistochemical studies on human gastric tissue have shown that MMP-9 immunoreactivity is predominantly expressed by inflammatory cells, including macrophages and fibroblasts [135, 136], by parietal cells [137], and to a lesser extent by epithelial cells with higher expression in *H. pylori* positive tissue compared to *H. pylori* negative tissue [135, 136]. Antral mucosa of *H. pylori* positive individuals showed approximately 20-fold higher MMP-9 activity compared to that of uninfected individuals, when measured by quantitative gelatin-zymography, probably by an increased number of macrophages containing a higher amount of MMP-9. Macrophages secrete MMP-9 in response to *H. pylori* [136, 138] and it has been demonstrated that *H. pylori* can induce activation of NF-κB in gastric epithelial cell lines leading to MMP-9 gene transcription [135].

In histologically normal, *H. pylori* negative human gastric tissue MMP-2 immunoreactivity was observed in parietal cells and to a lesser extent in epithelial cells [137]. MMP-2 activity was elevated in *H. pylori* positive individuals compared to uninfected individuals, but at lower levels than MMP-9 [136].

H. pylori positive individuals expressed higher levels of MMP-7 at the protein and mRNA levels in their antrum and corpus when compared to uninfected individuals. MMP-7 immunoreactivity in epithelial cells of *H. pylori* positive individuals was more intense than in uninfected persons [138, 139]. MMP-7 expression was strongly related to the infestation of cag$^+$ *H. pylori* strains since MMP-7 expression was demonstrated in gastric epithelial cells in 80% of *cag*$^+$ colonized persons but in none of *cag*$^-$ or uninfected individuals. *Cag*$^+$ *H. pylori* strains augment the risk for gastric cancer. In *in vitro* studies the increased levels of MMP-7 in inflamed gastric mucosa appeared to be induced by *cag*$^+$ *H. pylori* strains dependent on activation of extracellular signal-regulated kinase (ERK) 1/2 mitogen–activated protein kinase [140]. *H. pylori* infection has also been reported to stimulate MMP-1, MMP-3, TIMP-3, and MMP-3/TIMP-3 complex formation in gastric epithelial cells and in gastric mucosa [141, 142]. A strong interaction of interleukin-1β and *H. pylori* on MMP-3 secretion has been found [142].

In *H. pylori*-induced ulcers higher concentrations of MMP-1 were found compared to NSAID (non-steroidal anti-inflammatory drug)-induced ulcers, possibly due to the anti-inflammatory effect of the NSAIDs used [143].

Gastric carcinoma

Despite the sharp decrease in the incidence rate of gastric cancer over the last 50 years [144, 145] gastric cancer is still the second most common cancer worldwide accounting for approximately 10% of all cancers and being responsible for approximately 12% of all cancer deaths [146-148]. In contrast to the decreased incidence rate of gastric cancer, the incidence rates of cancer of the distal oesophagus and of the cardia have increased significantly over the last decades [149]. In 1985, the age-standardized incidence rate per 100,000 population varied from 8.8 and 4.2 in North America to 74.8 and 35.2 in Japan, in men and women, respectively. The incidence rate for Western Europe in 1985 was 18.0 and 9.4 in men and women, respectively [146, 150]. The incidence rate in the Netherlands from 1989 until 1992 was 15.4 and 6.1 in men and women, respectively (World Standardized Rate) and in this period, 10,116 new cases of gastric cancer were registered indicating approximately 2,500 new cases annually [151]. In 1998, the incidence rate in the Netherlands ranged from 18 in men (1,392 new cases) to 10 in women (768 new cases) according to the Dutch Cancer Registry (www.kankerregistratie.nl).

The 5-year cumulative survival rate ranges from 91% in stage 1 (intramucosal) to 5% in stage 4 (metastatic) gastric cancer [152]. The overall 5-year survival rate ranges from 7.4% to 16.5% [153, 154]. In the southeast of the Netherlands, the relative 5-year survival rate of patients who underwent resection was 85% for stage I and 60% for stage II tumours [155]. Surgical resection remains the primary curative treatment option in gastric cancer with 5-year survival rates of 58%-78% and 34% reported for stage I and II disease, respectively [156]. Postoperative chemoradiotherapy, perioperative chemotherapy, and postoperative chemotherapy have been shown to decrease the risk for recurrence and to improve the outcome for patients fit to undergo these treatments [157]. The aetiology of gastric cancer seems to be multifactorial with different relative influence of causal factors in different geographical regions. Compared to U.S.-born individuals, immigrants had at least a 50% higher mortality from stomach cancer [158]. Epidemiological data suggest an increased risk for gastric carcinoma development in patients with *H. pylori*-associated gastritis [90, 159]. *H. pylori* infection was especially associated with non-cardiac carcinomas, and a stronger association was observed with diffuse rather than with intestinal-type tumours [160].

MMPs in gastric carcinoma

Immunohistochemical and *in situ* hybridisation studies, as well as quantitative methods like gelatin-zymography and activity assays, have demonstrated that gastric carcinomas contain enhanced amounts of MMP-1, -2, -3, -9 and TIMP-1 [161, 162], MMP-7 [163] and MT1-MMP [164]. Particularly MMP-1, MMP-7, MMP-9 and TIMP-2 were immunolocalized in carcinoma cells, whereas MMP-2 immunostaining was observed on advanced gastric carcinoma cells and correlated with vascular invasion by tumour cells [165, 166]. Pro-MMP-2 activation was present only on gastric carcinoma cells that expressed MT1-MMP, indicating MT1-MMP-assisted activation of pro-MMP-2 in human gastric carcinomas. The MMP-2 genotype appeared to influence the susceptibility to develop gastric cardiac adenocarcinoma [166].

MMP-7 was reported to be produced by gastric carcinoma cells and significantly associated with aggressive pathological phenotypes of gastric cancer [167]. In gastric carcinomas, it has been shown that expression of E1AF/PEA3 (ETV4), an ets-family transcriptional factor, able to transactivate multiple MMP genes, correlates well with MMP-7 expression [168].

H. pylori infection of gastric carcinoma cells was reported to increase mRNA expression and protein levels of MMP-9 [169]. MT1-MMP [170] and MMP-1 [171] immunoreactivity in human gastric carcinomas were also found to be associated with worse prognosis, whereas increased TIMP-2 expression seems to be correlated with prolonged survival [172].

Outline of the studies described in this thesis

In this thesis, several studies are described on the putative role of the matrix metalloproteinases MMP-2, -7, -8 and -9, the tissue inhibitors of metalloproteinases TIMP-1 and -2, and of the lipocalin neutrophil gelatinase-associated lipocalin NGAL in chronic *H. pylori*-induced gastritis and in gastric carcinoma. MMPs are the main degrading enzymes of extracellular matrix proteins and basement membranes and are therefore involved in tissue remodelling and repair as well as recruitment of inflammatory cells and angiogenesis in many physiological and pathological processes, including inflammation and malignancy. As introduction, a short overview is given in **chapter 1** on MMPs, TIMPs and lipocalins in general and their role in *H. pylori*-induced gastritis and gastric carcinoma in particular.

H. pylori gastritis is recognized as an important pathogenetic factor in peptic ulcer disease and gastric carcinogenesis. **Chapter 2** reports on the influence of *H. pylori* infection on gastric mucosal MMP-2 and -9 expression. In gastric mucosal biopsies

of individuals with or without *H. pylori* infection, the levels, isoforms and activity of MMP-2 and -9 were determined by quantitative gelatin-zymography, bioactivity assays (BIAs), enzyme-linked immunosorbent assays (ELISAs) and immunohistochemistry. In addition, the relation between gastric mucosal MMP-2 and -9 expression and severity of inflammation was assessed.

The influence of *H. pylori* eradication therapy on mucosal MMP-2 and MMP-9 levels is described in **chapter 3**. Gastric biopsies from patients with *H. pylori*-associated gastritis, that were treated with a combination regimen of acid inhibitory therapy and antibiotics in order to eradicate *H. pylori* and to reduce the risk for peptic ulcer disease, were evaluated for the levels, isoforms and activity of MMP-2 and MMP-9 by quantitative gelatin-zymography, bioactivity assays (BIAs) and enzyme-linked immunosorbent assays (ELISAs).

In a number of human cancers, enhanced expression of MMPs has been described in primary tumours associated with tumour progression and poor prognosis. **Chapter 4** describes the initial study of levels, isoforms, and activities of MMP-2 and MMP-9 in gastric carcinomas and corresponding normal mucosa, as assessed by quantitative gelatin–zymography. These parameters were correlated with a number of clinicopathological parameters including TNM stage and histological classifications according to Laurén and WHO. In addition, the prognostic significance of the MMP-2 and MMP-9 levels for the overall survival of the patients was evaluated.

The results of a more comprehensive study that was carried out to endorse the findings as described in chapter 4 are presented in **chapter 5**. The MMP analyses in the same group of patients were extended and compared with those obtained with a new and more recent group of gastric cancer patients. Furthermore, instead of quantitative gelatin-zymography, bioactivity assays (BIAs) and enzyme-linked immunosorbent assays (ELISAs) for MMP-2 and MMP-9 were used. Moreover, the prognostic value of MMP-2 and MMP-9 was compared with those of MMP-7 and MMP-8 and the study was expanded by determination of the inhibitors TIMP-1 and TIMP-2. In addition, because of the increasing age of the patients and the length of the follow-up, tumour-associated survival was evaluated.

Neutrophil gelatinase-associated lipocalin (NGAL) is a lipocalin that has initially been discovered in specific granules of human neutrophils and was later shown to be expressed also by certain epithelial cells, especially in inflamed or malignant tissues. A part of the NGAL is present as a complex with MMP-9. In **chapter 6,** an analysis of the presence of MMP-9-NGAL complexes in tissue extracts from gastric cancers and their relation with survival is presented. In the same cohort of patients as used in the study described in chapter 5, MMP-9-NGAL complexes were measured by zymography and by ELISA. The tumour levels of MMP-9-NGAL complex, MMP-9 and NGAL were evaluated for correlations with established clinicopathological parameters of

the gastric carcinoma patients and for their predictive value to patients' outcome. In addition, immunohistochemical analysis of serial paraffin-embedded tissue sections and immunofluorescence double staining were used to establish the cellular origin of MMP-9 and NGAL.

Single-nucleotide polymorphisms (SNPs) within MMP genes are thought to influence the expression of MMPs and/or even seem to be associated with the susceptibility for the development of malignancy. The clinical impact of MMP and TIMP gene polymorphisms in our cohort of gastric cancer patients is described in **chapter 7**. The genotype distribution and allele frequencies of SNPs of MMP-2, -7, -8 and -9 and TIMP-1 and -2 were studied. In order to get insight into the functional and clinical contribution of these MMP-related gene polymorphisms, the relationship between the distribution of these SNPs and the respective protein levels in tumour and adjacent normal tissue, as well as the relation of the SNPs with established clinicopathological parameters and tumour-related survival was assessed.

The different studies are finally compiled as a summarizing discussion in **chapter 8**, including a discussion on the potential role of MMP inhibition in gastric cancer.

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