

PERITONEAL FIBRINOLYSIS DURING
PNEUMOPERITONEUM
AND LAPAROSCOPIC SURGERY

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ABSTRACT

BACKGROUND

Laparoscopic surgery is believed to induce less postoperative adhesion formation compared with open procedures, but information regarding biological impact of a laparoscopic approach is limited.

MATERIAL & METHODS

Peritoneal response to laparoscopic surgery was assessed in human peritoneal tissue in two clinical trials (paper I & V). Human mesothelial cell response to CO₂ was assessed in cell culturing media (paper II & III). Measurements of peritoneal pH was evaluated in an animal model (paper IV), and then performed in patients (paper V). Assays of the key fibrinolytic enzymes t-PA and PAI-1 were done at protein (paper I-III & IV) and mRNA levels (paper II & III).

RESULTS

The initial clinical study showed a similar decrease in peritoneal t-PA activity during both open and laparoscopic cholecystectomy. However, there was a higher initial peritoneal PAI-1 concentration in the laparoscopic group, which might be attributable to prior exposure to CO₂. The *in vitro* studies showed an up regulation of PAI-1 mRNA production in cells exposed to CO₂ or acidic conditions, and that acidification could be caused by CO₂. *In vivo* studies of peritoneal pH showed an immediate decrease to 6.5 during insufflation, reproducing the CO₂ effect during surgery. He did not affect peritoneal pH. When initial peritoneal exposure to CO₂ was controlled for, peritoneal levels of t-PA decreased and PAI-1 increased during laparoscopic surgery regardless of gas used. These findings are consistent with observations done in open surgery and indicate that the effect is related to the surgical trauma. However, the t-PA activity was better preserved using CO₂ suggesting that use of CO₂ might have less adverse effect on peritoneal fibrinolysis than He.

CONCLUSION

Peritoneal fibrinolytic response during laparoscopic surgery is similar to open surgery, but CO₂ elicits specific biological effects. Exposure of peritoneum and mesothelium to CO₂ leads to a local and systemic acidosis and seems to have direct effects on key fibrinolytic enzymes. The systemic acidosis is manageable through controlled ventilation. The clinical implication of the effect on peritoneal fibrinolysis is unclear, but the observations done in humans are consistent with a reduced propensity for adhesion formation.

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LIST OF PUBLICATIONS

- I. Peritoneal response to pneumoperitoneum and laparoscopic surgery.
Bergström M, Ivarsson ML, Holmdahl L,
Br J Surg 2002;89(11):1465-9
- II. CO₂ promotes plasminogen activator inhibitor type 1 expression in human mesothelial cells.
Bergström M, Falk P, Holmdahl L,
Surg Endosc 2003;17(11):1818-22
- III. Effect of acidosis on expression of mesothelial cell plasminogen activator inhibitor type-1.
Bergstrom M, Falk P, Holmdahl L,
Surg Endosc 2006;20(9):1448-52
- IV. Peritoneal and systemic pH during Pneumoperitoneum with CO₂ and helium in a pig model.
Bergstrom M, Falk P, Park PO, Holmdahl L,
Surg Endosc 2007, *e-pub ahead of print*
- V. Clinical evaluation of peritoneal acidification and fibrinolytic response during laparoscopy, a randomized parallel group study comparing Helium and Carbon dioxide.
Bergstrom M, Falk P, Park PO, Bengtsson JP, Haglind E, Holmdahl L,
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ABBREVIATIONS

c-DNA	copy of DNA used in RT-PCR
CO₂	Carbon Dioxide
CV	Coefficient of Variance
ELISA	Enzyme Linked ImmunoSorbent Assay
FDP	Fibrin Degradation Products
FGF	Fresh Gas Flow
He	Helium
HIF	Hypoxia Inducible Factor
MMPs	MatrixMetalloProtinases
mRNA	messenger RNA
PAI-1	Plasminogen Activator Inhibitor -1
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction
t-PA	tissue type Plasminogen Activator
uPA	urokinase type Plasminogen activator
uPAR	uPA receptor
VCO₂	CO ₂ elimination
V_E	Expiratory minute ventilation

INTRODUCTION

LAPAROSCOPIC SURGERY

Laparoscopy was first attempted in the early 20th century by Kelling, who used a cystoscope to visualise the abdominal cavity in a dog model (1). A Swedish internist, H-C Jacobaeus learned the technique and used it for diagnostic procedures in both the peritoneal and pleural cavities in patients with tuberculosis. During the 20's and 30's, laparoscopy remained a diagnostic tool for internists, and was not used for surgery until the 1940's when it was taken up by gynaecologists. A development of tools and insufflators started, and in 1980, the first laparoscopic appendectomy was carried out by Kurt Semm (2). His technique was then adopted and adjusted by Eric Muhe who performed the first laparoscopic cholecystectomy in 1985 (3). Although questioned at the time, these events started the laparoscopic expansion in general surgery. However, it wasn't until Philip Mourau, a French general surgeon, who performed the first modern laparoscopic cholecystectomy in 1987, that laparoscopic technique and applications started to evolve rapidly. Today the laparoscopic technique is well established, and is the standard of care for several procedures, such as cholecystectomy, Nissen fundoplication, gastric by-pass and now also increasingly for colectomies.

The clinical advantages of laparoscopic surgery are well known, including shorter hospital stay, less postoperative pain, more rapid recovery and shorter periods of sick leave being the most important (4-6). The laparoscopic technique seems to induce a milder systemic immune response than open procedures (6-10), and is even considered to be beneficial in cases with peritonitis (5, 11). A reduced incidence of postoperative wound infections after non-contaminated surgery has been reported in several studies (9). The impact of laparoscopy on postoperative adhesion formation is also clinically relevant. Adhesions form to a lesser extent after laparoscopic surgery and are thinner and filmier in quality, (12-15) than after open procedures. Adhesions formed after laparoscopic surgery are more localised to port-site incisions and to the actual operating area, but less to distant sites in the abdominal cavity. However, the clinical significance of this is unclear and there are varying reports on the incidence of bowel obstruction after laparoscopic procedures compared with open procedures. A French retrospective study reported the same incidence of postoperative bowel obstruction after corresponding open and laparoscopic procedures (16). On the other hand, there is a recent study reporting less bowel obstruction after laparoscopic than open appendectomy (17), and a long term follow up of 150 patients operated on for an acute abdomen reporting less frequent occurrence of bowel obstruction after laparoscopic procedures (18).

POSTOPERATIVE ADHESION FORMATION

Postoperative adhesion formation is a well-known consequence of abdominal surgical procedures, recognised since the early days. The major complications of adhesions are bowel obstruction and difficulties during re-operations, with risk of organ perforation, increasing patient morbidity and mortality, and cost of surgical care (19, 20). A Scottish retrospective study reported 30000 patients with no prior surgical history, who underwent abdominal or pelvic surgery in 1986. Thirty-five percent of patients were readmitted, on average 2.1 times over 10 years, for a disorder that could directly or possibly be related to adhesions (21).

Adhesions form in the peritoneal cavity, mainly at sites of trauma and are the result of tissue damage from a variety of sources: surgery, bacterial infection, irradiation, chemical injury and tissue ischemia. As a response to trauma, fibrin is deposited initiating tissue remodelling. Fibrin deposits can either be completely or partially resolved by the fibrinolytic process, or developed into adhesions. Fibrin degradation takes place within two to three days post operatively, and fibrin deposits remaining later on will be invaded by fibroblasts and organised into adhesions. Fibrous adhesions join tissue surfaces in abnormal locations, forming potential sites of bowel obstruction and creating surgical difficulty during re-entry of the abdominal cavity.

THE PERITONEUM

The peritoneal cavity is lined by the peritoneum, a large organ consisting of a monolayer of mesothelial cells standing on a basal membrane (22). This lining minimises friction within the cavity and facilitates organ movements. The peritoneum acts as an active membrane, transporting fluids and residues from the cavity, an activity of mechanism utilised in the peritoneal dialysis technique. It also plays an important active role in the local host defence against bacteria and infections, presenting antigen and foreign material for phagocytosis by other cells (23, 24).

The mesothelial cells of the peritoneal membrane are highly active metabolically, producing proteins and regulating biological processes in the abdominal cavity (22). They have a sophisticated system of vesicles and vacuoles and experimental studies indicate that particles up to 100 nm can be transported through the mesothelial cells via micro pinocytosis. The interstitial area between the mesothelial cells is complex with adjacent cells often overlapping as well as widely spread junctions between the cells. There are also gaps of varying size between the cells, known as lacunae, where transportation and absorption of liquid occurs. The mesothelial cells are capable of producing a variety of mediators, such as t-PA and PAI-1, and are considered the main source of these key

enzymes within the abdominal cavity (25, 26). They also produce other proteins such as growth factors, MMPs and elements of the extracellular matrix (27, 28). Structurally and functionally mesothelial cells resemble the vascular endothelium.

The peritoneum rapidly regenerates after injuries. The peritoneal serosal layer heals in a size-independent way, where small and large injuries heal in approximately the same time-period. Free floating mesothelial cells adhere to the denuded areas and initiate further cell proliferation and restoration (29). During laparoscopy the peritoneum is distended together with the abdominal wall. Studies using electron microscopy (EM) showed bulging and detachment of rat mesothelial cells, exposing the basal lamina, after laparoscopic procedures (30). These changes peaked after 12 hours and the peritoneal lining was then restored with macrophages migrating in and regenerating cells covering the denuded areas. At 96 hours the mesothelial lining appeared intact (30). However, all injuries and changes of the mesothelial cell layer during operative procedures may constitute sites of trauma where tissue remodelling might take place.

THE FIBRINOLYTIC SYSTEM

Fibrinolysis in the abdominal cavity, as well as in other tissues, depends on the balance between the local plasminogen activating capacity and its inhibition. In order to initiate fibrin degradation, the inactive precursor plasminogen, present in the systemic circulation needs to be activated. Activation is performed by the two plasminogen activating enzymes t-PA and uPA (Fig 1). Both are equally efficient as activators (31), and t-PA is the predominant activator in the abdominal cavity (32). They can both be inhibited by complex binding to PAI-1 in a one-to-one relation (Fig 1).

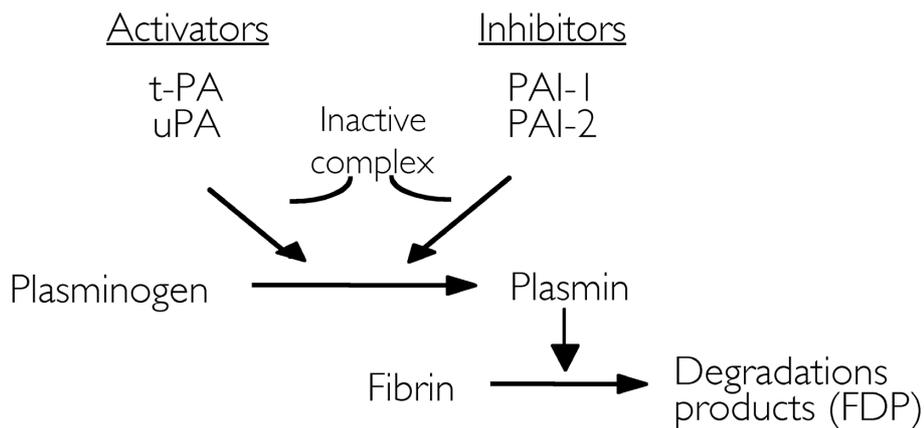


Fig 1. The fibrinolytic system

t-PA

The tissue-type Plasminogen Activator (t-PA) is a serine protease enzyme and the main plasminogen activator in plasma. It is highly specific for the fibrinolytic process, and its activity has been shown to increase 1000-fold in the presence of fibrin (33, 34). The presence of t-PA has been documented in many tissues (35, 36), and the most t-PA production takes place in the vascular endothelium (35, 37). There is also growing evidence of production and local regulation of t-PA in other cells, including macrophages (38) and mesothelial cells (25, 26). Like many other enzymes, t-PA circulates as an inactive precursor that is activated when needed, and the main stimulator to activate t-PA is fibrin.

uPA

The urokinase type Plasminogen Activator is also a serine protease enzyme and the second activator in plasma. It is the main activator in urine and has also been shown to be present in other tissues (39, 40). In the peritoneal cavity, uPA can be found in the mesothelial cells, and has been shown to be produced and secreted locally (25, 41). There is evidence that uPA and t-PA are equally efficient in terms of fibrin degradation capacity (31), but t-PA dominates fibrin degradation in the peritoneal cavity (32). It has been suggested that uPA has a variety of functions involving inflammation, and that it plays an important role in the spreading of cancer metastasis (42, 43), making invasion of cancer cells into other tissues possible. It is also known as a negative prognostic marker in breast cancer (44).

PAI-1

The Plasminogen Activator Inhibitor-1 is a large multifunctional protein, a serine protease inhibitor (serpin), capable of blocking both uPA and t-PA by complex binding in a 1:1 relation, forming biologically inactive complexes. PAI-1 is produced and secreted by many cells, mainly the vascular endothelium (26, 45), but also from fibroblasts, platelets, macrophages (46) and mesothelial cells (25, 26). It is present in low concentrations in normal peritoneum, but seems to be recruited when inflammation occurs (47). On these occasions it is probably released from many sources, including macrophages. PAI-1 is released in its active inhibitory conformation, but undergoes spontaneous conformational change to the latent state unless it is bound to the cofactor Vitronectin (48). Thus PAI-1 contains an "auto-inactivation" mechanism and has a half-life of 2 hours at physiologic pH 7.4 and 37°C (48). Interestingly, the half-life of active PAI-1 is prolonged in acidic conditions, reaching 8 hours at pH 6.5 and 18 hours at pH 5.5 (49).

PAI-1 also plays an important role in secondary tumour-take, making detachment of target organ cells possible. By binding to a uPA-uPAR complex on a cell membrane, PAI-1 detaches integrins and the target cells can disconnect, making tumour invasion possible (50). These events seem to be crucial, as PAI-1 knock-out mice have been shown to lack the capability of secondary tumour-take (51).

PERITONEAL FIBRINOLYSIS DURING SURGERY

It is conceivable that fibrinolysis in the abdominal cavity to a large degree depend upon local peritoneal conditions, putting the scope of studies of postoperative tissue remodeling into the cavity. The systemic fibrinolytic response does not appear to represent peritoneal conditions (52). During open surgical procedures, local peritoneal fibrinolytic capacity has been shown to decrease (32). In a clinical trial peritoneal levels of t-PA and the peritoneal t-PA activity both decreased during surgery in a time-dependant manner (53). This was shown for samples obtained from the wound edges, and from peritoneal tissue distant to the incision line or area of surgery (53), indicating a global peritoneal effect. Interestingly, after 90 minutes of surgery, there was significantly less t-PA activity in tissue from traumatised areas when compared with tissue from distant areas, indicating a more severe fibrinolytic depression at sites of trauma (53). This also indicates that the t-PA activity might be a marker for the extent of the local peritoneal trauma.

In peritonitis, peritoneal levels of t-PA have been shown to be lower from the start of the procedure, compared with non-inflamed conditions, indicating a reduced fibrinolytic capacity in the abdominal cavity already before surgery. Starting at this reduced level, t-PA decreased during surgery, further diminishing the peritoneal fibrinolytic capacity (32, 52). Concomitantly, PAI-1 levels were higher at start of the procedure, but did not change during surgery (52). Additionally, external PAI-1 from macrophages and platelets, seem to be recruited during peritonitis (52, 54), altogether generating a situation with low fibrin clearing capacity and high risk of adhesion formation.

LAPAROSCOPY

Laparoscopic surgery is performed within the abdominal cavity in a closed environment, making the exposure of organs and tissues within the cavity different from that during open surgery. The creation and maintenance of a pneumoperitoneum causes peritoneal distension, as well as peritoneal exposure to the gas used and the flow of this gas, creating a specific peritoneal milieu. The laparoscopic technique also involves visualisation of the operating field through video techniques, and the lack of manual manipulation, factors reducing the mechanical peritoneal damage.

POSSIBLE BIOLOGIC EFFECTS OF LAPAROSCOPY

THE LAPAROSCOPIC GAS

Insufflation of gas is used to expand the peritoneal cavity, a procedure exposing the peritoneum to both the flow of gas and to the chemical properties of the gas. The flow has been shown to induce a jet-cooling effect on the peritoneal surface (55), causing desiccation and so called “thin-film evaporation”, possibly leading to peritoneal damage (56). If the gas is heated (35°C) and humidified to physiologic conditions, this effect can be avoided (56).

Carbon dioxide (CO₂) has been the preferred gas since the introduction of laparoscopic techniques. It is endogenous, easily soluble in tissues and blood, non-combustible and readily eliminated through pulmonary ventilation. However, CO₂ has a side effect as a result of its acidifying capacity, in reacting with water to form carbonic acid. Systemic acidosis is a consistent finding with CO₂ pneumoperitoneum (57), and local peritoneal pH as low as 6.2 has been observed in humans (unpublished data from pilot-study). Alternative gases have been considered (58), and helium (He) seems to be safe, as it is a small molecule that easily penetrates tissue, apparently without creating gas embolism (59). It is also non-combustible and does not create acidic conditions in contact with water. Furthermore, it is believed to be advantageous concerning tumour growth (60), inhibiting tumours cell progression in vitro.

PERITONEAL HYPOXIA

The use of a monomolecular non-oxygen gas for creation of pneumoperitoneum changes intra abdominal conditions and possibly creates a hypoxic environment on the peritoneal surface. This has been investigated by Molinas et al. In experimental models in rabbits and mice, the addition of oxygen (O₂) to the insufflation gas resulted in less adhesion formation than CO₂ or He alone (61, 62). Adhesion scores also increased with prolonged exposure time and increased intra abdominal pressure. In these studies no differences in adhesion formation was seen comparing CO₂ and He. Molinas also showed that the positive effect of O₂ was absent in mice deficient for hypoxia inducible factor (HIF), adding evidence to the hypoxia theory (63).

PERITONEAL DISTENSION

Peritoneal distension undoubtedly occurs during pneumoperitoneum, and may create local peritoneal hypoxia, possibly inducing ischemic trauma for the mesothelial cells. However, there is evidence of increased peritoneal blood-flow during CO₂ pneumoperitoneum, an effect not present using He (64). There is also evidence of increased oxygen tension in the rat abdominal wall during pneumoperitoneum with CO₂, but

not with air or at laparotomy (65). These effects might potentially balance the hypoxic effect of abdominal wall distension, at least when a moderate intra abdominal working pressure is used. Both studies used low working pressures, relevant to the respective animal model. In the studies by Molinas, quite high intra abdominal pressures were used; 11 mm Hg for mice and 15 mm Hg for rabbits. In modern laparoscopic surgery in patients, intra abdominal pressure is not allowed to exceed 12 mm Hg, and 10-11 mm Hg is often used to minimise circulatory side effects. However, abdominal wall oxygen tension during laparoscopy has not been investigated and correlated to different intra abdominal working pressures in man.

PERITONEAL pH

Peritoneal pH has been shown to decrease during laparoscopy using CO₂, but not using He or N₂O. Changes in pH are typically significant biologic events, but the impact of a mild acidosis on peritoneal mesothelial cell function, or on the integrity and function of local proteins and enzymes is not known. However, both t-PA and PAI-1 seem to be stabilised into active conformations during acidification (49, 66). The choice of laparoscopic gas might therefore be important for postoperative tissue repair and adhesion formation.

LAPAROSCOPIC SURGERY FOR MALIGNANCY

During the initial stage of laparoscopy, the technique was thought to induce more secondary tumour take than open surgery, indicated by the occurrence of port-site metastasis (67-69). The use of laparoscopic surgery for cancer was questioned, and officially only to be performed within trials (70). Since then, numerous experimental studies have been performed trying to mimic the laparoscopic situation, mainly in animals but also in vitro. Different gases have been utilised, which showed that CO₂ seemed to enhance tumour cell growth both in vitro and when free floating malignant cells were injected into the peritoneal cavity of animals (71-74), whereas helium seemed to inhibit the growth of malignant cells (60, 74). Increased flow of gas or increased intra peritoneal working pressure seemed to intensify tumour take (75, 76). On the other hand, other studies demonstrate a toxic effect of CO₂ on rat colonic cancer cells (77) and an equal spread of cancer cells injected into the portal vein in rats prior to CO₂ or He pneumoperitoneum (78). Lecuru et al showed no differences in circulating tumour DNA in rats comparing laparotomy with CO₂ or gasless laparoscopy (79). These studies were all performed in animal models or in vitro, therefore postulating clinical outcome remains a challenge. The better preserved immune response in man after laparoscopy, as compared with open surgery, also needs to be taken into account (8, 10). Due to conflicting results from experimental studies evaluating the oncological con-

sequences of laparoscopic surgery, a conclusive inference on tumour growth or spread cannot be made.

Several recent clinical studies and series, where colonic/rectal resections for colorectal cancer have been performed with laparoscopic technique or laparoscopic assistance, show encouraging results (80-85). Technical and short-term results show that laparoscopic resections for colorectal cancer is feasible, safe and with quicker patient recovery. Sufficient patients and follow-up time support the conclusion that laparoscopic surgery also is oncologically safe in colonic cancer surgery (85). In addition, data from the German register for incidental diagnosis of gallbladder cancer show slightly longer survival for patients operated laparoscopically (86), compared with those operated with open surgery.

The sequence following upon the reports of "port-site metastasis" was scientifically sound. Four different large randomised trials of the effects and safety of laparoscopic surgery for colonic or colorectal cancer were undertaken. Thus, this important clinical area has undergone trials in a way that is very unusual for new surgical techniques. Paper II and III can be regarded as part of this scientific activity.

LAPAROSCOPIC SURGERY AND PERITONEAL FIBRINOLYSIS

When the current investigations were initiated, little was known about peritoneal fibrinolysis during laparoscopic surgery. For this thesis, we sought to investigate the impact of different aspects of the laparoscopic milieu, including time, on peritoneal fibrinolysis. Since then, and during this work, a small number of human studies have been published on the subject, showing somewhat contradictory results (87, 88). These data will be presented together with our in vivo findings in the discussion section.

HYPOTHESIS

Laparoscopic surgery induces a specific environment in the peritoneal cavity, possibly influencing peritoneal biology.

For investigations, this hypothesis was broken down into the following sub-hypotheses:

- Laparoscopic surgery induces a specific peritoneal fibrinolytic response
- CO₂ influences mesothelial cell fibrinolysis
- CO₂ has biological effects and the inert gas He could therefore be favourable

AIMS

The aims of this thesis were to investigate the peritoneal and mesothelial cell fibrinolytic response to biologic conditions created during pneumoperitoneum and laparoscopic surgery.

- I. To compare peritoneal fibrinolytic response during open and laparoscopic cholecystectomy in patients.
- II. To investigate secretion of fibrinolytic proteins from mesothelial cells *in vitro*, exposing them to CO₂ during experimental conditions.
- III. To investigate the effect of acidic conditions on mesothelial cell PAI-1 expression and production *in vitro*.
- IV. To create a reliable method for measuring peritoneal pH and to validate it comparing peritoneal and systemic pH during pneumoperitoneum using CO₂ or Helium in a porcine model.
- V. To investigate and compare peritoneal fibrinolytic response and peritoneal pH during gas insufflation and laparoscopic cholecystectomy in patients, randomised to CO₂ or He as working gas.

MATERIAL AND METHODS

TISSUE SAMPLING

PAPER I & V

Human peritoneal tissue was sampled for protein assays in both studies. In study I, tissue samples at start and conclusion of each open or laparoscopic procedure were obtained. In the open surgery group sampling took place immediately after opening of the abdominal cavity and at the end of surgery, just before closure. In the laparoscopic group tissue sampling took place immediately after establishment of pneumoperitoneum and just before exsufflation. Samples were taken with scissors in the open procedures and with biopsy forceps in the laparoscopic procedures. This sampling method for laparoscopy was chosen, as it was swift and easy to perform. However, it rendered smaller samples and resulted in differences in tissue handling, possibly confounding comparisons between the two groups. In study V all patients were operated laparoscopically, using different gases and all tissue sampling was performed using scissors. The initial tissue samples were taken with open technique in a small incision at the umbilicus, to create a baseline before entering the abdominal cavity. After port placement in the incision, gas insufflation was performed and the subsequent samples during the laparoscopic procedure were obtained using laparoscopic scissors. Thus, we believe the sampling methodology in the second clinical study was improved, as there were no systematic methodological differences in sampling technique between the groups.

TISSUE PROCESSING

PAPER I & V

Further tissue processing was required for biochemical analyses of the obtained samples. All tissue samples were immediately snap-frozen in liquid nitrogen and stored at -70°C until processed in batches. Batch processing was used both to increase the reproducibility of the results and the laboratory efficiency.

After thawing, samples were weighed and homogenised using an Ultra-Turrax homogeniser and the protein fraction was extracted as previously described (32, 89). Homogenisation of tissue and extraction of proteins is a well-established method in our laboratory (32, 89), performed on a daily basis by an experienced lab technician, and has been used in many studies. It enables evaluation of the amount of proteins present at a certain site at a certain time, giving a snapshot of local conditions. In previous studies (32, 52, 90), as well as in the current studies, tissue sampling has been used to compare the peritoneal fibrinolytic capacity at different stages of surgery, in different procedures,

and using different operating techniques. The method of tissue homogenisation and measuring of different target molecules in the supernatant is well established, and commonly used by research groups analysing tissue proteins.

MESOTHELIAL CELL CULTURE

PAPER II & III

For *in vitro* cell culture experiments, human peritoneal mesothelial cells were isolated from peritoneal fluid to create a human primary cell culture, as close to physiological conditions as possible. Our technique for harvesting and culturing of mesothelial cells has developed over a decade (25), and has been used in several studies (91, 92).

Briefly, peritoneal mesothelial cells are harvested during open elective surgery and cultured under standardised conditions until the 2nd or 3rd passage, when they are used for experiments. To confirm the mesothelial characteristics of the cell culture, cell identification is performed using standard immunofluorescence technique (25, 93, 94). Methodological details are described in the present papers II and III and in the work by Ivarsson et al (25).

For both experiments, cells were sub-cultivated on 8-well plates, and one plate was used for each exposure. Medium for protein analyses was sampled separately from each well, creating eight different observations.

All cells used in the current studies were primary cultures of human cells, and all experiments were performed at cell confluency after the 3rd passage, aiming at a relevant *in vitro* model. Primary culture of human cells, as compared to transformed continuous cell-lines, is the closest an *in vitro* model can get to the clinical human situation. Cells should be allowed to grow and expand reaching confluency, contacting each other and forming a continuous mono-layer, before experiments. This allows them to change properties from regeneration and repair into their physiological steady state and more normal functions (26). It is also important to use cells from early passages. If mesothelial cells are allowed to grow into further passages their phenotype will approach that of peritoneal fibroblasts, and will change characteristics (25, 26). The fibroblasts can be identified by microscopy and are also tested for in the immunofluorescence panel. To make sure a pure cell culture was used for experiments, all cells were manually checked in the microscope on a daily basis, allowing for the detection of bacterial contamination or fibroblast ingrowths. Mesothelial cells look similar to endothelial cells by microscopy (26). To eliminate a potential mix-up, an antibody against the v.Willebrand factor was used. Having taken all these precautions, we judged our cultures to be pure mesothelial cells at the start of the experiments.

Despite all measures to create a realistic cell-model, the *in vitro* situation can only show what cells are capable of doing without surrounding regulations and physiologic interactions. However, *in vitro* experiments may give answers about cell functions and clues about clinical reactions impossible to deduct in the complex “real” situation. This information increases our basic knowledge, leading us to sharpen our hypotheses and to direct our questions. In this sense, the “simplified” situation of cell culture experiments may lead to improved understanding of biological mechanisms.

IN VITRO CELL MODELS

PAPER II & III

In both studies cells were sub-cultivated onto 8-well plates at the 3rd passage and experiments were performed when confluency was achieved.

In paper II, fresh medium was supplied just before the exposures and the cells were divided into several subgroups. To mimic laparoscopic ambient conditions, two sets of cells were placed in a specially built, airtight plastic box attached to a Wolf automatic laparoscopic CO₂ insufflator. One group was exposed to a continuous flow (1l/min) of CO₂ during 90 minutes, the other to CO₂ with a constant pressure of 14 mm Hg, also for 90 min. One set of cells placed on the lab-bench and another left in the incubator served as controls. Measurements of medium pH, sampling of medium for protein analyses and harvesting of separate cells for mRNA detection, was performed at three time-points. (For details see paper II)

When designing this experiment in 1998, local recommendations for laparoscopic procedures were taken into account. Intra abdominal pressure levels as high as 14 mm Hg were often used clinically, and even considered standard of care. It was also the preset level of the insufflators at our department. The flow rate of 1l/min may seem to be low, but during a period of 90 minutes the cells were exposed to 90 litres of CO₂, an amount similar to what is used during a laparoscopic procedure. An exposure time of 90 minutes was chosen, as this actually was the mean operating time for laparoscopic procedures at our hospital in 1997.

Peritoneal gas exposure during laparoscopy *in vivo* can be divided into the flow of gas with its cooling and dehydrating effect, and the chemical exposure to gas. This *in vitro* experiment was undertaken to explore the fibrinolytic response of the isolated mesothelial cell when exposed to flowing or pressurised CO₂. One of the interesting but not surprising findings in paper II was the significant acidification of cell culturing media caused by exposure to CO₂. Given this might be an important factor affecting mesothelial cell fibrinolytic response, the next *in vitro* experiment was setup.

In paper III, cells were exposed to medium of differing pH (6.0 -8.0) for 90 minutes. Three samples for protein assays were taken during this time. The altered exposure-medium was replaced by fresh neutral medium and the plates returned to the incubator. Recovery samples of the medium were obtained at 1, 5 and 24 hours. Monitoring of the mesothelial cell response during the recovery phase is not possible *in vivo*. *In vitro* samples taken in the immediate recovery phase (1 h), at an intermediate phase (5h) and in the late phase (24 h) made assessment of this period possible. The chosen time-points also enabled comparisons with previous studies. Based on the results from this initial phase, and to better compare physiologic and laparoscopic peritoneal ambient conditions, a second set of experiments was undertaken. Mesothelial cells were exposed to medium with pH 6.4, to mimic laparoscopy, or 7.4, which is physiologically neutral. Sampling for protein analyses took place twice during the exposure, after which the medium was changed as in the first phase. Follow up samples were only withdrawn at 5 hours of recovery as the first phase showed no differences at 1 h or at 24 hours. Sampling from cell cultures at 24 hours might be questioned, as there is a situation of accumulation in the culturing well where no elimination of proteins or other substances takes place. Results from the first phase at 24 hours of recovery revealed 10 times higher PAI-1 concentrations than at 5 h, with no differences between groups. A similar situation was shown at 24 hours in paper II. Separate sets of cells were also harvested at 90 minutes of exposure and at 5 hours post-exposure. They were used for c-DNA preparation aiming at mRNA detection. We assumed that any changes in coding for protein production would take place in the immediate post exposure period and chose the time-points accordingly.

PROTEIN ASSAYS *PAPER I - III & V*

All assays of fibrinolytic proteins were performed using commercially available ELISA kits, following measures previously established in our laboratory (89), which also adhered to established quality assurance policies. The assays were run by two experienced laboratory technicians and all samples were analysed in duplicates. The standard curve for assay was evaluated using internal control samples. Final absorbance was read in an automatic plate reader, which was the same for all analyses (V-max from Molecular Devices). To standardise the results, and to make comparisons with other studies possible, concentrations of fibrinolytic proteins and their activities, were normalised to the total protein contents of each sample (89). Assays of the total proteins were performed using a chromogenic assay (DC Protein assay, Bio-RAD Hercules, CA, USA).

T-PA, PAI-1, uPA

PAPER I–III & V

The same commercially available ELISA assays were used in all studies. They were chosen because of our considerable experience with them, and they had been shown to give reproducible results. The accuracy of these assay results stay within a variance of 5-8% (CV). When the obtained results are used for statistical comparisons, the inference of them is also dependent on the degree of significance. The implication of this is that with a p-value below 0.01 a variance in assay results of 5-8% does not interfere, but with p-values close to 0.05 conclusions should be made more cautiously.

T-PA ACTIVITY AND PAI-1 ACTIVITY

PAPER I, III & V

To analyse the active fraction of t-PA, the t-PA activity, and the active fraction of PAI-1, the PAI-1 activity, chromogenic ELISA methods were used; the Chromolyze tPA and PAI-1 from Biopool Umeå. In these activity assays, the active site of the protein is not blocked by primary binding but exposed to the substrate for enzymatic action added in the next step, thus creating an assay where the targeted enzymatic activity is first completed and then quantified.

In paper V, peritoneal PAI-1 activity was analysed but omitted from further inference, as most of the results were below the lower limit of detection. After completion of ELISA analyses all values are related to the standard curve of the assay and results below the curve are considered non-conclusive. With low values, small differences in light absorbance generate unreliable differences in numerical values and the method cannot measure values within 2SD from zero, and not absorbances < 0.05. If just a few values fall below the detection limit, they can be estimated to zero, when compared to other values. However, if the majority of results gather below the detection limit, they are not to be considered as zero since zero actually is difficult to define. Typically they are referred to as being below the detection limit.

MRNA DETECTION

PAPER II & III

During the course of these experiments, the technique for detecting mRNA was refined, requiring less manual work, resulting in less time consuming analyses, and probably with higher accuracy in quantification. Detection was performed with two different techniques in the two studies: a more manual technique in paper II and an automatic technique in paper III. All mRNA detection was performed in our laboratory by a specialist technician, trained to perform these assays. The technical change was judged to improve the quantification of mRNA.

In both papers, the total cellular DNA (c-DNA) was isolated immediately after sampling and frozen at -70°C for later mRNA detection in batches. In paper II, mRNA detection was performed with the more manual technique where the PCR products are separated on an agarose gel, which is photographed and analysed digitally for band-intensities. For detail see paper II. In paper III a quantitative real time PCR was performed using the Smart Cycler system, details are presented in paper III.

Quantification of cell specific mRNA reveals the transcription of a protein not yet expressed in the tissue or *in vitro*. The analysis makes it possible to get clues about biologic processes being triggered by a certain stimuli or event, thus giving further clues about its physiologic impact.

ANIMALS

PAPER IV

In study IV, a total of 24 pigs (27-42 kg) were used. They were anesthetized and intubated using ketamine and midazolam and general anaesthesia was maintained using inhalation with a predetermined standardized ventilation; tidal volume of 0.225 litres, frequency 15/min, to allow for monitoring and comparison of changes in systemic pH. The first four pigs were used for an exploratory phase of this study, where different methods of measuring pH on the peritoneum were tried out. The next 20 pigs were used for the evaluation study, comparing peritoneal pH using CO_2 or He for induction and maintenance of pneumoperitoneum. All animals were sacrificed at the end of the procedure.

MEASUREMENTS OF PERITONEAL pH

PAPER IV & V

The task of measuring pH on the peritoneal surface was actually a greater challenge than anticipated. Firstly, there are no devices or probes intended for this purpose or for sterile human use available. Secondly, the probe has to be attached to the peritoneum in order to reliably measure a specific location during laparoscopy. The attachment should be quick and easy to perform, with minimal operating time, give a location of the probe-eye on to the peritoneum and stay in place during the entire procedure, despite insufflation and flush-out. For these reasons we designed a pilot study to establish a way of performing measurements of peritoneal pH. We also wanted to evaluate our method comparing laparoscopy using CO_2 as a potentially acidifying gas and He as a non-acidifying gas.

A pH electrode with external reference intended for measurements of 24-hours-oesophageal-pH (Slimline, 1 channel, Medtronic Synectics), was evaluated in liquids holding different pH. As it was considered stable in the anticipated pH range, animal studies with different electrode placements were undertaken. Two electrode placements, allowing pH measurements during both gas insufflation and laparoscopy, one preperitoneal and one on the peritoneal surface, were selected.

In paper IV, the two electrode placements were used simultaneously in all animals. Initially, a small incision was made at the umbilicus, allowing further manipulations. Following this, the first electrode aimed for preperitoneal placement was inserted through the abdominal wall in the lower right quadrant. It was introduced until digitally detectable from the inside of the cavity and placed just beneath the peritoneum. The second electrode, for measuring pH on the peritoneal surface, was introduced through the umbilical incision and held in place facing the peritoneum. After completion of gas insufflation, it was relocated and placed through the abdominal wall and laparoscopically fixated with clips onto the peritoneal surface. Measurement of peritoneal pH was carried out during gasinsufflation, pneumoperitoneum (30 min) and recovery period (30 min), using CO₂ in the first ten pigs, followed by He in the subsequent 10 pigs.

Animals were not randomised for logistical reasons. The animal facility is located far from our laboratory. He use required the He-gas-container to be transported with special transpo-tation across town. As the purpose of these measurements mainly was to evaluate electrodes and electrode placements comparing an acidifying gas with a non-acidifying, we decided that with a standardised set-up, a protocol with operation in two cohorts would suffice.

As the two electrode placements showed similar results, the preperitoneal placement was chosen for study V. This placement was technically easy and quick to achieve and the electrode stayed in place during the procedure. The reason for not choosing the peritoneal placement was the more complicated placing, taking considerably longer time to perform.

In paper V, an electrode for measuring pH was inserted and placed in the preperitoneal space before start of gas insufflation, using the method described above. The electrode was then left in place during both gasinsufflation and laparoscopic cholecystectomy. For human use, the pH electrodes were sterilized using the Sterrad™ technique, as it was considered better to use a technique with lower temperature, not to melt the plastic sheath of the probes. However, the magnetic field utilised for dissolving the peroxide gas seemed to affect electrode calibration causing a parallel shift in the returned numerical

values. After in vitro evaluation of the sterilized electrodes showed reproducible values, we decided to use them, but to present data as a percent of initial value in each case.

MEASUREMENT OF ARTERIAL pH

PAPER IV & V

Arterial blood sampling was performed in order to investigate the systemic reflections of changes in peritoneal pH. Available clinical automatic blood gas readers were utilised in both studies. Arterial blood was sampled at specific time-points and immediately analysed according to the manufacturers instructions. For paper IV a Radiometer ABL 725 blood gas analyser available at the animal facility was used. For paper V, we used a Radiometer ABL 700 Blood gas analyser system available in the ICU at the hospital.

RANDOMISATION AND SURGICAL SET-UP

PAPER V

Thirty patients on the waiting list for elective cholecystectomy were randomized to laparoscopic surgery using either CO₂ or He as working gas. The two groups were comparable in age, morbidity and body surface area.

To be able to use He for gas insufflation, several technical devices as well as the gas-container had to be exchanged before start of surgery. Therefore, the patients were randomised to the use of either CO₂ or He on the morning of the day of surgery before any preoperative preparations began. The group allocation then became obvious in the OR, which was acceptable as the surgeons needed to be aware of which equipment was used, as the insufflator worked differently with the two gases. The different physical properties of the gases, with He being volatile, filling the abdominal cavity at a slow rate and continuously diffusing into tissues, also made the gas-assignment evident during surgery. The anaesthetist was not aware of which gas was used, but could determine this from the equipment used. Given the different properties of the gases during surgery, blinding would not have been possible. The knowledge of group allocation was assessed not to have influence on the results as the entire set-up with measurements and sampling was strictly standardised. The patient was not informed of group allocation, before or after the procedure. Group allocation was not disclosed until the postoperative follow-up, long after samplings and measurements were taken.

Initial calibration of the pH electrode, initial tissue sampling and placement of the electrode was performed by the two participating surgeons together, before one of them proceeded with the laparoscopic cholecystectomy, using a standard four-trochar setting with hook electro-cautery.

ELIMINATION OF CO₂

PAPER V

In all patients, controlled ventilation was maintained using a technique with low-flow (95) of fresh gas, keeping the end-tidal VCO₂ at 5%. During laparoscopy, this usually means increasing ventilation during the procedure to compensate for the increasing uptake of CO₂, and subsequent changes in acid-base balance. To control this factor and to be able to evaluate the trans peritoneal uptake of CO₂, we quantified the ventilatory elimination of CO₂ (VCO₂), for patients in both treatment groups.

For these measurements, a canister was inserted in the expiratory line to obtain mixed gas concentrations. Ports permitted sampling of inspired, end-tidal and mixed expired gas. Gas sampling was performed every 5 minutes during stabilisation of the anaesthesia, before start of surgery and during the entire procedure. The VCO₂ was then calculated for each patient and normalised to body surface area (litres/min/m²), using the method described by Mosteller et al (96).

One of the questions regarding peritoneal acidification during laparoscopy has been whether it is a primarily local CO₂ effect, or an effect secondary to peritoneal distension and hypoxia. In this study, the comparison between pneumoperitoneum created with CO₂ and He makes it possible to suggest a theory. For both groups, the same intra abdominal pressure was maintained (11 mmHg), creating comparable degrees of abdominal wall distension and the same degree of peritoneal distension. Both gases were equally hypoxic. Changes in peritoneal pH were immediate upon CO₂ exposure and were followed by a slightly delayed increase in CO₂ elimination rate, differing from He at 10 minutes of gas exposure. There was also a slight decrease in systemic pH after completion of CO₂ insufflation. All of these factors point towards immediate local peritoneal formation of carbonic acid when exposed to CO₂, followed by a trans peritoneal uptake of CO₂, affecting systemic pH and the CO₂ elimination rate.

STATISTICS

Non-parametric statistical methods were used for the majority of tests in all papers. For comparisons between related samples the Wilcoxon signed rank test was used and for non-related, the Mann-Whitney-U test. For multiple comparisons the non-parametric Kruskal-Wallis test was used in all papers but paper II, where the parametric analyses of variance, the ANOVA, was used followed by the post-hoc Fischer's PLSD test. Least square linear regression was used to investigate correlations with time and other factors. Values are presented as mean \pm 2SD.

RESULTS

PAPER I

To get an understanding of potential differences or similarities with conventional open surgery, the peritoneal fibrinolytic response in laparoscopic surgery was investigated. Peritoneal fibrinolytic capacity in tissue samples obtained at the beginning and conclusion of open and laparoscopic cholecystectomy was compared. A significant peroperative decrease in t-PA activity was recognized in both groups, with no differences between open and laparoscopic surgery at start or conclusion of the procedures (Fig 2). The laparoscopic procedures were quite lengthy, operation time averaged 90 minutes during the study, which might influence the t-PA results. The peritoneum responded similarly to the two techniques from a fibrinolytic activation perspective.

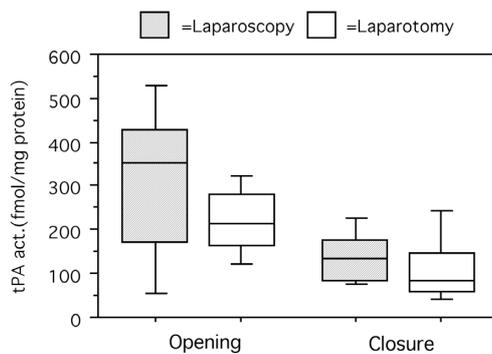


Figure 2: Peritoneal t-PA activity in tissue samples obtained at start and conclusion of laparoscopic or open cholecystectomy. A significant decrease during surgery was shown in both groups ($p=0.02$ for laparoscopy and $p=0.01$ for laparotomy).

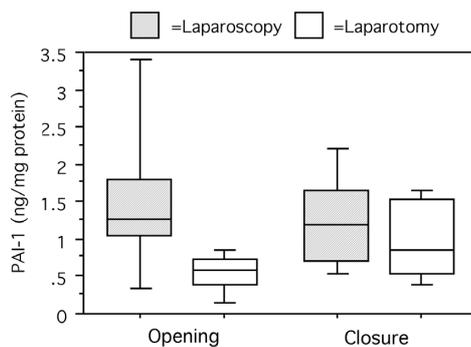


Figure 3: Peritoneal PAI-1 in tissue samples obtained at start and conclusion of laparoscopic or open cholecystectomy. At start of surgery there were higher levels of PAI-1 in the laparoscopic group ($p=0.004$), but at the end of surgery PAI-1 in the open group had increased and no remaining differences could be shown ($p=0.29$).

We also found higher tissue concentrations of PAI-1 at the start of the laparoscopic cholecystectomy than at the start of the open procedure (Fig 3). However, at the end of the procedures PAI-1 levels in the open group had increased and there were no remaining differences. This study was designed to give an overall observation of the peritoneal response during laparoscopy and the cause of these findings remained unclear. However, as the first peritoneal sampling in the laparoscopy group (opening sample) was performed after the intervention of CO₂ insufflation, the gas exposure might be an explanation. To address this, the experiment presented in paper II was designed.

PAPER II

During laparoscopy, the peritoneum is exposed both to the flow of the insufflated gas and the gas itself with its chemical and physical properties. To investigate the mesothelial cell response to these two challenges, the following in vitro experiment was conducted. Cultured human mesothelial cells were exposed to either flowing or pressurised CO₂ while untreated control cells were kept on the lab bench or in the cell incubator. These results show that flowing CO₂ induces an immediate release of both t-PA and PAI-1 from the mesothelial cells (Fig 4 & 5). At 5 hours post exposure, most release of t-PA came from cells exposed to either flowing or pressurised CO₂, but most PAI-1 release came from the untreated cells kept in the incubator.

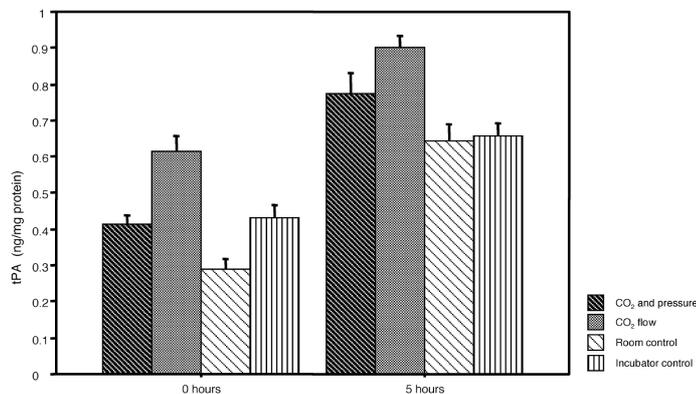


Figure 4: t-PA concentrations in culture media immediately post exposure and 5 hours post exposure. Immediately post exposure the highest t-PA levels were found in the CO₂ flow group ($p < 0.001$). At 5 hours post exposure the highest t-PA levels were still found in the CO₂ flow group ($p < 0.001$).

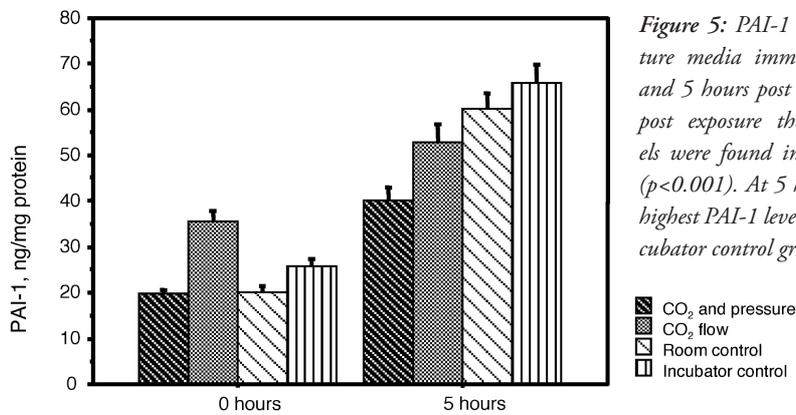


Figure 5: PAI-1 concentrations in culture media immediately post exposure and 5 hours post exposure. Immediately post exposure the highest PAI-1 levels were found in the CO₂ flow group ($p < 0.001$). At 5 hours post exposure the highest PAI-1 levels were found in the incubator control group ($p < 0.001$).

Analysing mRNA coding for PAI-1 at 5 hours post exposure, cells exposed to flowing CO₂ showed 20 times more mRNA copies than both control groups. In comparison, cells exposed to pressurised CO₂ showed only the double amount of mRNA copies as the controls. These observations indicate that the flow of CO₂ or large amounts of CO₂ induce PAI-1 transcription. The amount of t-PA mRNA did not change during the experiment, indicating that neither the flow of gas nor the gas itself stimulate t-PA transcription. The t-PA results imply that CO₂, by itself or by its flow, stimulates release of t-PA from the mesothelial cells, but does not stimulate to an increased t-PA production. These findings suggest that CO₂ initially adds t-PA to the abdominal cavity, but does not further interfere with plasminogen activation

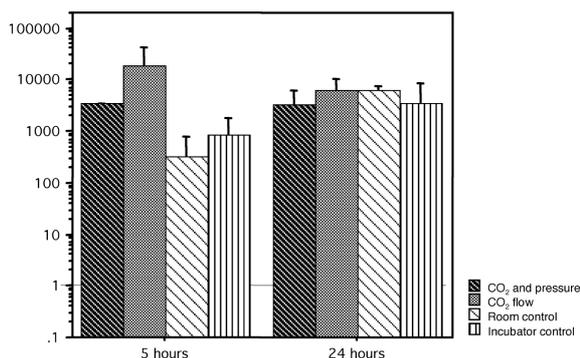


Figure 6: Specific mRNA expression/ μ g total RNA. mRNA for PAI-1 in the CO₂ flow group was up regulated at 5 hours compared with all the other groups. At 24 hours post exposure, the PAI-1 expression did not differ between groups.

Another finding in this study was the differences in pH in culturing medium after the different exposures. An immediate acidification was recorded in cells exposed to CO₂, which was most pronounced in the CO₂ flow group, indicating an acidification in the mesothelial environment by CO₂ (Fig 7). Moreover, the results suggest that the degree of acidification is related to the amount of CO₂, since the acidification was most pronounced in the group exposed to the largest amount of gas. These results also show a recovery in medium pH after returning the cultures to the incubator, indicating a buffering potential even in vitro.

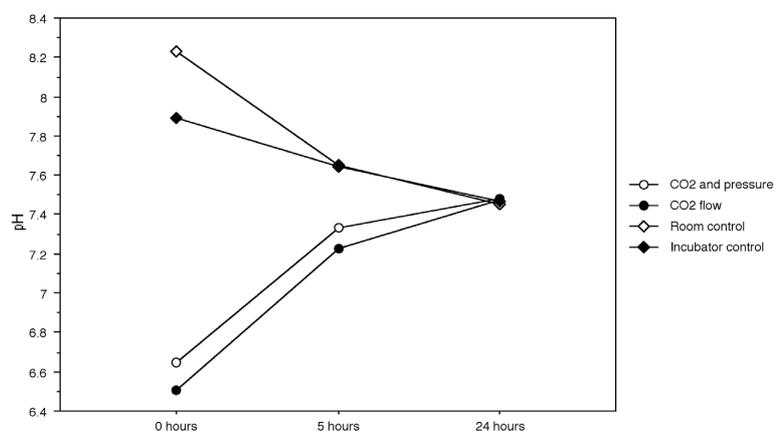


Figure 7: pH in culture media immediately post exposure, at 5 and 24 hours post exposure.

PAPER III

Considering the changes in pH after exposure to CO₂, we sought to investigate the role of acidification as a stimulant for PAI-1 release and production. Cultured mesothelial cells were exposed to medium that had been acidified using HCl. This experiment was undertaken in two phases. In the first phase, medium with pH range between 6.0 and 8.0 were tested. To investigate whether there were differences between acidic, neutral and alkalotic environments acidic treatment-groups were put together (pH 6.0, pH 6.5 and pH 7.0) and compared with pH 7.4 (neutral) and 8.0 (alkalotic). This revealed an increased PAI-1 secretion to conditioned media at 5 hours post-treatment from the mesothelial cells exposed to an acidic environment ($p=0.040$ Fig. 8), but no differences could be detected at any of the other time-points.

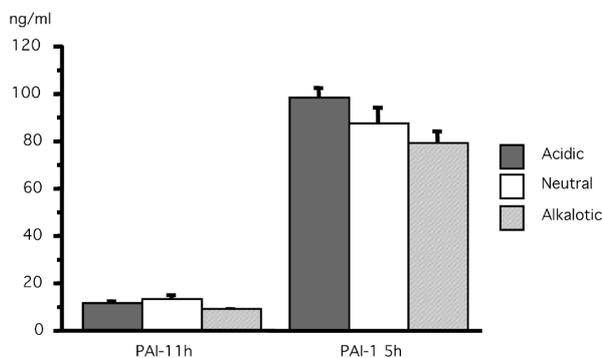


Figure 8: PAI-1 concentration in media comparing acidic environment (pH 6.0, pH 6.5, pH7.0) with neutral (pH 7.4) and alkalotic (pH 8.0). There was an increased PAI-1 secretion into the media from cells exposed to acidic pH at five hours post exposure, as compared to neutral and alkalotic pH ($p=0.040$).

In the second phase, mesothelial cells were exposed to medium conditioned to pH 6.4, to mimic the laparoscopic situation and compared with the physiologic pH 7.4. Both concentrations of PAI-1 antigen as well as the PAI-1 activity were investigated. At 60 and 90 minutes of exposure, there was more PAI-1 secreted in medium of cells treated with medium of a physiologically neutral pH, but at 5 hours post exposure there was no difference (Fig. 9). The PAI-1 activity in medium from cells treated with pH 7.4 exceeded that of cells treated with pH 6.4 at 60 min of exposure. Interestingly, at 90 min this difference had disappeared and at 5 hours post-exposure the initial difference was reversed, with PAI-1 activity in medium from cells treated with pH 6.4 exceeding those treated with pH 7.4. (Fig. 10)

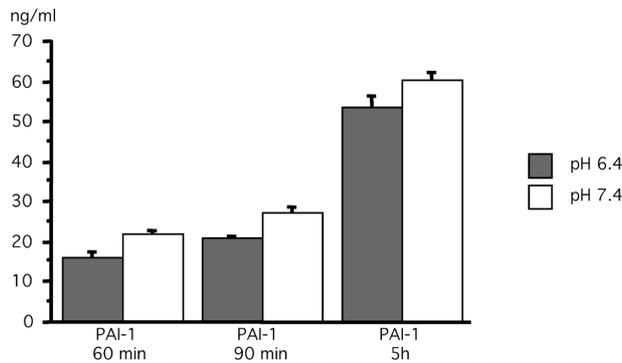


Figure 9: PAI-1 in cell culturing media at 60 and 90 minutes of exposure and at five hours post exposure. There was significantly more PAI-1 in media from cells exposed to pH 7.4 at 60 and 90 minutes of treatment ($p=0.02$ at 60 min. and $p=0.003$ at 90 min.). At five hours post treatment there was no remaining difference between the groups.

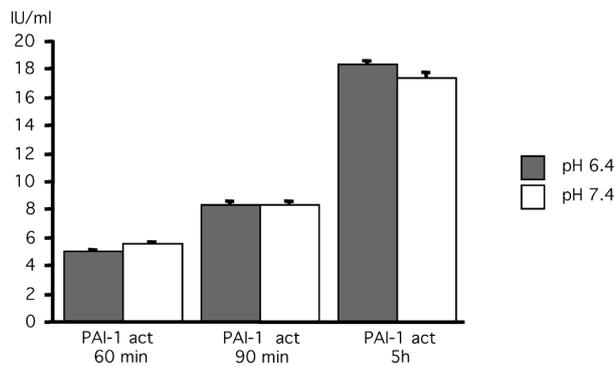


Figure 10: PAI-1 activity in cell culturing media at 60 and 90 minutes of exposure, and at five hours post-exposure. At 60 minutes there was more PAI-1 activity in cells treated with pH 7.4 ($p=0.03$), at 90 minutes this difference had disappeared and at five hours post treatment there was more PAI-1 activity in cells treated with pH 6.4 ($p=0.04$).

At completion of the 90 minutes exposure period, the mRNA copies coding for PAI-1 in cells treated with pH 6.4 were more than twice as many as those from cells treated with pH 7.4. This difference remained at 5 hours post exposure.

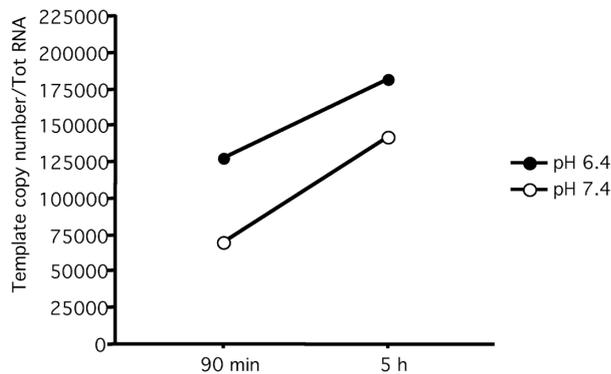


Figure 11: Template copy number/total RNA from real time PCR reaction. More than twice as many mRNA copies for PAI-1 were seen in cultured mesothelial cells treated with pH 6.4, compared with cells treated with pH 7.4 at 90 min. The difference remained at five hours post exposure.

These in vitro findings (papers II and III) of an increased production of mRNA coding for PAI-1 in cells exposed to large amounts of CO₂ (paper II) or to medium of lower pH (paper III) is interesting, but difficult to correlate to in vivo findings. An increased mRNA quantity for a certain protein shows that synthesis of this protein has been initiated. It is well known that mesothelial cells in vitro can secrete large amounts of PAI-1 into medium (25), but we know little about what happens in the peritoneum after surgery. Investigations on drain fluids have been performed to address this question. In two recent randomised studies fibrinolytic markers were analysed immediately after surgery and at 24 and 48 hours post open or laparoscopic procedures (97, 98). Both studies showed increasing PAI-1 levels post surgery, with no differences between groups. These results are both in line with our in vitro findings and somewhat conflicting, as PAI-1 levels increased to a similar extent regardless of CO₂ exposure.

PLANNING FOR PAPER IV AND V

To further characterise peritoneal fibrinolysis during laparoscopy, a human study was planned. As acidification might influence the peritoneal fibrinolytic response, we sought to design a study also controlling for peritoneal acidification. Patients were randomised to the use of He, a non-acidifying gas versus CO₂, and simultaneous peritoneal pH measurements were planned. During this work we realised that measurement of peritoneal pH was technically difficult and decided to perform an animal trial evaluating methods for these measurements.

PAPER IV

The first aim of this study was to determine positioning of the pH probe on the peritoneal surface that would be mechanically stable and give reproducible pH values both during gas insufflation and laparoscopic surgery. Two different placements were judged to function well for measurements and to be relatively easy to accomplish. The first placement involved initial measurements on the peritoneal surface through the small umbilical incision during insufflation, followed by attachment of the electrode with clips onto the peritoneal surface (Fig. 12a) when pneumoperitoneum was established. The second placement was preperitoneal, where the probe was inserted before gas insufflation through the abdominal skin, fat and muscle layers and placed just underneath the thin peritoneal surface (Fig. 12b).



Figure 12a: Pre-peritoneal pH electrode placed in the pre-peritoneal space.

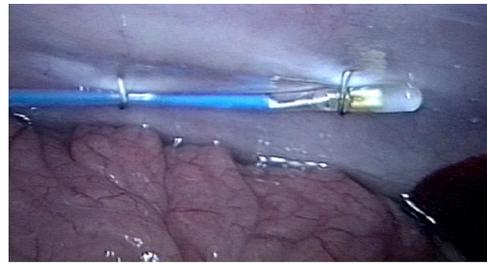


Figure 12b: Peritoneal pH-electrode attached to the peritoneum with laparoscopic clips.

To evaluate the measurements and results achieved by these electrode placements, they were used simultaneously during gas insufflation, 30 minutes of pneumoperitoneum and 30 minutes of recovery, with either CO₂ or He.

Continuous monitoring of peritoneal pH showed an immediate drop in pH, down to 6.7 within 2 min during gas insufflation using CO₂, and a slight increase in pH using He (Fig. 13). The decrease was attenuated with CO₂ during the period of pneumoperitoneum (reaching pH 6.4), but after exsufflation of gas, pH recovered. With He, peritoneal pH was slowly normalised (Fig.13). The two placements of electrodes gave similar results, therefore the swift preperitoneal placement was chosen for further investigations. Peritoneal changes in pH were mirrored in the arterial pH values achieved before and after gas insufflation, at the end of the pneumoperitoneum period and at the end of the recovery period (Fig. 14).

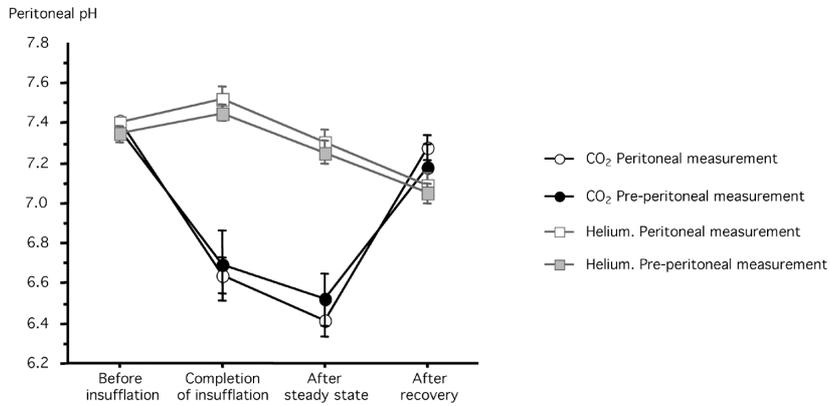


Figure 13: Peritoneal pH in both groups at four time-points, showing both peritoneal and pre-peritoneal measurements. The two measuring techniques gave similar values, showing that pre-peritoneal pH well reflects peritoneal pH. There was no difference between groups before gas insufflation. After gas insufflation and after steady state, peritoneal pH was significantly lower in pigs exposed to CO₂ ($p=0.001$), a difference that disappeared during the recovery period.

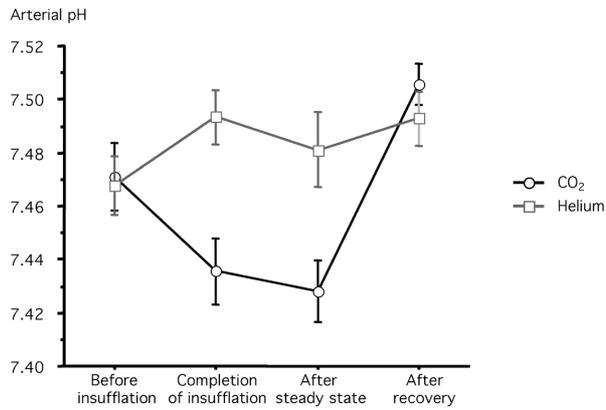


Figure 14: Arterial pH in both groups at four time-points. There was no difference between groups before gas insufflation. After gas insufflation and after steady state, arterial pH was significantly lower in pigs exposed to CO₂ ($p=0.004$ and $p=0.02$), a difference that disappeared during the recovery period.

PAPER V

Peritoneal pH was monitored during both gas insufflation and the laparoscopic procedure. As expected, there was a significant decrease in peritoneal pH during gasinsufflation using CO₂, but no changes using He (Fig. 15). The drop in pH noticed during CO₂ insufflation continued during the cholecystectomy, stabilising after 20 minutes at about 92% of initial pH values.

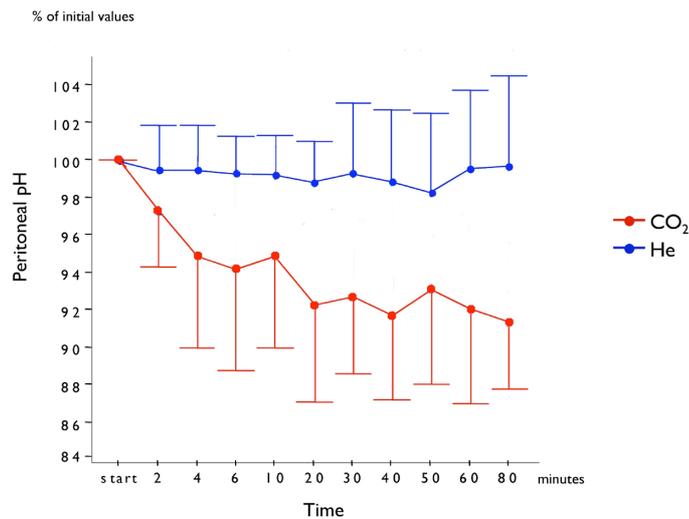


Figure 15: Peritoneal pH during gas insufflation and laparoscopic surgery. pH is given as percent of initial values. In patients operated with CO₂ there was an immediate drop in peritoneal pH to 94% of initial value during insufflation. Pneumoperitoneum was established at the four minutes time-point. During surgery pH stabilised around 92% for the duration of the operation. Please note that the time scale is non-linear, the first ten minutes were more closely monitored.

The rate of ventilatory elimination of CO₂ was calculated. Patients operated with CO₂ showed a continuous increase in CO₂ elimination rate during the first 30 minutes of laparoscopic surgery, when stabilisation occurred at 0.09 l/min/m². Meanwhile, the CO₂ elimination rate in patients operated with He did not change during the entire procedure (Fig. 16). During the first hour of laparoscopic surgery patients exposed to CO₂ pneumoperitoneum eliminated 3.7 litres more CO₂ than those exposed to He, representing trans peritoneal uptake. This finding adds an explanation to the known need for increased ventilation during laparoscopy and also shows that CO₂ passes through the peritoneal membrane.

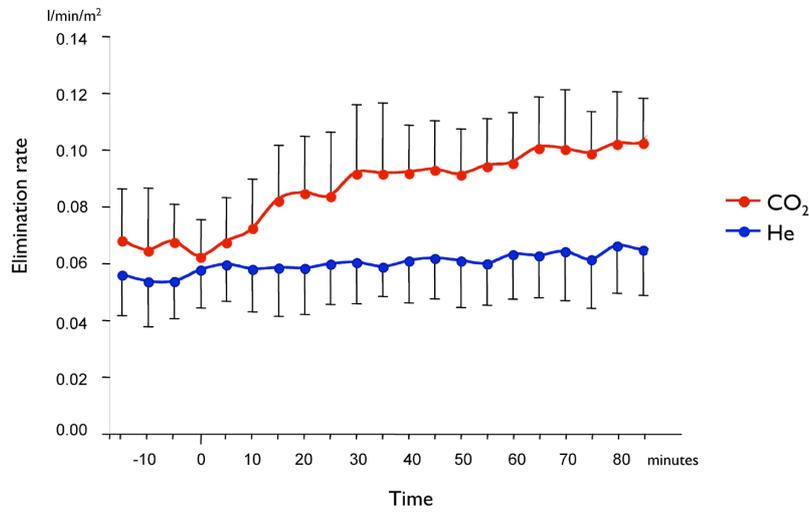


Figure 16: Ventilatory elimination of CO₂ during gas insufflation and laparoscopic surgery. Elimination rates are given as litres/min/body surface area. The CO₂ elimination rate started to increase immediately after initiation of CO₂ insufflation and continued to increase during the first 30 minutes of surgery. At the 10 min time-point respiratory elimination of CO₂ was significantly higher when CO₂ was used as insufflation gas compared to He and remained significantly elevated for the duration of the procedure

Comparing peritoneal samples taken before and after gas insufflation, peritoneal concentrations of the assayed fibrinolytic proteins did not change. Changes did however occur during the laparoscopic cholecystectomy. Although operating time varies between patients, pre-procedural samples, obtained after establishment of pneumoperitoneum were compared with samples obtained at the end of each procedure, illustrating the net-effect for each patient during surgery and enabling comparisons with previous studies. Making this evaluation, there was a significant net decrease in tissue t-PA concentrations during surgery in both groups (Fig. 17). Aggregating data obtained at different time-points during the procedure and correlating them with time, showed that there was a time-dependant decrease in peritoneal t-PA during the procedure in both groups (Fig. 18). These results are consistent with the findings in paper I and also in line with the decreasing peritoneal t-PA levels seen during open surgery.

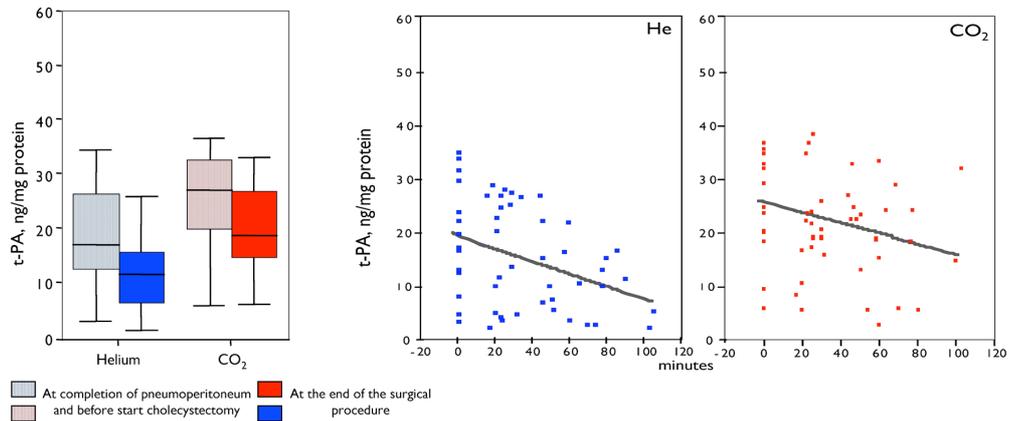


Figure 17: Peritoneal concentrations of t-PA at start of the cholecystectomy and at end of surgery. Both groups showed significant decreases during surgery (He: $p=0.006$, CO_2 : $p=0.026$). However, at end of surgery there were significantly higher t-PA concentrations in the CO_2 group ($p=0.005$) compared with He.

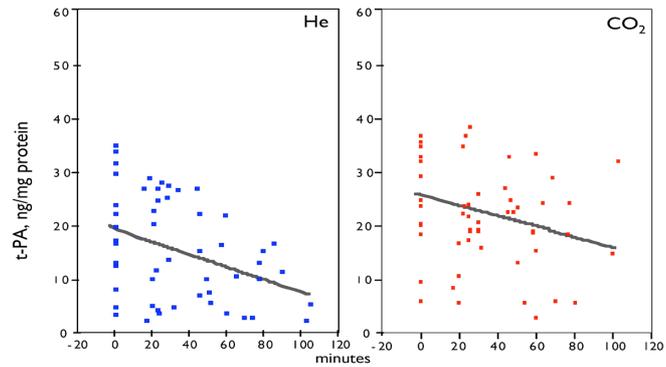


Figure 18: Peritoneal concentrations of t-PA in samples taken at different time-points during the procedure correlated with time. Both groups showed a time dependant decrease during laparoscopic surgery (He $p=0.006$, $r^2=0.140$, CO_2 $p=0.05$, $r^2=0.073$).

However, the same comparisons for the active fraction of t-PA, the t-PA activity, showed that there was a net decrease during laparoscopic surgery for the patients operated with He, but not for those operated with CO₂ (Fig. 19). When the t-PA activity of samples obtained at different time-points during the procedure was correlated with time, there was a time dependant decrease in the He group, but not in the CO₂ group (Fig. 20). Evaluating these results, it seems that CO₂ preserves the enzymatic activity of t-PA, despite decreasing t-PA concentrations. This effect might be due to stabilisation of t-PA in its active conformation by the induced acidification.

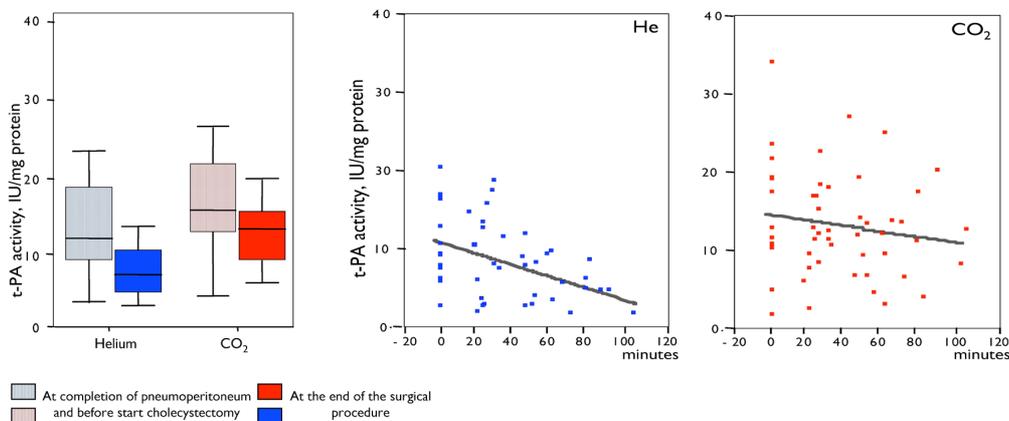


Figure 19: Peritoneal t-PA activity at start of the cholecystectomy and at end of surgery. There was a significant decrease during surgery in patients operated with He ($p=0.004$), but there was a preservation of t-PA activity when using CO₂ and at end of surgery more t-PA activity was present in the CO₂ group than in the He group ($p=0.008$).

Figure 20: Peritoneal t-PA activity in samples taken at different time-points during the procedure correlated with time. There was a significant time dependant decline in t-PA activity during laparoscopic surgery in patients operated with He ($p=0.001$, $r^2=0.196$), but not in patients operated with CO₂ ($p=0.3$, $r^2=0.023$).

The time from establishment of pneumoperitoneum until the end of each procedure, which is the time frame comparable with previous studies, was an average of 60 minutes. During this period, a decrease in t-PA antigen but not in t-PA activity was seen in patients inflated with CO₂. There is preliminary data from laparoscopic gastric bypass procedures using CO₂, showing that peritoneal t-PA activity stays the same at 45 minutes of surgery and then declines, showing significantly lower levels of t-PA activity at 90 and 135 min (M Reijnen personal communication). Our present data together with

the data by Brokelman et al suggest preservation of peritoneal plasminogen activation capacity during the first 45-60 minutes of laparoscopic surgery using CO₂, followed by decreasing capacity.

The differences between the gases that have an impact on peritoneal fibrinolytic capacity is not clear. The slight acidification caused by CO₂ does not seem to influence local fibrinolysis in a negative way. The different physical properties of He which is more volatile, slow at filling up the abdominal cavity and constantly diffusing into tissues, may also influence the peritoneal fibrinolytic response.

For PAI-1, the same comparisons were performed, revealing a statistically significant net increase during the procedure in both groups. In samples obtained at different time-points during the procedure, there were increasing PAI-1 concentrations, correlating with time, in patients operated with He, but not in those operated with CO₂. However, all concentrations were low and in the CO₂ group many measurements were close to zero. This adds an explanation to why there was a significant increase during surgery in PAI-1, but no correlation with time could be shown.

In total, it seems that CO₂ results in a greater net fibrinolytic capacity after laparoscopic surgery than does He, indicating a beneficial role for this gas. However, there was a decrease in total peritoneal t-PA concentrations during surgery, regardless of gas used. This finding is in line with our previous results and also mimics fibrinolysis during open surgery.

DISCUSSION

We are in the era of minimal invasive surgery, where not only laparoscopy, but also other endoluminal and transluminal techniques are continuously being developed. The idea of minimising both abdominal access and operative trauma has guided this fundamental change in surgical approach. Laparoscopy creates a specific intra abdominal milieu where the peritoneal surfaces are distended and exposed to gas. Moreover, the surgical traumatic manipulation is minimised with small abdominal wall incisions, and surgical mechanical manipulation is restricted to the site of surgery, thus creating a specific environment for the peritoneal cavity, possibly influencing peritoneal biology and provoking a specific peritoneal and systemic response.

PERITONEAL FIBRINOLYSIS DURING LAPAROSCOPIC SURGERY

When the current investigations were planned, hardly anything was known about peritoneal fibrinolysis during laparoscopic surgery. Our first study indicated a similar per-operative response in peritoneal fibrinolytic capacity, with decreasing levels of t-PA activity and increasing PAI-1, during both open and laparoscopic cholecystectomy. However, due to the rapid change in standard of care concerning laparoscopy, this study was not randomised. Additionally, the first peritoneal samples in the laparoscopic group were obtained after peritoneal exposure to CO₂. We therefore sought to investigate this further, and undertook the in vitro experiments, as well as a clinical study where changes in peritoneal fibrinolysis before and during laparoscopic surgery using CO₂ and He were investigated (paper V). Here, initial samples were obtained before gas exposure, and the results indicated preserved peritoneal t-PA activity after 60 minutes of laparoscopic surgery using CO₂. Others have come to seemingly contradictory conclusions on this subject (87, 88). In a series of 50 patients, Brokelman et al found no significant changes in peritoneal concentrations of t-PA or PAI-1, comparing samples obtained at start and conclusion of laparoscopic cholecystectomy using CO₂ (87). However, the concluding samples were taken at 45 minutes of surgery in all patients, which is shorter than 60 min. Yet unpublished data from the same group, investigating peritoneal fibrinolysis in patients undergoing laparoscopic surgery for obesity, reproduced this observation at 45 minutes. However, at 90 and 135 min significantly lower t-PA levels were observed (M. Reijnen, personal communication). These results support the observations done in this thesis. The totality of data indicate that operation time influences peritoneal fibrinolysis, but that no significant changes are evident during the first 45-60 minutes.

There is, to our knowledge only one published study except for paper I of this thesis, comparing peritoneal fibrinolysis in open and laparoscopic surgery. In a randomised study comparing open and laparoscopic colonic resection, Neudecker et al found a perioperative decrease in t-PA activity in both groups (88). No differences could be detected between groups, or between start and end of the surgical procedure for either t-PA or PAI-1 concentrations. These findings are quite unexpected, as operation time was long (220 minutes) which should, if our assumptions are correct, result in a more pronounced impairment of the peritoneal fibrinolytic capacity, at least for open surgery. However, there is a confounding factor in Neudecker's study; all patients included were first operated with diagnostic laparoscopy exposing the peritoneum to CO₂ before randomisation to open or laparoscopic colonic resection. This initial laparoscopy could have changed conditions in the peritoneal cavity, biasing the comparison between the different surgical approaches.

The variability of the results from tissue analyses may depend upon many factors including operation time, gas insufflation rate, warming and humidification of the gas, gas exposure, intra abdominal working pressures, timing of sampling, sampling techniques and more. The present studies indicate that operation time, gas exposure and thereby timing of sampling has an impact on the peritoneum and that these factors need to be taken into consideration in future studies. Presently, the accumulated data indicate that peritoneal fibrinolysis may be better preserved during laparoscopy using CO₂ than during open surgery, supporting the hypothesis of a specific peritoneal milieu during laparoscopy.

LAPAROSCOPIC GAS AND FIBRINOLYSIS

When designing further experiments there were many details in the laparoscopic technique to take into consideration and many experimental studies pointed towards an adverse effect of CO₂. To investigate the influence of the gas itself on mesothelial cell fibrinolysis, we went on to perform the in vitro experiments. They showed an increased cell release of both t-PA and PAI-1 after gas exposure, more pronounced with a flow of gas or larger amounts of gas, but the experimental design did not allow for assessing whether this was specific to CO₂ or just the flow of gas. Both exposure to CO₂ and acidic conditions induced mesothelial cell PAI-1, but not t-PA, mRNA transcription, indicating a specific in vitro response to acidification. These experiments support the hypothesis of a specific effect of the gas itself on the mesothelial cells, but further studies are needed.

CARBON DIOXIDE

During the work with paper II, we realised how quickly acidification occurs when the cells are exposed to CO₂. The same finding was made by Ziprin et al (99), exposing mesothelial cells to CO₂ in vitro, he reported an immediate drop in media pH reaching 6.5. Interestingly we found a similar degree of acidification in vivo in pigs (paper IV), comparing the two gases, a finding later reproduced in the subsequent clinical study (paper V). The acidifying capacity of CO₂ is well known, so these findings were not surprising. They do however show that the effect is immediate and that local buffering mechanisms in vivo are not sufficiently compensating.

The interesting question is how acidification affects peritoneal biology in general, and peritoneal fibrinolysis in particular. Although acidosis in general is believed to be an adverse effect, it might even be that it facilitates several beneficial local effects of CO₂. Recent studies show that CO₂ helps preserving antimicrobial defence in the peritoneal cavity during peritonitis, enhancing macrophage opsonisation (11, 100). Acidification has also been shown to increase peritoneal blood flow (64) and peritoneal oxygen tension during laparoscopy (65). Moreover, the results described in paper V indicate that both peritoneal t-PA concentrations and the t-PA activity are better preserved with CO₂. Here the use of He, preserving pH by not inducing acidification, resulted in a more pronounced impairment of the peritoneal fibrinolytic capacity, thus rejecting our initial hypothesis of a potential beneficial effect with regards to adhesion formation.

Both the present studies and most of the accumulated literature seem to agree that CO₂ is a good choice as a laparoscopic gas, and that the use of CO₂ might add advantages not previously considered. The use of He does not seem to improve peritoneal fibrinolysis (paper V). Moreover, He does not seem to carry any clinical advantages, except for in respiratory impaired patients (101) where the reduced need for ventilatory elimination of CO₂ might be a benefit. However the literature indicate that laparoscopic surgery could be made less traumatic by heating and humidifying the CO₂, (65), by keeping low working pressure (62, 102), and a low flow rate (56).

Although surgery by a laparoscopic approach has been demonstrated to have many benefits, it is still a surgical intervention, imposing a surgical trauma. It is well documented that adhesions do form after laparoscopic surgery, but the clinical impression is that they are of less severity than after open surgery. The present studies demonstrate that peritoneal fibrinolysis is affected during laparoscopic surgery. However, the effect seems to be less pronounced and observations done in this thesis indicate a reduced adverse effect on peritoneal fibrinolytic capacity compared to open surgery. This would offer a biological explanation for the clinical observation of a reduced adhesion formation with a laparoscopic approach.

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REFERENCES

1. Kelling G. Uber Oesophagoscopie, Gastroscopie, and Koelioskopi. *Munchen Med Wochenschr* 1902;52:21.
2. Semm K. Endoscopic appendectomy. *Endoscopy* 1983;15(2):59-64.
3. Muhe E. Long-term follow-up after laparoscopic cholecystectomy. *Endoscopy* 1992;24(9):754-8.
4. Baird DR, Wilson JP, Mason EM, Duncan TD, Evans JS, Luke JP, et al. An early review of 800 laparoscopic cholecystectomies at a university-affiliated community teaching hospital. *Am Surg* 1992;58(3):206-10.
5. Clary EM, Bruch SM, Lau CL, Ali A, Chekan EG, Garcia-Oria MJ, et al. Effects of pneumoperitoneum on hemodynamic and systemic immunologic responses to peritonitis in pigs. *J Surg Res* 2002;108(1):32-8.
6. Le Blanc-Louvry I, Coquerel A, Koning E, Maillot C, Ducrotte P. Operative stress response is reduced after laparoscopic compared to open cholecystectomy: the relationship with postoperative pain and ileus. *Dig Dis Sci* 2000;45(9):1703-13.
7. Redmond HP, Watson RW, Houghton T, Condron C, Watson RG, Bouchier-Hayes D. Immune function in patients undergoing open vs laparoscopic cholecystectomy. *Arch Surg* 1994;129(12):1240-6.
8. Sietses C, von Blomberg ME, Eijsbouts QA, Beelen RH, Berends FJ, Cuesta MA. The influence of CO₂ versus helium insufflation or the abdominal wall lifting technique on the systemic immune response. *Surg Endosc* 2002;16(3):525-8.
9. Boni L, Benevento A, Rovera F, Dionigi G, Di Giuseppe M, Bertoglio C, et al. Infective complications in laparoscopic surgery. *Surg Infect (Larchmt)* 2006;7 Suppl 2:S109-11.
10. Novitsky YW, Litwin DE, Callery MP. The net immunologic advantage of laparoscopic surgery. *Surg Endosc* 2004;18(10):1411-9.

11. Hanly EJ, Fuentes JM, Aurora AR, Bachman SL, De Maio A, Marohn MR, et al. Carbon dioxide pneumoperitoneum prevents mortality from sepsis. *Surg Endosc* 2006;20(9):1482-7.
12. Lundorff P, Hahlin M, Källfelt B, Thorburn J, Lindblom B. Adhesion formation after laparoscopic surgery in tubal pregnancy: a randomized trial versus laparotomy. *Fertil Steril* 1991;55:911-915.
13. Krahenbuhl L, Schafer M, Kuzinkovas V, Renzulli P, Baer HU, Buchler MW. Experimental study of adhesion formation in open and laparoscopic fundoplication. *Br J Surg* 1998;85(6):826-30.
14. Gutt CN, Oniu T, Schemmer P, Mehrabi A, Buchler MW. Fewer adhesions induced by laparoscopic surgery? *Surg Endosc* 2004;18(6):898-906.
15. Cainzos M, Rodriguez-Segade F, Martinez-Castro J, Prieto D, Becker MR, Aneiros F, et al. Intra-abdominal adhesions after open and laparoscopic cholecystectomy: an experimental model. *J Laparoendosc Adv Surg Tech A* 2006;16(2):108-12.
16. Duron JJ, Hay JM, Msika S, Gaschard D, Domergue J, Gainant A, et al. Prevalence and mechanisms of small intestinal obstruction following laparoscopic abdominal surgery: a retrospective multicenter study. French Association for Surgical Research. *Arch Surg* 2000;135(2):208-12.
17. Tsao KJ, St Peter SD, Valusek PA, Keckler SJ, Sharp S, Holcomb GW, 3rd, et al. Adhesive small bowel obstruction after appendectomy in children: comparison between the laparoscopic and open approach. *J Pediatr Surg* 2007;42(6):939-42; discussion 942.
18. Majewski WD. Long-term outcome, adhesions, and quality of life after laparoscopic and open surgical therapies for acute abdomen: follow-up of a prospective trial. *Surg Endosc* 2005;19(1):81-90.
19. Ivarsson M-L, Holmdahl L, Franzén G, Risberg B. Cost of bowel obstruction resulting from adhesions. *Eur J Surg* 1997;163:679-684.

20. Al-Fozan H, Dufort J, Kaplow M, Tulandi T. Cost analysis of adhesion-related admissions in a Canadian teaching hospital. *Int J Gynaecol Obstet* 2003;82(2):227-8.
21. Ellis H, Moran BJ, Thompson JN, Parker MC, Wilson MS, Menzies D, et al. Adhesion-related hospital readmissions after abdominal and pelvic surgery: a retrospective cohort study. *Lancet* 1999;353(9163):1476-80.
22. Mutsaers SE. Mesothelial cells: Their structure, function and role in serosal repair. *Respirology* 2002;7(3):171-91.
23. Muijsken MA, Heezius HJ, Verhoef J, Verbrugh HA. Role of mesothelial cells in peritoneal antibacterial defence. *J Clin Pathol* 1991;44(7):600-4.
24. Topley N, Mackenzie RK, Williams JD. Macrophages and mesothelial cells in bacterial peritonitis. *Immunobiology* 1996;195(4-5):563-73.
25. Ivarsson ML, Holmdahl L, Falk P, Molne J, Risberg B. Characterization and fibrinolytic properties of mesothelial cells isolated from peritoneal lavage. *Scand J Clin Lab Invest* 1998;58(3):195-203.
26. van Hinsbergh VW, Kooistra T, Scheffer MA, Hajo van Bockel J, van Muijen GN. Characterization and fibrinolytic properties of human omental tissue mesothelial cells. Comparison with endothelial cells. *Blood* 1990;75(7):1490-7.
27. Saed GM, Diamond MP. Modulation of the expression of tissue plasminogen activator and its inhibitor by hypoxia in human peritoneal and adhesion fibroblasts. *Fertil Steril* 2003;79(1):164-8.
28. Martin J, Yung S, Robson RL, Steadman R, Davies M. Production and regulation of matrix metalloproteinases and their inhibitors by human peritoneal mesothelial cells. *Perit Dial Int* 2000;20(5):524-33.
29. Mutsaers SE, Whitaker D, Papadimitriou JM. Mesothelial regeneration is not dependent on subserosal cells. *J Pathol* 2000;190(1):86-92.

30. Volz J, Koster S, Spacek Z, Paweletz N. Characteristic alterations of the peritoneum after carbon dioxide pneumoperitoneum. *Surg Endosc* 1999;13(6):611-4.
31. Lu HR, Wu Z, Pauwels P, Lijnen HR, Collen D. Comparative thrombolytic properties of tissue-type plasminogen activator (t-PA), single-chain urokinase-type plasminogen activator (u-PA) and K1K2Pu (a t-PA/u-PA chimera) in a combined arterial and venous thrombosis model in the dog. *J Am Coll Cardiol* 1992;19(6):1350-9.
32. Holmdahl L, Eriksson E, al-Jabreen M, Risberg B. Fibrinolysis in human peritoneum during operation. *Surgery* 1996;119(6):701-5.
33. Ranby M. Studies on the kinetics of plasminogen activation by tissue plasminogen activator. *Biochim Biophys Acta* 1982;704(3):461-9.
34. Ranby M, Bergsdorf N, Nilsson T. Enzymatic properties of the one- and two-chain form of tissue plasminogen activator. *Thromb Res* 1982;27(2):175-83.
35. Risberg B, Eriksson E, Bjork S, Hansson GK. Immunohistochemical localization of plasminogen activators in human saphenous veins. *Thromb Res* 1986;41(3):301-8.
36. Vipond MN, Whawell SA, Thompson JN, Dudley HAF. Peritoneal fibrinolytic activity and intraabdominal adhesions. *Lancet* 1990;335:1120-1122.
37. Kooistra T, Schrauwen Y, Arts J, Emeis JJ. Regulation of endothelial cell t-PA synthesis and release. *Int J Hematol* 1994;59(4):233-55.
38. Freyria AM, Paul J, Belleville J, Broyer P, Eloy R. Rat peritoneal macrophage procoagulant and fibrinolytic activities. An expression of the local inflammatory response. *Comp Biochem Physiol A* 1991;99(4):517-24.
39. Vassalli JD, Wohlwend A, Belin D. Urokinase-catalyzed plasminogen activation at the monocyte/macrophage cell surface: a localized and regulated proteolytic system. *Curr Top Microbiol Immunol* 1992;181:65-86.

40. Falkenberg M, Tom C, DeYoung MB, Wen S, Linnemann R, Dichek DA. Increased expression of urokinase during atherosclerotic lesion development causes arterial constriction and lumen loss, and accelerates lesion growth. *Proc Natl Acad Sci U S A* 2002;99(16):10665-70.
41. Ivarsson ML, Falk P, Holmdahl L. Response of visceral peritoneum to abdominal surgery. *Br J Surg* 2001;88(1):148-151.
42. Dano K, Behrendt N, Hoyer-Hansen G, Johnsen M, Lund LR, Ploug M, et al. Plasminogen activation and cancer. *Thromb Haemost* 2005;93(4):676-81.
43. Blasi F. Molecular mechanisms of protease-mediated tumor invasiveness. *J Surg Oncol Suppl* 1993;3:21-3.
44. Duffy MJ, Duggan C, Mulcahy HE, McDermott EW, O'Higgins NJ. Urokinase plasminogen activator: a prognostic marker in breast cancer including patients with axillary node-negative disease. *Clin Chem* 1998;44(6):1177-1183.
45. Sprengers ED, Kluft C. Plasminogen activator inhibitors. *Blood* 1987;69(2):381-7.
46. Kuraoka S, Campeau JD, Rodgers KE, Nakamura RM, diZerega GS. Effects of interleukin-1 (IL-1) on postsurgical macrophage secretion of protease and protease inhibitor activities. *J Surg Res* 1992;52(1):71-8.
47. Holmdahl L, Falkenberg M, Ivarsson ML, Risberg B. Plasminogen activators and inhibitors in peritoneal tissue. *Apmis* 1997;105(1):25-30.
48. Lindahl TL, Sigurdardottir O, Wiman B. Stability of plasminogen activator inhibitor 1 (PAI-1). *Thromb Haemost* 1989;62(2):748-51.
49. Sui GC, Mangs H, Wiman B. The role of His(143) for the pH-dependent stability of plasminogen activator inhibitor-1. *Biochim Biophys Acta* 1999;1434(1):58-63.

50. Czekay RP, Aertgeerts K, Curriden SA, Loskutoff DJ. Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J Cell Biol* 2003;160(5):781-91.
51. Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, et al. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* 1998;4(8):923-8.
52. Ivarsson M-L, Holmdahl L, Eriksson E, Söderberg R, Risberg B. Expression and kinetics of fibrinolytic components in plasma and peritoneum during abdominal surgery. *Fibrinolysis & Proteolysis* 1998;12(1):61-67.
53. Holmdahl L, Eriksson E, Eriksson BI, Risberg B. Depression of peritoneal fibrinolysis during operation is a local response to trauma. *Surgery* 1998;123:539-44.
54. Ivarsson ML, Diamond MP, Falk P, Holmdahl L. Plasminogen activator/plasminogen activator inhibitor-1 and cytokine modulation by the PROACT System. *Fertil Steril* 2003;79(4):987-92.
55. Gray IR, Ott DE, Courtney Henderson A, Cochran SA, Roth EA. Severe local Hypothermia from Laparoscopic Gas Evaporative Jet Cooling: A Mechanism To Explain Clinical Observations. *JLS* 1999;3:171-177.
56. Ott DE. Desertification of the peritoneum by thin-film evaporation during laparoscopy. *Jls* 2003;7(3):189-95.
57. Galizia G, Prizio G, Lieto E, Castellano P, Pelosio L, Imperatore V, et al. Hemodynamic and pulmonary changes during open, carbon dioxide pneumoperitoneum and abdominal wall-lifting cholecystectomy. A prospective, randomized study. *Surg Endosc* 2001;15(5):477-83.
58. Neuhaus SJ, Gupta A, Watson DI. Helium and other alternative insufflation gases for laparoscopy. *Surg Endosc* 2001;15(6):553-60.
59. Jacobi CA, Junghans T, Peter F, Naundorf D, Ordemann J, Muller JM. Cardiopulmonary changes during laparoscopy and vessel injury: comparison of CO₂ and helium in an animal model. *Langenbecks Arch Surg* 2000;385(7):459-66.

60. Neuhaus SJ, Ellis TS, Barrett MW, Rofe AM, Jamieson GG, Watson DI. In vitro inhibition of tumour growth in a helium-rich environment: implications for laparoscopic surgery. *Aust N Z J Surg* 1999;69(1):52-5.
61. Molinas CR, Koninckx PR. Hypoxaemia induced by CO₂ or helium pneumoperitoneum is a cofactor in adhesion formation in rabbits. *Hum Reprod* 2000;15:1758-63.
62. Molinas CR, Mynbaev O, Pauwels A, Novak P, Koninckx PR. Peritoneal mesothelial hypoxia during pneumoperitoneum is a cofactor in adhesion formation in a laparoscopic mouse model. *Fertil Steril* 2001;76(3):560-7.
63. Molinas CR, Campo R, Elkelani OA, Binda MM, Carmeliet P, Koninckx PR. Role of hypoxia inducible factors 1alpha and 2alpha in basal adhesion formation and in carbon dioxide pneumoperitoneum-enhanced adhesion formation after laparoscopic surgery in transgenic mice. *Fertil Steril* 2003;80 Suppl 2:795-802.
64. Brundell SM, Tsopelas C, Chatterton B, Touloumtzoglou J, Hewett PJ. Experimental study of peritoneal blood flow and insufflation pressure during laparoscopy. *Br J Surg* 2002;89(5):617-22.
65. Bourdel N, Matsuzaki S, Bazin JE, Pouly JL, Mage G, Canis M. Peritoneal tissue-oxygen tension during a carbon dioxide pneumoperitoneum in a mouse laparoscopic model with controlled respiratory support. *Hum Reprod* 2007;22(4):1149-55.
66. Ranby M, Sundell IB, Nilsson TK. Blood collection in strong acidic citrate anticoagulant used in a study of dietary influence on basal tPA activity. *Thromb Haemost* 1989;62(3):917-22.
67. Rae D, Bowyer R, Wharton R, Paterson I. Port site metastasis after laparoscopic colorectal surgery for cure of malignancy. *Br J Surg* 1995;82(8):1142.
68. Neuhaus SJ, Texler M, Hewett PJ, Watson DI. Port-site metastases following laparoscopic surgery. *Br J Surg* 1998;85(6):735-41.

69. Martinez J, Targarona EM, Balague C, Pera M, Trias M. Port site metastasis. An unresolved problem in laparoscopic surgery. A review. *Int Surg* 1995;80(4):315-21.
70. Lundholm K, Hulten L, Engaras B, Nordgren S, Svaninger G. [Laparoscopy is not suitable in malignant tumors. Risk of neoplasm seeding by instrument]. *Lakartidningen* 1994;91(46):4262-5.
71. Dorrance HR, Oien K, O'Dwyer PJ. Effects of laparoscopy on intraperitoneal tumor growth and distant metastases in an animal model. *Surgery* 1999;126(1):35-40.
72. Mathew G, Watson DI, Ellis T, De Young N, Rofe AM, Jamieson GG. The effect of laparoscopy on the movement of tumor cells and metastasis to surgical wounds. *Surg Endosc* 1997;11(12):1163-6.
73. Yamaguchi K, Hirabayashi Y, Shiromizu A, Shiraishi N, Adachi Y, Kitano S. Enhancement of port site metastasis by hyaluronic acid under CO₂ pneumoperitoneum in a murine model. *Surg Endosc* 2001;15(5):504-7.
74. Gupta A, Watson DI, Ellis T, Jamieson GG. Tumour implantation following laparoscopy using different insufflation gases. *ANZ J Surg* 2002;72(4):254-7.
75. Wittich P, Steyerberg EW, Simons SH, Marquet RL, Bonjer HJ. Intraperitoneal tumor growth is influenced by pressure of carbon dioxide pneumoperitoneum. *Surg Endosc* 2000;14(9):817-9.
76. Ishida H, Murata N, Idezuki Y. Increased insufflation pressure enhances the development of liver metastasis in a mouse laparoscopy model. *World J Surg* 2001;25(12):1537-41.
77. Takiguchi S, Matsuura N, Hamada Y, Taniguchi E, Sekimoto M, Tsujinaka M, et al. Influence of CO₂ pneumoperitoneum during laparoscopic surgery on cancer cell growth. *Surg Endosc* 2000;14(1):41-4.
78. Gutt CN, Gessmann T, Schemmer P, Mehrabi A, Schmandra T, Kim ZG. The impact of carbon dioxide and helium insufflation on experimental liver metastases, macrophages, and cell adhesion molecules. *Surg Endosc* 2003;17(10):1628-31.

79. Lecuru FR, Agostini AF, Coulet FP, Robin FP, Aggerbeck MS, Jais JP, et al. Effect of pneumoperitoneum on circulating tumor DNA. *Anticancer Res* 2001;21(3B):2029-32.
80. Lacy AM, Garcia-Valdecasas JC, Delgado S, Castells A, Taura P, Pique JM, et al. Laparoscopy-assisted colectomy versus open colectomy for treatment of non-metastatic colon cancer: a randomised trial. *Lancet* 2002;359(9325):2224-9.
81. Veldkamp R, Kuhry E, Hop WC, Jeekel J, Kazemier G, Bonjer HJ, et al. Laparoscopic surgery versus open surgery for colon cancer: short-term outcomes of a randomised trial. *Lancet Oncol* 2005;6(7):477-84.
82. Capussotti L, Massucco P, Muratore A, Amisano M, Bima C, Zorzi D. Laparoscopy as a prognostic factor in curative resection for node positive colorectal cancer: results for a single-institution nonrandomized prospective trial. *Surg Endosc* 2004;18(7):1130-5.
83. Weeks JC, Nelson H, Gelber S, Sargent D, Schroeder G. Short-term quality-of-life outcomes following laparoscopic-assisted colectomy vs open colectomy for colon cancer: a randomized trial. *Jama* 2002;287(3):321-8.
84. Jayne DG, Guillou PJ, Thorpe H, Quirke P, Copeland J, Smith AM, et al. Randomized trial of laparoscopic-assisted resection of colorectal carcinoma: 3-year results of the UK MRC CLASICC Trial Group. *J Clin Oncol* 2007;25(21):3061-8.
85. Bonjer HJ, Hop WC, Nelson H, Sargent DJ, Lacy AM, Castells A, et al. Laparoscopically assisted vs open colectomy for colon cancer: a meta-analysis. *Arch Surg* 2007;142(3):298-303.
86. Goetze T, Paolucci V. Does laparoscopy worsen the prognosis for incidental gallbladder cancer? *Surg Endosc* 2006;20(2):286-93.
87. Brokelman WJ, Holmdahl L, Bergstrom M, Falk P, Klinkenbijn JH, Reijnen MM. Peritoneal fibrinolytic response to various aspects of laparoscopic surgery: a randomized trial. *J Surg Res* 2006;136(2):309-13.

88. Neudecker J, Junghans T, Ziemer S, Raue W, Schwenk W. Effect of laparoscopic and conventional colorectal resection on peritoneal fibrinolytic capacity: a prospective randomized clinical trial. *Int J Colorectal Dis* 2002;17(6):426-9.
89. Holmdahl L, Eriksson E, Risberg B. Measurement of fibrinolytic components in human tissue. *Scand J Clin Lab Invest* 1997;57(5):445-51.
90. Ivarsson ML, Bergstrom M, Eriksson E, Risberg B, Holmdahl L. Tissue markers as predictors of postoperative adhesions. *Br J Surg* 1998;85(11):1549-54.
91. Reijnen MM, van Goor H, Falk P, Hedgren M, Holmdahl L. Sodium hyaluronate increases the fibrinolytic response of human peritoneal mesothelial cells exposed to tumor necrosis factor alpha. *Arch Surg* 2001;136(3):291-6.
92. Falk P, Ma C, Chegini N, Holmdahl L. Differential regulation of mesothelial cell fibrinolysis by transforming growth factor beta 1. *Scand J Clin Lab Invest* 2000;60(6):439-47.
93. Potzsch B, Grulich-Henn J, Rossing R, Wille D, Muller-Berghaus G. Identification of endothelial and mesothelial cells in human omental tissue and in omentum-derived cultured cells by specific cell markers. *Lab Invest* 1990;63(6):841-52.
94. Pronk A, Leguit P, Hoynck van Papendrecht AA, Hagelen E, van Vroonhoven TJ, Verbrugh HA. A cobblestone cell isolated from the human omentum: the mesothelial cell; isolation, identification, and growth characteristics. *In Vitro Cell Dev Biol* 1993;29A(2):127-34.
95. Bengtson JP, Sonander H, Stenqvist O. Gaseous homeostasis during low-flow anaesthesia. *Acta Anaesthesiol Scand* 1988;32(7):516-21.
96. Mosteller RD. Simplified calculation of body-surface area. *N Engl J Med* 1987;317(17):1098.

97. Neudecker J, Junghans T, Raue W, Ziemer S, Schwenk W. Fibrinolytic capacity in peritoneal fluid after laparoscopic and conventional colorectal resection: data from a randomized controlled trial. *Langenbecks Arch Surg* 2005;390(6):523-7.
98. Tarhan OR, Barut I, Akdeniz Y, Sutcu R, Cerci C, Bulbul M. Fibrinolytic responses of human peritoneal fluid in laparoscopic cholecystectomy: a prospective clinical study. *Surg Endosc* 2007.
99. Ziprin P. Laparoscopic-type environment enhances mesothelial cell fibrinolytic activity in vitro via a down-regulation of plasminogen activator inhibitor-1 activity. *Surgery* 2003;134(5):758-65.
100. Hanly EJ, Mendoza-Sagaon M, Murata K, Hardacre JM, De Maio A, Talamini MA. CO₂ Pneumoperitoneum modifies the inflammatory response to sepsis. *Ann Surg* 2003;237(3):343-50.
101. Neuberger TJ, Andrus CH, Wittgen CM, Wade TP, Kaminski DL. Prospective comparison of helium versus carbon dioxide pneumoperitoneum. *Gastrointest Endosc* 1996;43(1):38-41.
102. Dexter SP, Vucevic M, Gibson J, McMahon MJ. Hemodynamic consequences of high- and low-pressure capnoperitoneum during laparoscopic cholecystectomy. *Surg Endosc* 1999;13(4):376-81.