

**Extracellular matrix remodelling proteases
in acute appendicitis and their impact on
appendiceal perforation**

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Crea diem - skapa dagen

*To
Joban and my tutors*

Abstract

Background

Appendicitis is associated with varying degradation of extracellular matrix involved in tissue injury. The principal aim of this study was to investigate whether immunoreactive techniques could illustrate the course and severity of appendicitis and separate the different inflammatory grades, phlegmonous, gangrenous and perforated appendicitis, from each other and from uninflamed appendix. This could lead to early identification of patients with appendicitis that have or are at risk of a perforation, thereby improving their treatment and outcome.

Materials and Methods

In Papers I-II, tissue biopsies were taken from 40 appendectomized patients. Ten patients who had a hemicolectomy served as controls. In Paper III, proteolysis from tissue biopsies at and adjacent to the perforation was studied in 15 patients operated on for perforated appendicitis. In Paper IV, plasma samples taken prior to surgery and 4 weeks postoperatively and biopsies from the appendix were taken in 57 patients operated on for suspected appendicitis. Protein levels of matrix metalloproteinase (MMP) -1, -2, -3, -9 and tissue inhibitor of metalloproteinase (TIMP-1) (Papers I, III and IV), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) (Papers II, III and IV) were determined by ELISA and localised by immunohistochemistry.

Results

MMP-9 was the most abundant protease in all groups of appendicitis compared with controls. The expression of MMP-1 and PAI-1 was significantly higher in perforated appendicitis compared with phlegmonous appendicitis and controls while MMP-2 showed an opposite pattern. uPA was twice as high in all groups of appendicitis compared with controls while no differences were found in MMP-3 and TIMP-1 expression between the groups. Immunohistochemically a scattered distribution of MMP-9 and TIMP-1 was demonstrated in the appendiceal wall in gangrenous and perforated appendicitis. When investigating the proteases in relation to the sites of perforation, MMP-9 and PAI-1 were found to be highest at the perforation sites while MMP-1 was higher close to them and MMP-2 decreased gradually away from them. No difference was seen in TIMP-1 and uPA. The individual differences in plasma for TIMP-1 were higher in patients with perforated appendicitis than in them who had phlegmonous and gangrenous appendicitis.

Conclusions

ECM remodelling proteases and anti-proteases could be demonstrated in appendicitis. A local imbalance between MMP-9 and TIMP-1 in combination with an over-expression of PAI-1 participated in the ECM degradation, leading to tissue injury in appendiceal perforation. TIMP-1 in plasma could be an inflammatory diagnostic marker for patients with appendicitis and at risk for perforation.

Keywords; Appendicitis, Perforation, Extracellular matrix, MMP-9, TIMP-1, uPA, PAI-1

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List of publications

This thesis is based on the following publications and manuscripts, which are referred to in the text by their Roman numerals (I-IV).

- I. A local imbalance between MMP and TIMP may have an implication on the severity and course of appendicitis.
Solberg A, Holmdahl L, Falk P, Palmgren I, Ivarsson ML.
Int J Colorectal Dis. 2008 Jun;23(6):611-8

- II. Progress of tissue injury in appendicitis involves the serine proteases uPA and PAI-1.
Solberg A, Holmdahl L, Falk P, Willén R, Palmgren I, Ivarsson ML.
Scand J Gastroenterol. 2009;44(5):579-84

- III. Tissue proteolysis in appendicitis with perforation.
Solberg A, Holmdahl L, Falk P, Willén R, Palmgren I, Ivarsson ML.
Accepted for publication in Journal of Surgical Research, Jan 2010

- IV. Plasma MMP-9, PAI-1 and TIMP-1 as diagnostic markers for the severity of acute appendicitis.
Solberg A, Holmdahl L, Falk P, Wolving M, Palmgren I, Ivarsson M-L.
in manuscript

Reprints of tables and figures were made with kind permission from the respective publisher.

Abbreviations

BM	Basement membrane
CRP	C-reactive protein
CT	Computer tomography
CTDA	Citratetheofyllaminediaminic acid
DIC	Disseminated intravascular coagulation
DL	Diagnostic laparoscopy
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FFA	Free fatty acid
IH	Immunohistochemistry
IL-8	Interleukin-8
IL-6	Interleukin-6
IL-1 beta	Interleukin-1beta
LA	Laparoscopic appendectomy
LPS	Lipopolysaccharid
mRNA	Messenger ribonucleic acid
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
OA	Open appendectomy
PAD	Pathological anatomical diagnose
PAI	Plasminogen activator inhibitor type 1
PLA2	Phospholipase A2
PBS	Phosphated buffered saline
TIMP	Tissue inhibitor of metalloproteinase
TNF-alpha	Tumour necrosis factor-alpha
tPA	Tissue plasminogen activator
US	Ultrasound
uPA	Urokinase plasminogen activator
WBC	White blood cell count

GENERAL INTRODUCTION

Appendix vermiformis and appendicitis

Epidemiology - introduction

Appendicitis is the most common cause of emergency abdominal surgery in the Western world. The lifetime risk of developing this condition is approximately 7 -12 % (1) while the risk for appendectomies has been reported to 12% in men and 25% in women (2). The risk for appendicitis peaks between 13 – 40 years of age (1).

Anatomy and histology

Appendix vermiformis is thought to be a vestigial organ and its location varies as shown in Figure 1. Its most common position is retrocecal and this location may influence the presentation of appendicitis with atypical symptoms and signs. There are also reports of retroperitoneal locations (3). Histologically the appendix vermiformis is similar to the colon but the lymphoid tissue resembles the small intestine. Moreover, the lymphoid tissue in the appendix wall degenerates and fibrous tissue increases with age (4, 5).

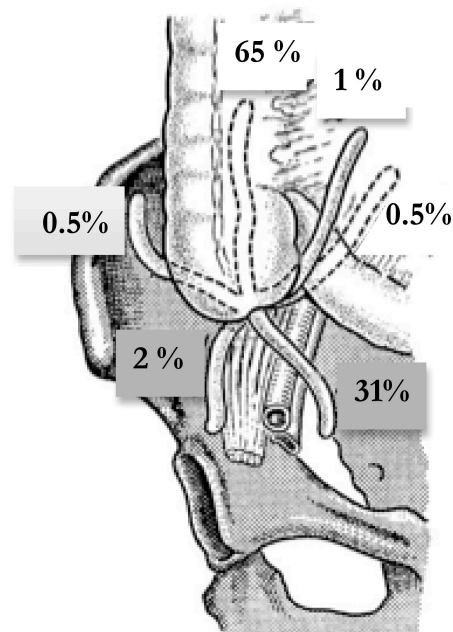


Figure 1: The different locations of appendix vermiformis in relation to ceacum and peritoneum.

Macro- and microscopic appearance

Appendicitis is divided into different inflammatory grades or stages, referred to as phlegmonous, gangrenous and perforated appendicitis (Table 1). Perforated appendicitis is considered to be the most severe grade or stage of the inflammation (6). However there is an ongoing discussion about whether perforated appendicitis can be considered an entity of its own or the final stage of the disease (7).

Table 1: Definitions used for classification

<i>Grade of inflammation:</i>	<i>Macroscopic appearance</i>	<i>Microscopic appearance</i>
Phlegmonous appendicitis	Appendix may have an increased diameter and/ or dilated, dull serosa, dilatation and congestion of surface vessels; fibropurulent exsudate	Neutrophilic infiltration in muscularis propriae, transmural inflammation, intramural abscesses and mucosal ulceration
Gangrenous appendicitis	Friable appendix wall, green, black or purple and fibrin on surface	Areas of necrosis in transmural inflammation, extensive mucosal ulceration and vascular thrombosis
Perforated appendicitis	Often as in gangrenous appendicitis and visible perforation	The site of perforation might be possible to demonstrate if it is in the specimen, transmural inflammation, extensive ulceration and necrosis

Appendicitis and historical notes

Perforated appendicitis was first described in an autopsy report during the 16th century when the mortality rate for appendicitis could reach 70%. It remained high until the end of the 19th century and declined further in all groups of severity after the mid 20th century (8, 9). Surgical treatment of appendicitis was introduced during the 19th century and consisted in the beginning of an incision of the lower abdominal wall in order to empty an intra-abdominal abscess. The experience of spontaneous resolution in some patients together with fear of operative mortality delayed this procedure until day 5-12 of the illness (8).

Appendectomy through an abdominal "muscle fibre-splitting incision" in the right lower quadrant was demonstrated around the 1880s. Reginald H. Fitz, professor of Pathology at Harvard University, stated in his paper from 1886 that "The inflammatory process once excited, its course and results show extreme variations" and he recommended that appendectomy should be performed within the first three days of the illness (10). The treatment in the current circumstances was then focused on the approach "If you are in doubt take it out" in order to avoid perforations and a

negative appendectomy-rate became accepted (11, 12). However, there is now evidence of a higher mortality rate in patients operated on with negative appendectomies compared with those operated on for appendicitis (13). The reason for this could be that these patients suffer from another serious illness, that will remain undetected during a negative appendectomy performed through a minor incision and then concealed by the postoperative pain (14). Furthermore, a correct diagnosis with an indication for surgery seems more important than a high frequency of appendectomies (11).

Incidence, mortality and morbidity

The incidence of appendicitis in Sweden is 118/100 000 and perforated appendicitis constitute approximately 20%, with a higher rate found in the youngest and elderly patients (1, 7, 15, 16). This higher rate of perforations in older patients is thought to be a consequence of the lower incidence of nonperforated appendicitis in this group (17). The mortality rate for appendicitis over all declined to approximately 1% after the improved treatment of peritonitis during the 1940s (18) and is currently below 1% for nonperforated appendicitis. However, it has been reported to increase to 5% due to a perforation (13). Common complications after appendectomy are wound infections (7-11%), intra abdominal abscesses (10%) and development of adhesions with an increased risk of bowel obstruction (2-3%) (13). The rate of complications may rise to over 20% in perforated appendicitis and is described to be as high as 39% in patients over the age of 80 (19-22).

Pathogenesis

The pathogenesis of appendicitis is still not clear and it may be multi-factorial (6). The obstruction theory is currently the most accepted view where an obstruction caused by a fecalith or lymphoid reaction may precede mucosal ischemia and bacterial invasion (6, 23-25). However, other authors suggest that the inflammatory process itself leads to an obstruction (6, 26, 27). The decrease in lymphoid tissue and blood supply in the appendix has been discussed as possible causes to a more rapid progression to perforations in the elderly (28). A delay in presentation, increased body-mass index and the presence of a fecalith is associated with perforated appendicitis (29-34). A fecalith is also correlated with an increased risk for postoperative complications after appendectomy for perforated appendicitis and with recurrences after conservative treatment of perforated appendicitis (35, 36). The fact that appendices with fecaliths without signs of inflammation on autopsies have been identified (6), indicates that other factors correlate with its occurrence and might be of importance for variations in the inflammatory process. Many different bacteria are found in appendicitis

cultures, where *E-coli* and *Bacteroides fragilis* are most common (37). Appendicitis has been described as an infectious disease with clusters in the population getting ill at the same time (38) although the primary infectious agent is still unknown. The bacteria within the fecalith could influence the severity of appendicitis and results reveal that elevated antibodies against *B. fragilis* and *Helicobacter pylori* are related to gangrenous and perforated appendicitis (39-41).

Appendicitis - characteristics and clinical signs

The typical clinical presentation of appendicitis includes abdominal pain migrating from the pre-umbilical area towards the right lower quadrant with or without signs of local peritonitis such as rebound tenderness, low-grade fever, lack of appetite and elevated white blood cell count. Vomiting may occur after the initial abdominal pain but seldom before the onset of pain (23). Clinical signs of perforated appendicitis are abdominal distension, reduced bowel sounds, pale skin, generalised or severe abdominal tenderness and tachycardia (30). A typical presentation is not always obvious in patients with appendicitis and the bedside diagnostic accuracy is uncertain even though scoring systems and the surgeons' experience may increase the diagnostic accuracy (43). Moreover, there are difficulties separating perforating from nonperforating appendicitis on the basis of clinical signs (44) and it has been postulated that perforated appendicitis is an entity of its own (1, 7, 17, 30, 45, 46). Clinically, a prolonged time from symptom onset to surgery, high age, smoking and the presence of a fecalith (7, 20, 28-33) are all characteristics of patients with perforated appendicitis which may lead to increased postoperative morbidity and mortality (1, 13, 15, 20, 32, 47). The correlation between smoking and the increased risk of appendiceal perforation is not clear, although smoking is known to modulate the immune system (29). Most of the perforations are believed to occur pre-hospital and patient-related factors, socioeconomic aspects and availability of hospitals have been considered responsible for it (31, 32). An in-hospital delay can also increase the perforation frequency whereas the access to operating rooms, doctor-related factors such as uncertainty about the patient's diagnosis and the time required from imaging studies are suggested as possible causes of it (32, 48).

Diagnostic tools

Laboratory markers

Recent studies have investigated cytokines, neutrophil elastase, S-bilirubin, phospholipase A2 (PLA2), C-reactive protein (CRP) and white blood cell count (WBC) as diagnostic tools for acute appendicitis and as predictors for patients with

complicated appendicitis who need surgery at an early stage (49-54). CRP and WBC are valuable for the diagnosis of appendicitis and should be repeated during the active observation phase and may also discriminate between nonperforated and perforated appendicitis (12, 55, 56). This is consistent with the results reported earlier, where CRP alone was correlated with the severity of appendicitis (57-59). CRP may be used as a marker suggesting of surgery as the optimal choice (55, 60). Nevertheless, CRP is an acute phase reactant that peaks after 24 hours and is unreliable in the early course of appendicitis (52). Cytokines such as interleukin 6 (IL-6) and interleukin 8 (IL-8) have been found to be elevated locally in the peritoneal fluid and systemically in serum from patients with gangrenous and perforated appendicitis (53, 54). Moreover, a one hundred fold increase in IL-8 in the peritoneal fluid from patients with perforated appendicitis compared with nonperforated appendicitis has been described (61).

Radiology

Computer tomography (CT), ultrasound (US) and magnetic resonance imaging (MRI)

Presentations are equivocal in approximately one third of patients with appendicitis and they may benefit from CT or US imaging. The accuracy has been reported to be 94% for CT and 91% for US in diagnosing appendicitis with a sensitivity of 96% for CT and 76% for US while the specificity was more equal, 89% versus 91% (62). A recent study on MRI and diagnosing appendicitis showed a sensitivity of 100% and a specificity of 99% (63). Thus MRI could be a better alternative in imaging for pregnant women with suspected appendicitis (64).

Other recent studies have revealed that patients operated on who have a negative appendectomy are at risk of an increased fatality rate compared with those with appendicitis. This has led to an increased frequency of preoperative radiological imaging in order to enhance diagnostic accuracy. However, the rate of perforations has not declined despite clinical observation, laboratory tests and modern radiological interventions (15, 32) and controversial results of CT-investigations have been presented (65). CT or US may not improve diagnostic accuracy or the negative appendectomy rate but may instead delay appendectomy (66). In atypical cases, diagnostic laparoscopy may have a better impact on the accuracy.

Diagnostic laparoscopy

Diagnostic laparoscopy (DL) has been described since 1910 (67) and introduced for appendectomies in the 1990s (68). The advantages are the possibility of ruling out appendicitis, localising the inflammatory area before a conversion to open surgery and investigating the entire abdominal cavity and pelvis for other diagnoses. Furthermore, there is a minor trauma of the abdominal wall as well as of intra-abdominal organs

(68). DL is reported to be safe with a sensitivity of 92% for diagnosing appendicitis (67) and it does not compromise the rate of perforations (69).

A disadvantage with DL is that it is an invasive procedure with risk for perioperative complications such as injuries to large vessels, reported in 0.003-0.08% of cases, and visceral injuries in 0.04 – 4%, although reduced in open access to the abdominal cavity (71). DL has the additional benefit of being therapeutic compared to CT and US but there is a risk for unnecessary surgery when no pathology is found. Premenopausal women benefit the most from this procedure (72, 73).

Management options

Surgery

Open appendectomy (OA) is one of the first abdominal operations a surgeon has to perform by him or herself. There are fewer complications with intra-abdominal abscesses after OA compared with laparoscopic appendectomy (LA) and the reason for this may be a difference in the environment for bacteria, as discussed below (74). Whether an increased rate of LA for perforated appendicitis is associated with this complication remains unclear (75). OA has been advocated during pregnancy even though LA is performed in this group (76).

LA was first introduced in 1983 by Semm a gynaecologist and there is evidence that it leads to less postoperative pain, faster return to active life, less wound infections and a reduced risk of bowel obstruction when compared with OA (77). However, another recent study showed no difference in the risk for bowel obstruction between the two techniques (78). Meeks et al. (2008) discuss the results in two recent meta-analysis performed in the 2004 and 2007 where the laparoscopic approach was found to be to a better alternative than OA (75). The choice between OA and LA often depends on the surgeon's experience of laparoscopy and in a 2009 meta-analysis it seem to appeared to be an important factor for the results (79). Another recent study concluded that LA is safe for perforated appendicitis (80) and there is also evidence that it has an advantage compared with OA in obese patients and in the elderly (81, 82).

Disadvantages related to LA consist of longer operation times, higher treatment costs and increased risk of deep abscess, particularly in patients with perforated or gangrenous appendicitis (77, 79). This increased risk may be related to overgrowth of anaerobic bacteria such as the *B. fragilis*, and the inflow of CO₂ as described in an experimental study on rats (83). The authors suggest that patients with perforated appendicitis ought to be operated on without pneumoperitoneum (83).

Based on a local regime, all patients in our hospital receive preoperative prophylactic antibiotics against aerobic and anaerobic bacteria, and in case of a perforated appendix they will continue with antibiotics for at least five days.

Expectancy and observation

There is long clinical experience of patients with suspected appendicitis that resolves spontaneously during active and close observation (84). In Sweden approximately 30 000 patients are kept under observation in hospital annually for abdominal pain located in the right fossa, of whom about one third of them will have an appendectomy (85). An expectant attitude may lead to a recovery in some of the patients with appendicitis during their in-hospital observation, although with risk for a recurrence rate of up to 30 - 40% within a year (84).

Conservative treatment with antibiotics

Antibiotics versus surgery as the primary treatment for nonperforated appendicitis has been tried in randomised trials (86, 87, 88) with successful results for nonperforating appendicitis. A recurrence rate between 14 - 35%, the fact that 10 – 15% of the patients treated with antibiotics require emergency surgery and the lower rate of complications after antibiotic-treatment compared with surgery highlight the need for a serious reflection (86, 87, 88). However, antibiotics do not prevent appendix perforations, nor their sequelae such as the development of adhesions with an increased risk for bowel obstruction (78). Perforated appendicitis with an obvious abscess is commonly treated conservatively with antibiotics and drainage (70, 89). A recent meta analysis shows that further studies on this subject are needed, but conservative treatment with antibiotics with or without drainage is still advocated (89). If a delayed appendectomy should be performed or not is controversial as well as which patients should undergo further colon examinations. A follow-up with a colonoscopy or virtual CT colonoscopy for patients over age 40 has been suggested, as the risk of malignancy compared to an appendiceal abscess has been reported to be 1 - 4 % after successful primary conservative treatment with antibiotics (70, 89).

Diagnostic and therapeutic dilemmas in appendicitis

Acute appendicitis constitutes a diagnostic and therapeutic dilemma, as not all patients present with typical signs or symptoms (62). Despite the fact that sophisticated imaging with ultra sound, CT-scan and MRI has high sensitivity and specificity in clinical studies, the value of these investigations in every day practice has been found to be of limited value (65). The individual course of appendicitis is unpredictable and the inflammation may proceed to a perforation after the admission to hospitals, even

though perforations are thought to occur pre-hospital in most cases (11, 90). It would be desirable to identify patients with appendicitis and a perforation, or at risk for it, at an early stage by separating the different grades of inflammation. Surgery or conservative treatment for acute appendicitis is still controversial and the possibility to diagnose the severity of appendicitis preoperatively could facilitate choosing the best treatment on an individual basis. Analysis of the molecular process involved in acute appendicitis and at the sites of perforation may improve the understanding of its pathogenesis (46). Furthermore, it could lead to the identification of reliable biochemical markers that reflect the course of appendicitis, which can be helpful in predicting which patients have or are at risk of having a perforation.

This project – translational research

The clinical task was to investigate whether immunoreactive techniques could illustrate the course and severity of appendicitis and separate the different inflammatory grades; phlegmonous, gangrenous and perforated appendicitis, from each other and from uninflamed appendix. The thesis is comprised of explorative studies with a clinical and molecular perspective on acute appendicitis, in order to investigate whether proteases and anti-proteases are present in the extracellular matrix (ECM) degradation leading to tissue injury and perforation in appendicitis.

Proteases, such as the matrix metalloproteinases (MMP) are able to degrade all parts of the extracellular matrix (ECM) including the basement membrane (BM) (91) and were therefore considered important for an appendix perforation to occur. The presence of serine proteases urokinase plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) has earlier been demonstrated in appendicitis (92, 93) and they may have an effect on inflammatory cell recruitment and migration, thereby modulating the inflammatory response (94, 95). Moreover, they participate in the activation and inhibition of the plasminogen-plasmin system, whereas plasmin is of importance in the activation of MMPs (Fig. 2) (91). The focus was put on the investigation of MMPs, tissue inhibitor of metalloproteinase (TIMP) and the serine proteases uPA and PAI-1 in appendiceal tissue and plasma in patients with appendicitis.

Molecular perspectives

The extracellular matrix

Proteolysis of the appendix wall may precede a perforation with degradation of all components in the ECM. The ECM consists of a loose meshwork of giant molecules such as collagens, proteoglycans and elastic fibres located between the cells in all our organs, vital for sustaining the individual architecture and structural integrity of all

tissues. The different cells are anchored to the ECM through adhesion proteins such as fibronectin, laminin and the surface receptors integrins. Collagen is important for the strength of an organ and divided in different types (96). Type I collagen is the most abundant collagen except in hyaline cartilage while the type IV collagen with laminin constitutes the BM, which is a thin sheet of specialized ECM composed of a web-type network (97).

ECM has effects on cell migration and differentiation and may interact with the inflammatory response in several ways (98). Degrading proteases, which may continuously increase during inflammation, remodels the ECM. Therefore proteases are of crucial importance for the tissue damage seen in several inflammatory diseases such as rheumatoid arthritis, periodontitis and inflammatory bowel diseases (91, 99-101).

There is also growing evidence that cleavage products from the degradation of the ECM may serve as chemokines for the neutrophils and other inflammatory cells (102). The MMPs, their tissue inhibitors of metalloproteinases (TIMPs) and the serine protease system with uPA and its inhibitor PAI-1 are involved in ECM degradation during the physiological remodelling of the ECM and during inflammation (103).

The MMPs and TIMPs - regulation and function

Such proteases as the MMPs are believed to be of importance in organ perforations (104-109). The MMP-family comprises of at least 25 different Zinc dependent endopeptidases (96), divided after their substrates into collagenases MMP-1, -8, and -13, gelatinases MMP-2 and MMP-9, stromelysins MMP-3, MMP-10 and -11, matrilysin MMP-7, membrane type MT-MMP -14 – 17 and MMP-24 and -25 (99) . MMP-1, -2, -3 and -9 can degrade all components of the ECM including BM (91).

To fulfil their physiological role in ECM remodelling, MMPs are strictly regulated on multiple levels. They occur in a latent pro-form and in active forms that require proteolytic degradation and they are also controlled on the transcription level through a number of cytokines and growth factors. Furthermore, they are inhibited by TIMPs and by humoral inhibitors such as alpha -2 macroglobulin (103).

MMPs can *activate* each other, by the serine protease plasmin (Fig. 2) and by other non-proteolytic compounds. The balance between the MMPs and TIMPs is of great importance for maintaining ECM homeostasis (91).

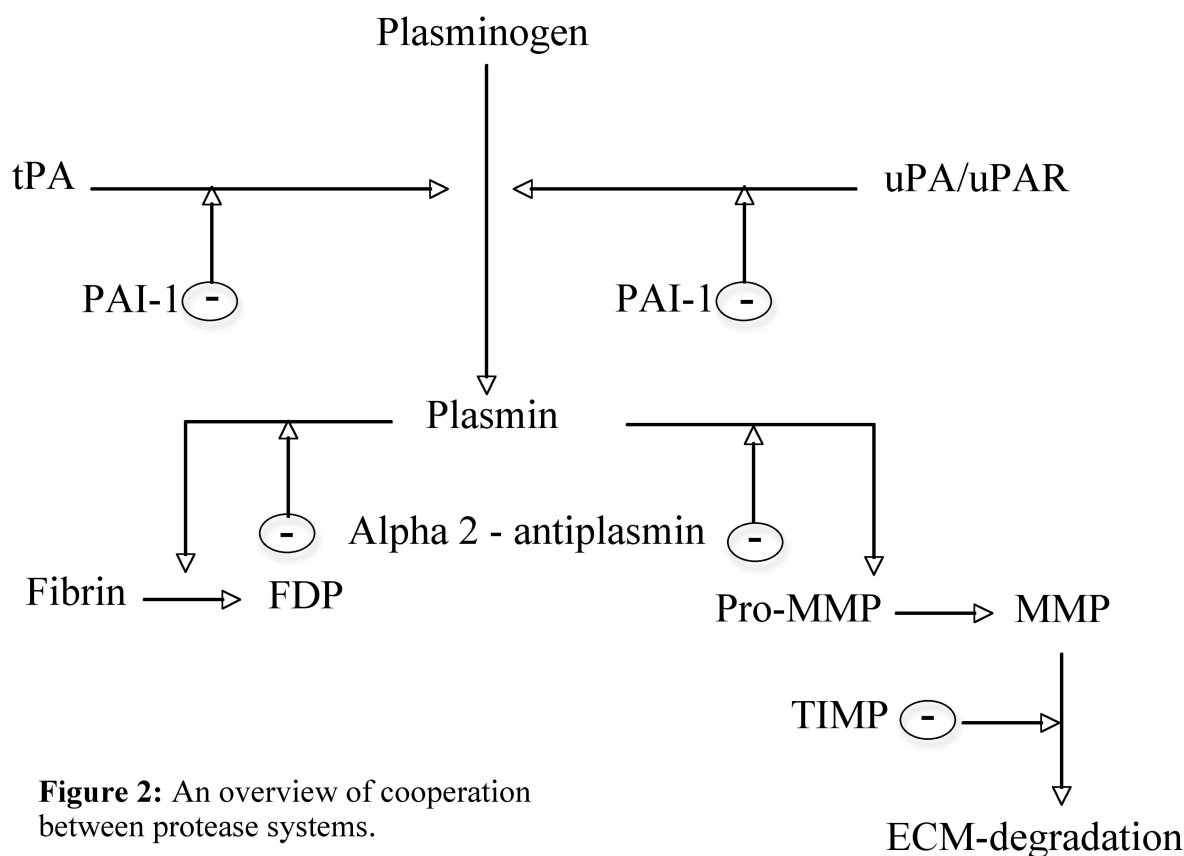


Figure 2: An overview of cooperation between protease systems.

Normally, endothelial cells and fibroblasts secrete MMPs but in the pathological state macrophages, lymphocytes and neutrophil granulocytes constitute an additional source (110). MMPs regulate physical barriers, modulating inflammatory mediators such as cytokines and chemokines and thereby establishing chemokine gradients in inflamed tissues that potentiate the migration of neutrophil granulocytes. They have a capacity to modulate immune responses in the gastrointestinal tract (98, 111). Pathological conditions such as inflammation interfere with the local balance between activators and inhibitors, resulting in ECM breakdown and tissue injury (91, 112). The imbalance between MMPs and TIMPs is suggested to be an important mechanism in inflammatory bowel diseases (99, 100, 113-116) and in other inflammatory conditions in the gut (112, 117, 118). It was therefore reasonable to assume that MMPs participate in the degrading of ECM and tissue injury in appendicitis.

The plasmin – plasminogen system

Urokinase plasminogen activator

Fibrinolytic activators and inhibitors may have an effect on inflammatory cell recruitment and migration, thereby modulating the inflammatory response. In particular, uPA and its receptor (uPAR) are involved in these processes and the expression of uPAR on leukocytes is associated with their migratory and tissue-invasive potential. The two plasminogen activators, tissue plasminogen activator (tPA)

and uPA, activate plasminogen to the active serine protease plasmin. Plasminogen is produced in the liver and released in this inactive form. The plasminogen- plasmin system is of importance for the fibrinolysis; it dissolves clots in the circulatory system as plasmin degrades the fibrinmonomer to soluble products, fibrindegrading products. Plasmin is inactivated by alpha 2-antiplasmin, a serine protease inhibitor (Fig. 2). Apart from fibrinolysis, plasmin proteolyse proteins in various other systems and activates the MMPs such as the collagenases and gelatinases. uPA is mainly produced in the endothelial cells, but also in stromacells and inflammatory cells as the neutrophils (119). The release of uPA from the endothelial cells is stimulated by circulating pro-inflammatory cytokines such as TNF-alpha and IL-1beta (120) and uPA has an positive effect on neutrophil activation and migration (94, 95, 121-124). Grondal-Hansen et al. (1989) identified uPA in tissue from appendicitis (92). The main inhibitor of uPA is PAI-1.

Plasminogen activator inhibitor type -1

PAI-1 may act as an acute phase reactant (125) and is produced in the liver as well as in endothelial cells, adipocytes and platelets where it is stored (126). PAI-1 belongs to the serine proteases and exists in active and latent forms. Active PAI-1 is only metastable and spontaneously transforms into a latent, inactive conformation (127). The various forms of PAI-1 may explain some of its differentiated functions and it has both pro- and inhibitory functions on cellmigration. PAI-1 is of importance for the processes in fibrinolysis, thrombosis and artherosclerosis and is elevated in several thrombotic conditions such as deep vein thrombosis, myocardial infarction, septicemia and disseminated intravascular coagulation (DIC) (125). The increase of PAI-1 has been correlated with advancing age, serum triglyceride level, free fatty acids (FFA) and obesity. Acute and chronic stress has been identified together with elevated plasma PAI-1 and it is released with a circadian rhythm early in the morning (128). Whawell *et al.* (1993) demonstrated that PAI-1 is over-expressed in the mesothelium of acute appendicitis and they localised it to serosal blood vessel endothelium. PAI-1 mRNA was most strongly detected in thrombosed veins of inflamed tissue (93).

Neutrophil granulocytes and proteases

The presentation of neutrophils in the muscularis propriae is an early and important criterion in diagnosing appendicitis (6). The granulocytes occur in the peripheral blood and constitute 40 - 75% of the leukocytes, whereas the neutrophils represent the major fraction. The neutrophil granulocytes participate in the innate immunity and migrate early into infected tissue where they are the first cells to defend antigens through phagocytosis and degranulation. Their lifetime are short, they circulate in the blood for a few days and occur in inactive and active forms (129).

They enter the ECM through different steps including adhesion to activated endothelial cells, "rolling" and migration through their barriers. MMP-9 has been found to be important for the disassembly of the endothelial intercellular junctions (98). Neutrophils store MMP-9 in granules and may use MMP-9 for ECM degradation in order to reach the antigen target. Both uPA and PAI-1 participate in the activation of neutrophils, especially together with gram-negative bacteria with lipopolysaccharid (LPS) (94, 95). PAI-1 may also prevent the apoptosis of the neutrophils, giving them a longer life span in their fight against antigen (130).

Bacteria and proteolysis in appendicitis

A large variation in the microbial flora has been found in appendicitis and an infective aetiology was first proposed in the end of the 19th century (37, 131). However, there is no evidence yet for bacteria as the cause of appendicitis, but a special interest has been shown for *B. fragilis* and *H. pylori* and their association with gangrenous and perforated appendicitis (39, 40, 131, 132). *B. fragilis* produces an enterotoxin called Fragilysin with a matrix metalloproteinase structure and effect. Fragilysin is able to induce intestinal damage and secretion in animals (133, 134) and has been found to be present in appendicitis (132). Moreover, Fragilysin stimulates the epithelial cells to produce IL-8 with the following chemotaxis (135, 136).

Several bacteria, including *B. fragilis*, have a capacity to recruit plasminogen to their cell surfaces that gets activated by uPA to plasmin. In this way the bacteria's presents with a host derived proteolytic activity (137, 138). There are contradictory reports of helicobacter in appendicitis and cultures for *H. pylori* have been both negative and positive while seropositive patients are associated with gangrenous and perforated appendicitis (139, 140). However, there are other helicobacter such as the *Campylobacter jejuni* earlier described to be involved in appendicitis. Thus, the authors introduced a suggestion of conservative treatment with antibiotics for appendicitis in their paper published in 1983 (141).

THE AIM OF THIS THESIS

The aim of this project was to investigate and compare the presence and expression of proteases and anti-proteases such as the MMP -1, -2, -3, -9, TIMP-1, uPA and PAI-1 in tissue samples and in plasma from patients with phlegmonous, gangrenous, perforated appendicitis and macroscopic noninflamed appendices (controls) in order to evaluate whether these factors could be used as diagnostic markers for patients presenting with or at risk for appendix perforation.

MATERIAL AND METHODS

Patients

Papers I and II

In Paper I, forty patients (26 men and 14 women) who had surgery for acute appendicitis were enrolled and two biopsies were taken from each appendix. Seven (7) specimens were excluded due to an unclear macroscopic description when compared with routine PAD. The remaining 33 specimens were classified as phlegmonous (n=15), gangrenous (n=7) and perforated appendicitis (n=11) according to the criteria described in table 1. Macroscopic normal appendices, taken from patients who had undergone hemicolectomy for tumours in the ascending colon with distance (>10cm) from the appendix, were used as controls (n=10). Patients in studies I-IV are described in Table 2.

The patients in Paper II were the same patients as in Paper I. However, there were insufficient tissue sample sizes for protein analysis in four specimens, 2 from the perforated, 1 from the gangrenous and 1 from the control group. The remaining thirty patients in the appendicitis groups were classified as phlegmonous (n=15), gangrenous (n=6) or perforated appendicitis (n=9) and the hemicolectomy patients in Paper I constituted the control group (n=9).

Paper III

Fifteen patients (8 men and 7 women) with appendicitis and a surgically confirmed perforation were included in this study. Three or four biopsies were taken from each perforated appendix at the perforation site and further away. The locations of the perforations are described in figure 3.

Paper IV

Seventy-four patients with suspected appendicitis were eligible during routine surgery and two biopsies were taken from each appendix as well as blood samples, before surgery and after 4 weeks. 57/74 patients completed their second blood sample and were included while 17 patients with uncompleted second blood samples were excluded; 5/17 had a macroscopic uninflamed appendix diagnosed during diagnostic laparoscopy, 9/17 had surgery for appendicitis, 2/17 withdrew and 1/17 had perforated diverticulitis. In summary 57 patients (34 men and 23 women) were studied and grouped into noninflamed appendix/lymphadenitis (n=7), phlegmonous (n=30), gangrenous (n=11) and perforated appendicitis (n=9) based on the surgical and pathology results.

Table 2 summarises the number of patients, demographic data, length of hospital stay and postoperative complications for the patients included in Papers I-IV.

	Patients				Paper I and II (*)		Paper III		Paper IV			
	Control	Phlegmonous	Gangrenous	Perforated	Perforated	Perforated	Uninflamed appendix	Lymphadenitis/Uninfl app	Phlegmonous	Gangrenous	Perforated	
No:	10 (9*)	15	7 (6*)	11 (10*)	15		1	6	30	11	9	
Gender (M/F)	5/5	9/6	4/3	7/4	8/7		1/0	4/2	18/12	7/4	4/5	
Age (years)	74 (49-90)	28 (16-50)	24 (21-56)	43 (38-69)	35 (18-56)		24	21 (16-44)	24 (16-45)	34 (17-61)	40 (24-65)	
WBC (10 ⁹ /L)	7.5 (4.9-14.3)	13.1 (7.2-18.3)	17.9 (14.5-20.7)	14.8 (11.4-22.3)	13.9 (9.6-22.5)		9.6	11.5 (7.8-16.7)	12.4 (6.8-18.9)	17.8 (7.7-26)	14.6 (9.8-19.1)	
CRP (mg/L)	27 (5-83)	15 (5-85)	15 (5-88)	67 (5-99)	36 (5-550)		27	34 (8-89)	10 (5-100)	28 (5-94)	40 (8-290)	
Temp (°C)	36.5 (36.1-37.3)	37. (36.3-39.1)	37.8 (37-38.7)	37.5 (36.5-38.8)	37.4 (36.3-39.1)		37.4	37 (36.5-37)	37 (36-38)	37.8 (37-39.1)	37.8 (37-38.4)	
Local peritoniti preop (no/tot)	-	3/15	2/7	5/11	6/15		0/1	1/6	7/30	8/11	4/9	
Time sympt onset – op (hr)	-	28 (10-61)	24 (21-56)	31 (19-252)	48 (21-168)		65	64 (32-108)	37 (13-117)	31 (13-85)	52 (16-144)	
Hospital stay (days)	-	2.5 (1-6)	2 (2-8)	4 (2-8)	3 (2-8)		1	3 (2-7)	2 (1-3)	2 (1-4)	3 (2-7)	
Complication postop (nr/total)	-	2/15	0/7	1/11	5/15		0/1	0/6	0/30	0/11	4/9	

METHODOLOGICAL CONSIDERATIONS

Patients

Papers I-IV

This thesis summarises four explorative studies, where the two first partly include the same patients. The patients in studies I - IV were emergency cases prospectively included during routine surgery. They were not consecutively included because research nurses performed most of the sampling during daytime while some of the surgeons sampled during their on call duty.

The exclusion of patients in studies I and II was due to unclear macroscopic descriptions of the grade of inflammation by the surgeons when compared with routine PAD (study I), or an insufficient number of samples for further analysis (study II). The controls in studies I - II were patients who underwent hemicolectomy due to a colon cancer located more than 10 cm from caecum. The controls were chosen in order to obtain biopsies from macroscopic uninflamed appendix and to avoid unnecessary surgery. It could be argued that the controls were older than the patients with appendicitis and that the latter had a malignancy. However, the results of protein analysis in tissue biopsies from patients with a macroscopic uninflamed appendix included in study IV were similar to those results demonstrated in controls in studies I and II.

In study IV, 77% of patients completed their blood samples for this study by coming to the outpatient clinic 4 weeks postoperatively. During the study period from December 2007 through May 2009, 330 patients were operated on for appendicitis in our clinic and 22% of them were included in our study. The difficulties involved in clinical research in emergency surgical patients highlight the need to improve planning for their participation and for the tissue and blood sampling performed during on call hours in clinical studies in the future. Furthermore, in study IV we compared the individual protein differences in the blood samples taken before surgery and 4 weeks postoperatively. For comparison, it might have been more appropriate to also include a healthy control group, matched for gender and age. This will be done in a future study.

Pathological Anatomical Diagnosis

Papers I-IV

The microscopic diagnoses in studies I - III were based on routine PAD, which is mainly performed to rule out tumours. A determined pathologist made a comparison of collected study biopsies and routine PAD as a complement. The discrepancies between micro- and macroscopic diagnoses in studies I and II may be explained by the fact that routine PAD is based on three sections taken from the base, centre and apical

part of the appendices, and a perforation could have been localised in a different part of the appendix. Moreover, the assessments of the routine PAD in the studies I - III revealed that the terminology for the grades of inflammation in appendicitis by several pathologists varied. In study IV, a protocol for the pathological assessments was introduced to standardise the diagnosis of the grades of inflammation performed by a determined pathologist without clinical information (criteria as in table 1). The micro – and macroscopic diagnoses corresponded in 90% of cases in study IV.

TISSUE AND PLASMA SAMPLING AND PROCESSING

Tissue Samples

Papers I, II and IV:

Immediately after appendectomy, two circular 0.5 cm sections - one for protein analysis and one for immunohistochemistry (IH) - were sampled from the centre of the appendix according to a standardised protocol. The purpose of the standardised sampling from the centre of the appendix in studies I, II and IV was to avoid tissue samples taken from locally extended inflammation that can occur in the distal part (6).

Paper III:

Immediately after appendectomy three 0.5 cm circular biopsies were taken in a standardised way for protein analysis, one at the site of perforation and the others at an increasing distance (0.5 and 1.0 cm) from the perforation (Fig. 3). When feasible, depending on the location of the perforation, one additional distal biopsy at 1.5 cm from the perforation was taken. This was possible in seven out of the fifteen specimens and allowed for protein analysis further away from the perforation. To investigate and compare the distribution of the proteases and their inhibitors in different parts of perforated appendicitis, we used nonperforated and noninflamed appendices as controls. The appendices were divided along the sagittal plane, and half-circular biopsies were collected at distances approximately 0.5, 1.0 and 1.5 cm from the perforation, similar to the samples taken for the protein analysis (Fig. 3).

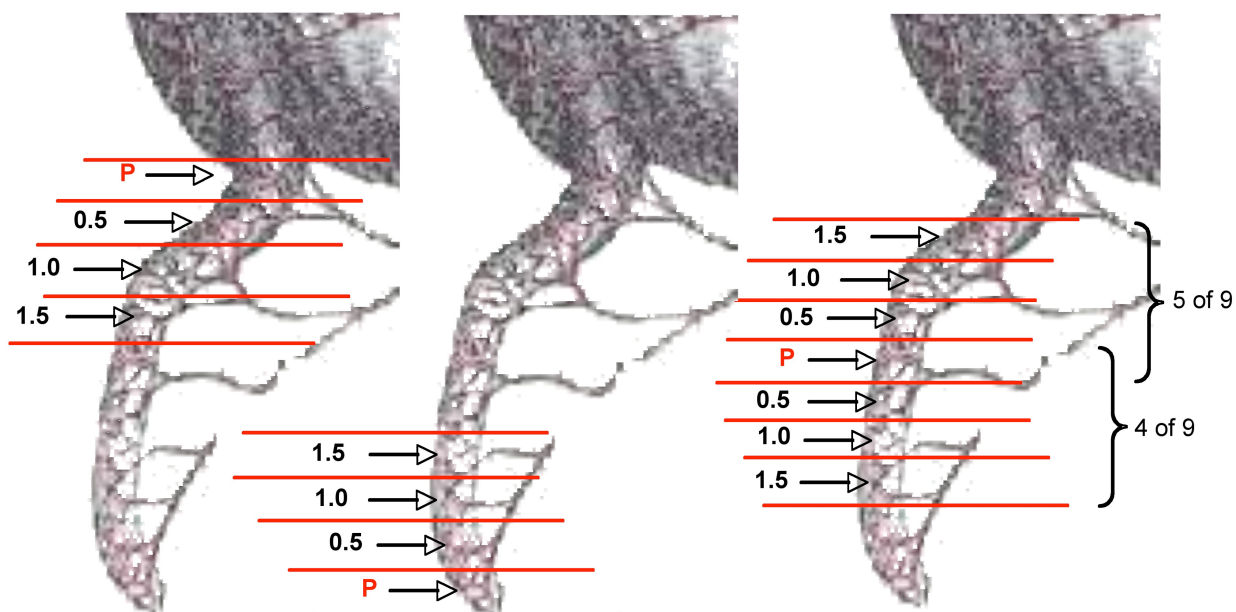


Figure 3: Locations of perforation in relationship to study samples. In 15 biopsies; 4 had a perforation close to the base, 2 were located in the distal part and 9 samples had the perforation in the centre of the appendix.

Handling of tissue samples

In the operation theatre all biopsies for protein analysis were snap frozen in liquid nitrogen, and then kept in freezer with -80°C until further analysis with Enzyme Linked ImmunoSorbent Assays (ELISAs). The second biopsy in studies I - II was taken to localise proteins by IH and put in fixation, dehydrated and embedded in paraffin until further immunostaining. The second biopsy in study IV was divided and sent for bacteria cultures.

Plasma samples

In study IV, venous blood samples were taken with vacutainer system for protein analysis and collected twice from each patient in a standardised manner. The first blood sample was taken before surgery after the induction of anaesthesia and the second after 4 weeks in the out patient clinic. Venous blood was collected in EDTA, citrate, Diatub (CTAD) and stabylite tubes. Blood samples were immediately put on ice and centrifugated at 10 000 g, in 4°C for 10 minutes. The supernatants were collected in aliquots and kept frozen at -80°C until further assay. Proteases and antiproteases were measured in plasma (142).

Protein extraction

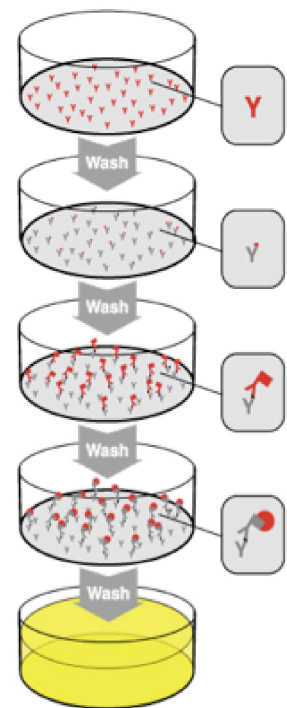
After thawing, samples were weighed and homogenised using an Ultra – Turrax (24 000 rpm) in phosphate buffer saline (PBS) buffer containing 0,01% Triton X-100 solution employing 1 ml buffer per 40 mg tissue. The homogenate was centrifugated (10 000 g, 3 minutes) and the supernatant collected was frozen at -80°C until further assayed. The homogenisation was performed on ice to prevent proteolysis.

Protein analysis

Protein assay with Enzyme Linked ImmunoSorbent Assay (ELISA) Papers I – IV

Methods:

Commercially available ELISA kits were used to detect and quantify tissue samples for MMP-1, -2, -3, -9, TIMP-1, uPA, PAI-1 and plasma samples for MMP-9, TIMP-1 and PAI-1. These methods are well established in our laboratory. The antigen is attached to pre-coated plates and excess antigen is washed. A secondary antibody specific to the antigen together with a linked enzyme is then attached to the plate. By using an antibody-antigen reaction as well as an enzyme fraction, this technique transforms a peroxidase sensitive substrate into a colour. The absorbtion at a specific wavelength was quantified by spectrophotometer (V-max, Molecular Devices) to measure concentration. Internal standards of known concentrations are used to quantify the optical densities of the samples.



MMPs and TIMP-1

The homogenised samples were analysed for MMP-1, -2, -3, -9 and TIMP-1 using ELISA kits from Amersham Pharmacia Biotech (Buckinghamshire, UK), as described above.

The plasminogen system

The levels of uPA were determined using an ELISA kit from Technoclone while the levels of PAI-1 were determined employing a kit from Biopool.

Total protein

The total protein level was measured with a kit from Bio-Rad (Hercules, CA). The final concentration of each protein was expressed as nanograms of target protein per milligram (ng/mg) of protein extract (143), and as nanograms of target protein per millilitre (ng/mL) for proteins assessed in plasma in Paper IV.

Variability in ELISA

All assays were run in duplicate by two experienced laboratory technicians. For the assays used in these studies the inter-assay variation was approximately 5 - 10% according to the manufacturer, which may be of importance during interpretation of results, especially results when the statistical significance is close to $p < 0.05$.

Protein distribution and morphology

Immunohistochemistry and monoclonal antibodies

Biopsies for immunohistochemical analysis were immediately fixed in Bouin's solution (Sigma Diagnostica, St Louis, MO, USA) left overnight, washed in phosphate buffered saline (PBS) (Sigma Diagnostica), pH 7.4, dehydrated in a graded series of ethanol, cleared and embedded in paraffin. Consecutive sections with a thickness of 5 - 7 μm mounted on slides were used, deparaffinised and rehydrated with xylene and a graded series of alcohol. After rinsing in distilled water, the immunostaining employing primary antibodies towards human agents started. All antibodies were mouse anti-human monoclonal IgG and used together with the DAKO Envision system (DAKO Cytomation, Glostrup, Denmark). The following concentrations and clones of antibodies were used, MMP-2 (1 mikrog/ml, clone 75 -F7), MMP-9 (2 mikrog/mL, clone 56-2A4) recognizing both latent and active MMP-2 as well as MMP-9 while the TIMP-1 antibody (4 mikrog/mL, clone 147-6D11) was derived from Calbiochem/Oncogen (Cambridge, MA, USA). The uPA antibody (American Diagnostica #3689, Stamford, CT, USA) recognises the inactive single-chain, active two-chain and receptor bound forms. The PAI-1 antibody (American Diagnostica #3785) recognises free PAI-1 and PAI-1 complex bound to tPA, without cross reactions with PAI-2 or PAI-3, according to the manufacturers' specification. Following incubation with peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin, the sections were incubated using 3,3'-diaminobenzidine as a chromogenic substrate, in line with the manufacturers's instructions (EnVision, DakoCytomation, Carpinteria, CA, USA). As negative control we used monoclonal mouse antibodies of isotype IgG, the specificity of which is directed towards *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in

mammalian tissues (DakoCytomation, Carpinteria, CA, USA). To visualise the morphology, the specimens were stained with haematoxylin and eosin (H&E). All slides were evaluated regarding localisation of the immunogen using Eclipse E800 (Nikon, Tokyo, Japan) together with Nikon Coolpix 995 digital photo equipment (Nikon Instruments Inc, Meville, N.Y, USA). Microscopically, areas of immunoreactivity were visualised as dark brown staining, demonstrating oxidation of the diaminobenzidine.

Statistical methods and considerations

We used nonparametric tests due to the small sample and the fact that patients could not be considered to have a normal distribution, which was also tested with descriptive statistics. In studies I, II and IV, Kruskal Wallis test was employed to detect statistically significant overall differences and the Mann-Whitney U test for comparison between groups. In study III, tests to identify differences were performed with the non-parametric Friedman test and analysis with the Wilcoxon signed rank test in case of paired observations. Spearman rank correlation in studies III and IV assessed potential correlations. All graphs are presented as box-plots showing the median (horizontal line), interquartile range (boxes) as well as the 10th and 90th percentile (error bars). Statisticians were consulted for Papers I – IV.

Multiple analyses are at risk for mass significances and will by chance alone make every twentieth test significant at the 5% level. Therefore compensation through a Bonferroni correction or by adjusting the p-value to a value below 0.05 according to the number of analyses could be done to confirm the differences. However, using a correction increases the risk for a beta-error with undiscovered differences in the results. The most important results in studies I, II and IV remained significant when a Bonferroni correction was made. In study III, the Spearman test showed that the expressions of MMP-9, -1, -2 and PAI-1 correlated significantly with the distance from the perforation sites.

Ethical aspects

The Regional Ethics Committee at Gothenburg University, Gothenburg Sweden approved these studies. All patients gave informed consent.

RESULTS AND COMMENTS

Tissue

Papers I, II and IV:

As described in Paper I, the protein expressions of MMP-1, -2 and -9 seemed to form a different pattern in gangrenous and perforated appendicitis, with a higher MMP-1 and -9 and lower MMP-2 in these groups compared with phlegmonous appendicitis and controls (Fig. 4). There were no differences for MMP-3 and TIMP-1 between the groups. A scattered distribution in the appendiceal wall of MMP-9 and TIMP-1 was demonstrated immunohistochemically in both gangrenous and perforated appendicitis (Fig. 5).

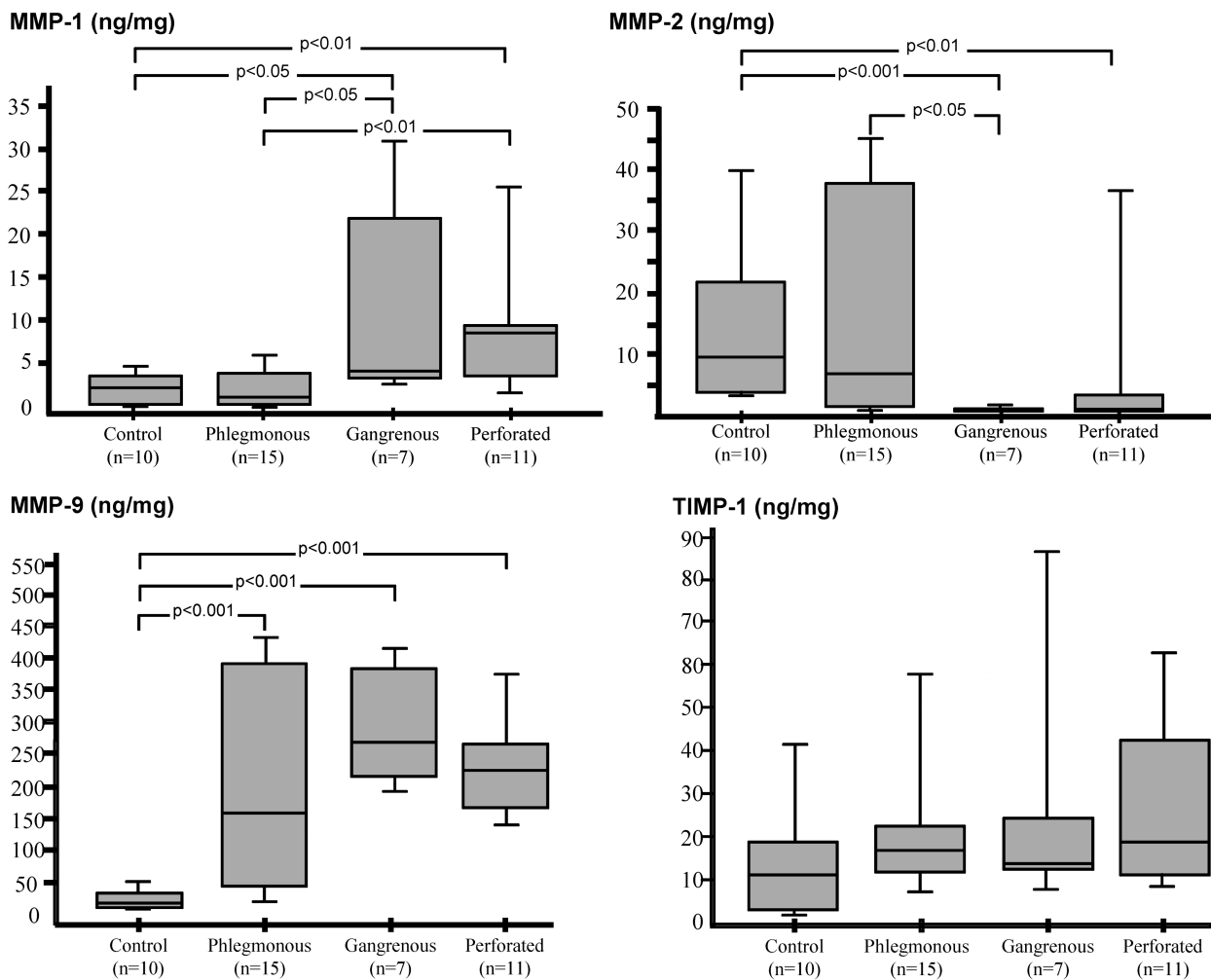
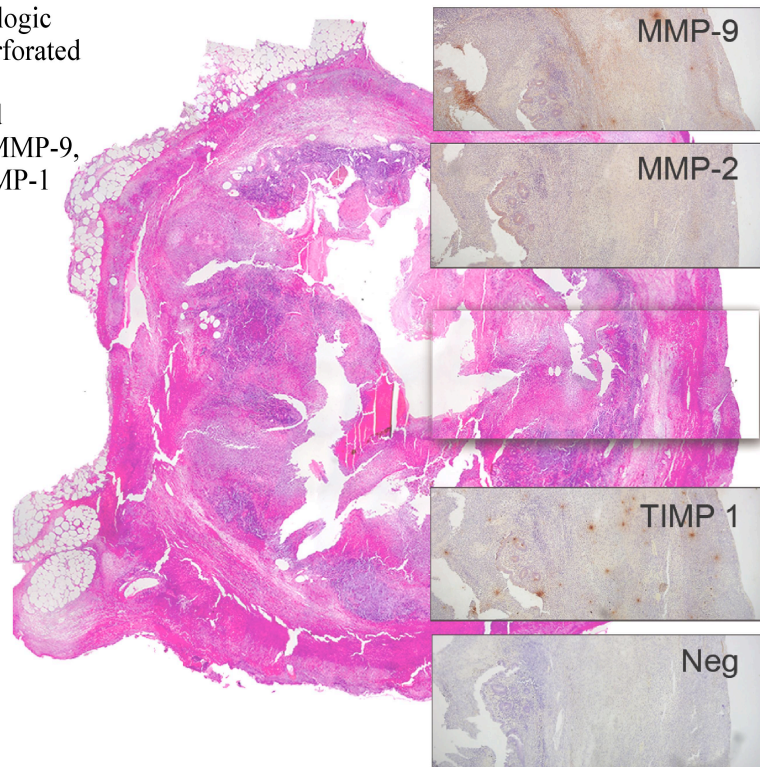


Figure 4: Summary of results in paper I.

This scattered distribution may consist of "hot spots" where the expression of protease and anti-protease were locally increased. One or more hot spots could lead to aggravated ECM degradation involved in tissue injury and a perforation. A similar pattern has been found in the wall of abdominal aortic aneurysms and in the Graaf's follicle before ovulation (107, 144). The results for the protein expressions of MMP-9 and TIMP-1 in gangrenous and perforated appendicitis supported this suggestion.

Figure 5: Histologic section from perforated appendicitis, morphology and localisation of MMP-9, MMP-2 and TIMP-1



The protein expressions of uPA and PAI-1, described in Paper II, demonstrated that they were involved in acute appendicitis, thus confirming earlier results (92, 93). In all groups of appendicitis uPA elevated twice, while PAI-1 elevated 18 times in phlegmonous, 34 times in gangrenous and 58 times in perforated appendicitis

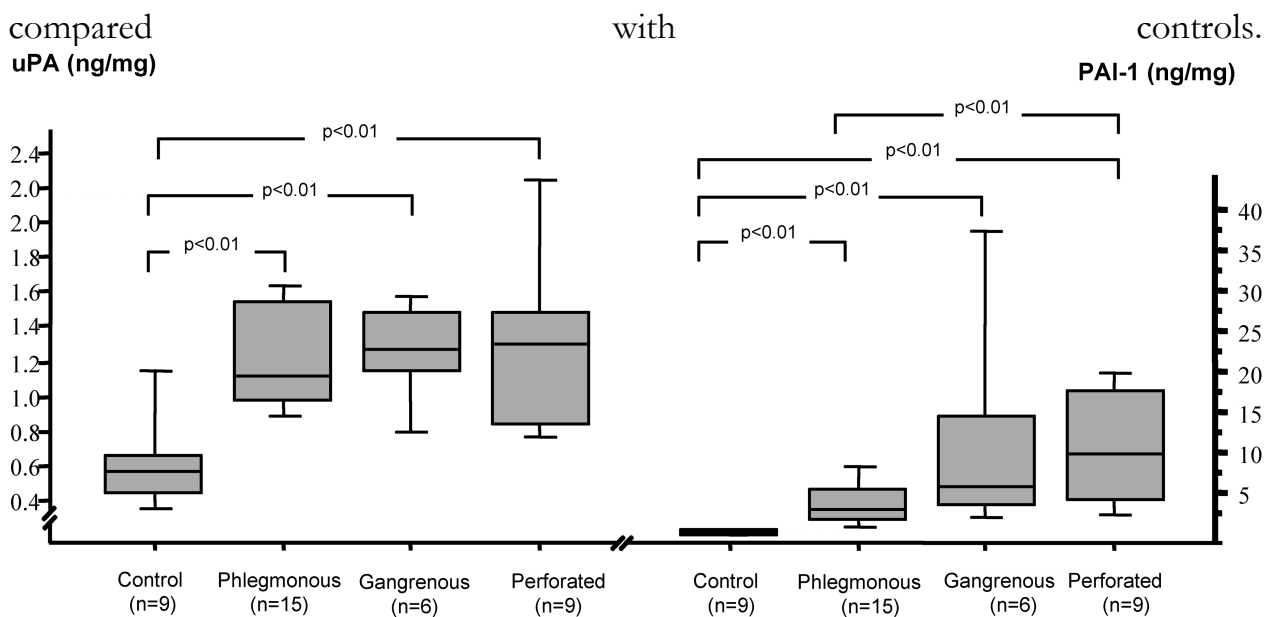


Figure 6: Summary of protein analyses in Paper II.

Moreover, PAI-1 was higher in perforated appendicitis when compared with phlegmonous appendicitis (Fig. 6).

The distribution of uPA showed a scattered pattern in all groups of appendicitis occurring in close connection with the vascular endothelial cells. The endothelial cells are the main producers of uPA, and the release of uPA is stimulated by proinflammatory cytokines (119, 121).

PAI-1 was most intense in the serosa and outer muscle layer, and PAI-1 has earlier been localised to thrombosed vessels in the serosa (93). The expressions of uPA, and especially those of PAI-1, seemed to correlate with the progression of the local inflammatory response in appendicitis.

The results for MMP-9, TIMP-1 and PAI-1 in tissue (ng/mg) in another cohort of patients with appendicitis described in Paper IV, confirmed the results in Paper I – II.

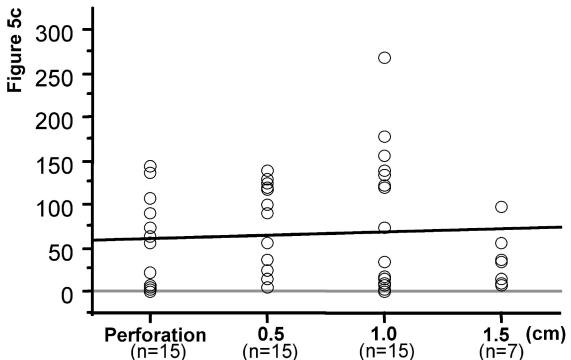
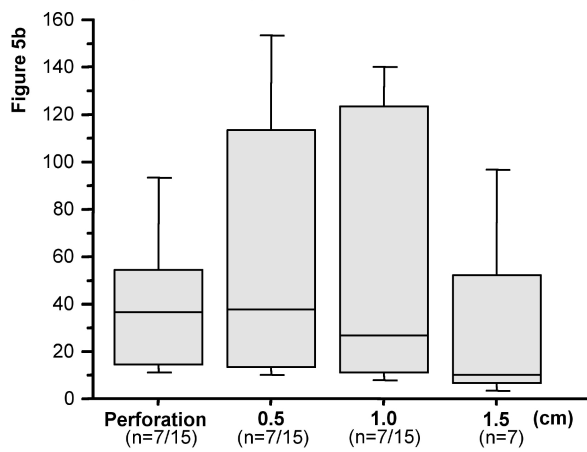
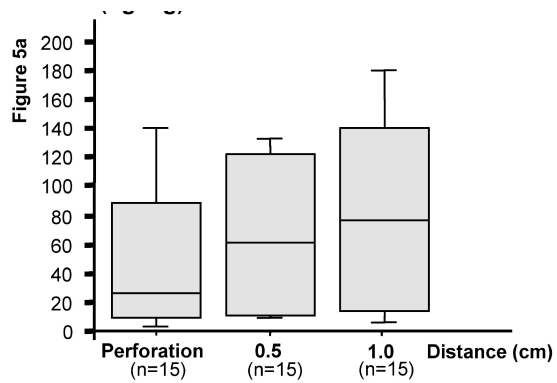
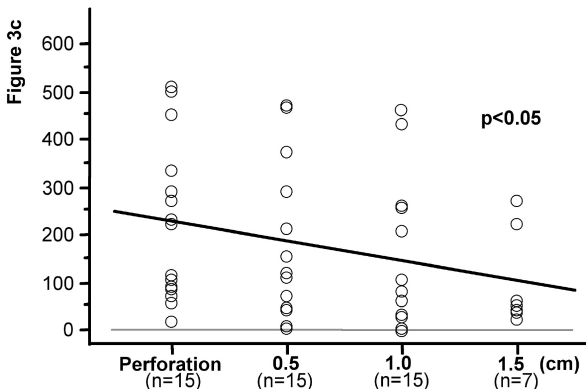
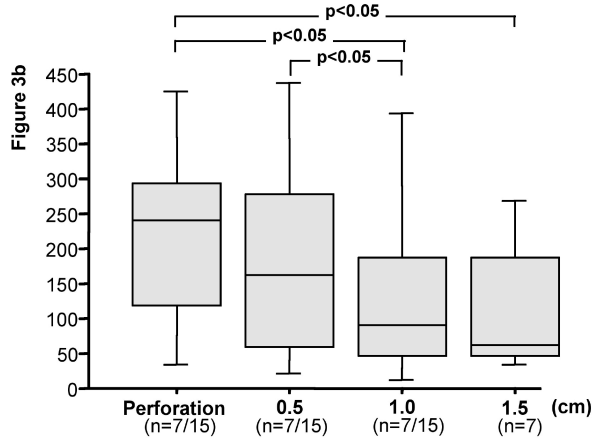
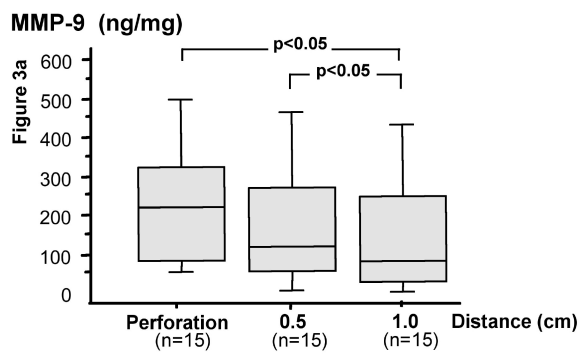


Figure 7a: MMP-9 and TIMP-1 in biopsies at the sites of perforation in 15 specimens (a) and in 7/15 specimens (b). The correlation between MMP-9 and TIMP-1 expression with the distance from the perforation (c).

Paper III:
Tissue from perforated appendix

The expressions of MMP-1, -2 and -9 and PAI-1 showed an individual variation in relation to the perforation sites and further away. MMP-9 and PAI-1 were found to be highest at the perforation sites, while MMP-1 was higher close to them and MMP-2 gradually decreased away from them (Fig. 7a and b). No difference was seen in TIMP-1 and uPA.

MMP-9 has been shown to be involved in perforation sites in other organs (106-109). The combination of the over expression of PAI-1 and local imbalance between MMP-9 and its inhibitor TIMP-1 present a potential pathway for ECM breakdown and seemed to have a strong impact on the tissue injury leading to an appendix perforation. The results for IH in Paper III demonstrated that the staining for MMP-9 as well as signs of severe ischemia with necrotic tissue became intensified towards the perforation site.

(Figures 7a and 7b are sequentially numbered according to Paper III).

Plasma

Paper IV:

The individual differences in plasma (ng/mg) between samples taken preoperatively and after four weeks lacked statistical significance for MMP-9 and PAI-1, while the differences for TIMP-1 were higher in patients with perforated appendicitis than in them who had phlegmonous and gangrenous appendicitis (Fig. 8).

Dalal et al. (2005) demonstrated a higher serum TIMP-1 in children with perforated appendicitis compared with them who had nonperforated appendicitis (145) while another study described that TIMP-1 in systemic blood reflected the severity of ulcerative colitis (146).

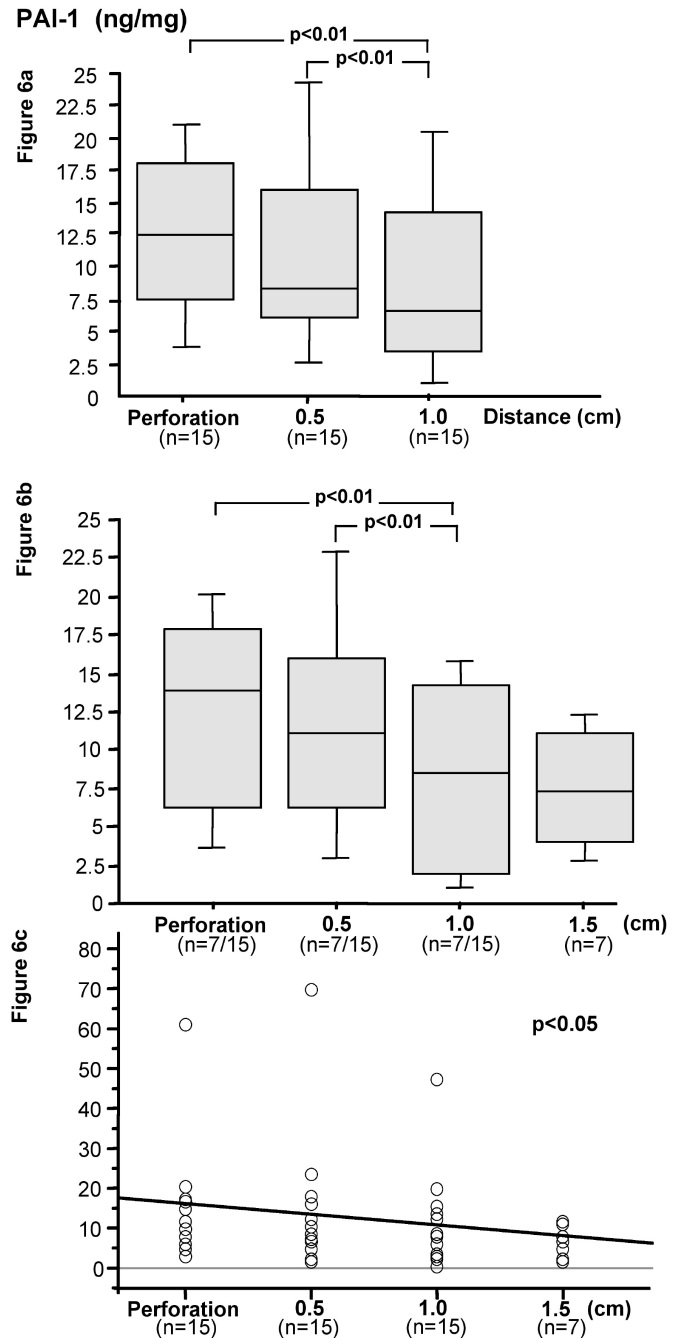


Figure 7b: PAI-1 in biopsies at the sites of perforation in 15 specimens (a) and in 7/15 specimens (b). The correlation between PAI-1 expression and the distance from the perforation (c).

Furthermore, Lorente et al. showed that the MMP-9/TIMP-1 ratio in serum in patients with septicemia correlated both with the severity and with survival (147). However, the MMP-9/TIMP-1 ratios in this study could not predict the severity of appendicitis as they were low in both perforated appendicitis and controls and further studies are required.

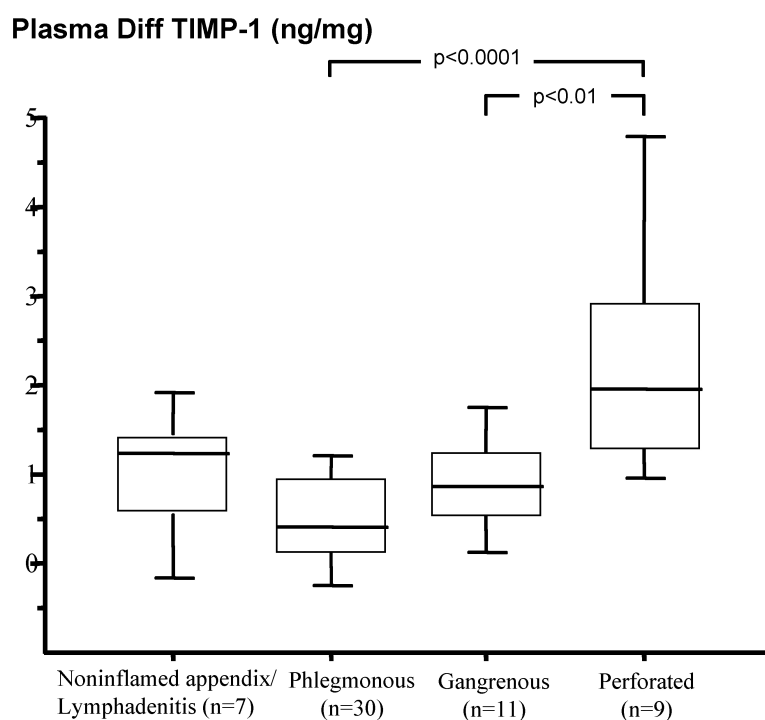


Figure 8: The individual differences in TIMP-1 (ng/mg) plasma preoperatively and in controls were higher in perforated appendicitis compared with phlegmonous ($p < 0.0001$) and gangrenous appendicitis ($p < 0.01$).

There was a positive correlation for PAI-1 (ng/mg) in plasma and in appendix tissue ($p < 0.05$).

Furthermore, plasma PAI-1 and TIMP-1 (ng/mg) correlated with increasing age ($p < 0.05$).

The prevalence of various bacterial species was similar in the different grades of inflammation, and antimicrobial resistance to commonly used antibiotics was found in over 50% of the cultures. The antibiotic resistance found in the appendix biopsies might mirror the resistance in fecal bacteria under in-hospital conditions. However, the results call for caution over the use of antibiotics as the only primary treatment for appendicitis in a larger population.

GENERAL DISCUSSION

Proteases and anti-proteases in appendicitis

The results of Papers I – IV, revealed that proteases and anti-proteases could be found in all grades of appendicitis. There are several aspects as to how these molecular processes might possibly lead to a perforation in appendicitis. An imbalance between MMP-9 and TIMP-1 in combination with an over expression of PAI-1 seem to participate in a perforation in appendicitis.

Neutrophil granulocytes are important in the innate immunity. They use proteases such as MMP-9 in their degradation of barriers in order to reach their antigen target (98). However, they may cause tissue injury in the inflammatory process and an up-regulation of MMP-9 in colonic and peripheral neutrophils has previously been shown to have effects on the severity in an experimental model of colitis in mice (113). MMP-9 was the most abundant protease in tissue in all groups of appendicitis compared with controls (Paper I and Paper IV) as well as at the perforation sites compared with further away (Paper III). Neutrophils might constitute the major source of MMP-9 in appendicitis. The severity of appendicitis has been linked to elevated level of IL-8, localised in neutrophil granulocytes and monocytes in the appendiceal tissue (148). The chemokine IL-8 binds to surface receptors on the neutrophils, attracts them to the site of inflammation (61, 149) and stimulates them to degranulate. MMP-9 from the granulae truncates IL-8 to become at least tenfold more potent leading to further chemotaxis (150, 151). Moreover, PAI-1 is also capable to modify IL-8 with a possible enhanced neutrophil recruitment as the result. PAI-1 may also affect the neutrophils to survive longer in the inflamed tissue, hence leading to increased tissue injury (130, 152). There is evidence that the neutrophils migrate as a result of chemokine gradients (61) and the individual variation of MMP-9 and PAI-1 in relation to the sites of perforation as demonstrated in Paper III, may reflect such a gradient leading to increased ECM degradation and tissue injury.

Proteases are produced and used by bacteria when they invade and infect tissues. *B. fragilis* produces an enterotoxin called Fragilysin with MMP structure and effect. Fragilysin is known to stimulate colonic epithelial cells to produce IL-8 (135) and *B. fragilis* could be a pathogen correlated to the risk of a more severe inflammation in appendicitis (37, 39, 40, 132). It occurred in cultures from all groups of appendicitis but most in gangrenous appendicitis, described in Paper IV. There were no positive cultures for *H. pylori*. However, it does not rule out its occurrence in appendicitis. *H. pylori* stimulates colonic epithelial cells to produce MMPs and other epithelial cells such as the gastric cells to produce PAI-1(153, 154). The presence of *H. pylori* and its possibly impact on the severity of appendicitis needs further study.

Clinical studies with ultrasound Doppler imply that the local circulation in the appendix is closely related to the severity in appendicitis. It appears that hyperaemia occurs in the appendix wall in phlegmonous appendicitis while the blood flow diminishes in gangrenous and perforated appendicitis (155, 156). There is also evidence that occlusion of the blood supply to the appendix results in appendicitis (157). PAI-1 inhibits the activation of plasminogen to plasmin and impairs the fibrinolysis and the simultaneous activation of MMP-1, -3 and -9. An impaired activation of MMP-9 may stimulate to an increase in the MMP-9 expression as a compensation for the increase in PAI-1, which may explain this paradox. The elevated expressions of PAI-1 in perforated appendicitis described in Papers II – IV, may cause microvascular thrombosis and ischemia resulting in weakening of the appendiceal wall. Furthermore, the ECM degradation of fibrous tissue in the appendix in appendicitis patients combined with increased age may lead to ECM products with an elevated potential for chemotaxis and an increase in neutrophil effects (102).

The release of PAI-1 by the mesothelial cells is known to lead to peritoneal hypofibrinolysis involved in adhesion formation (158, 159). When a perforation occurs in the abdominal cavity, adhesions takes place simultaneously in an attempt to “wall off” and restrict the inflammatory process. PAI-1 has pleiotrophic functions and may participate in the innate immunity, modulating the local and systemic inflammatory responses. There is also evidence of a polymorphism in PAI-1, which may influence its penetrating capacity associated with Crohns disease (160).

Patients with gangrenous and perforated appendicitis have earlier been found to have systemically elevated IL-6 (53, 161, 162) and a polymorphism in the gene for IL-6 has been associated with the severity of appendicitis (163). IL-6 has also been described as an important link between inflammation, coagulation and the fibrinolytic factors involved in tissue injury (123). The reason for the larger individual differences in plasmaTIMP-1 in patients with perforated appendicitis compared with them with phlegmonous and gangrenous appendicitis, described in Paper IV, is unknown. However, platelets have been identified as the main source of TIMP-1 in plasma (164) and neutrophils collaborate with platelets in innate and adaptive immunity (165). Hence, MMP-9, TIMP-1 and PAI-1 in appendicitis seem to participate in the dual interaction of coagulation and inflammation (123).

Future perspectives

The liberal indication for surgery established more than 100 years ago and associated with a high rate of negative appendectomies has changed. Today active observation and increased imaging with CT and US have become routine in order to achieve more

accurate diagnosis. However, the reduction in the negative appendectomy rate may be overturned by the increase in appendectomies in patients whose appendicitis may have resolved spontaneously if not imaged on CT or US. Despite CRP and CT as diagnostic tools for appendicitis and a growing frequency of appendectomies, appendix perforations seem to increase (46). According to Livingstone (46) one possible reason might be an infection with a pathogen or pathogens with a slowly increasing prevalence in the population. The bacteria within the fecalith could influence the severity of appendicitis and needs further study (166).

Appendicitis is not harmless and measuring the systemic inflammatory response with reliable diagnostic markers during the course of appendicitis could facilitate an individual treatment, avoiding unnecessary surgery and improving outcome (167). The TIMP-1 results seem to have the potential to mirror the severity of appendicitis systemically. Further prospective studies with consecutive sampling of TIMP-1 starting in the emergency ward and then continuing every fourth to sixth hours during the hospital stay is warranted.

The understanding of the crosstalk between inflammation, coagulation and fibrinolysis involved in the ECM-degradation leading to tissue injury will probably increase. Such understanding will provide insights into the pathogenesis in inflammatory conditions in the gut with risk for a perforation and future treatment may involve new biological drugs using this knowledge.

Conclusions

- MMP-9 was the most abundantly expressed MMP of those investigated in inflamed appendix
- The expression of uPA and especially the over-expression of PAI-1 correlated with the progression of the local inflammatory response in appendicitis
- The expressions of MMP-1, -2, -9 and PAI-1 showed an individual variation in relation to the perforation sites and further away
- An imbalance between MMP-9 and its inhibitor TIMP-1 in combination with the over expression of PAI-1 at the perforation sites seem to have a strong impact on the tissue injury leading to a perforation in appendicitis
- TIMP-1 in plasma could have a potential of becoming a possible diagnostic marker for the severity of appendicitis

Sammanfattning på svenska

Misstänkt appendicit är den vanligaste orsaken till bukoperation i Västvärlden och ca 12 500 patienter opereras varje år i Sverige. Appendicit kan ha ett oförutsägbart förlopp och 1/3 av patienterna har atypiska symptom. Ca 20 % av de som insjuknar drabbas av en perforation, vilket medför ökad morbiditet och mortalitet, medan andra insjuknar i ett lindrigare förlopp. Det är med dagens diagnostik svårt att veta om det föreligger risk för perforation och därmed svårt att välja lämplig behandling.

Det finns kliniska, epidemiologiska och immunologiska data som talar för att genesen till perforerad och ickeperforerad appendicit skiljer sig åt. Syftet med avhandlingsarbetet var att undersöka huruvida molekylära tekniker skulle kunna avspegla det inflammatoriska svaret såväl lokalt som systemiskt vid olika grader av appendicit och om möjligt kunna leda till en ökad förståelse till hur en perforation uppstår .

Extracellulär matrix, (ECM) utgör grundsubstansen i alla organ och den är uppbyggd av kollagen, proteoglykaner, laminin, elastin och adhesionsmolekyler som integriner och fibronectin. ECM remodeleras hela tiden genom vävnadsnedbrytande proteaser såsom matrixmetalloproteinaser (MMP). MMPs förekommer i latent och aktiv fas. De kontrolleras på transkriptionsnivå, proteolytisk nivå och genom direkt hämning av tissue inhibitor of metalloproteinase (TIMP) och de aktiveras bl a av varandra, serine proteaser, plasmin och av cytokiner. MMP-1, -2, -3 och -9 kan tillsammans bryta ned alla delar av ECM inklusive basalmembran. Serin proteaserna urokinas plasminogen activator (uPA) och dess hämmare plasminogen activator inhibitor typ 1 (PAI-1) interagerar med MMP systemet. UPA aktiverar plasminogen till plasmin, vilket i sin tur kan aktivera MMP-1, -3 och -9. Dessutom påverkar uPA och PAI-1 migrationen och aktivitet hos de neutrofila cellerna.

Ett tidigt mikroskopiskt tecken vid appendicit är nedvandring av neutrofila granulocyter i appendixväggen. Neutrofila granulocyter är bärare av granulae som innehåller bl a MMP-9 vilka degranuleras vid stimulering. Vid inflammatoriska tillstånd råder en obalans mellan proteaser och antiproteaser vilken kan leda till en ökad nedbrytning av ECM och vävnads skada.

Föreliggande avhandling har kartlagt förekomsten av proteaser och antiproteaser vid olika stadier av appendicit samt även i själva perforationsstället och i vävnaden intill. Vidare har studerats huruvida de i vävnad förekommande proteaserna avspeglas i blodet, samt om dessa är korrelerade till svårighetsgraden av appendicit.

Resultaten visade att en obalans mellan MMP-9 och TIMP-1 samt ett ökat uttryck av PAI-1 hade betydelse för uppkomsten av vävnads skada som kan leda till en

perforation vid appendicit. TIMP-1 speglade graden av inflammation systemiskt och skulle kunna ha en potential att utgöra en diagnostisk markör för patienter med perforerad appendicit eller med appendicit och risk för perforation. Detta skulle kunna vara ett stöd vid beslut om operation och leda till en minskning av komplikationer och sjukhusvård.

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