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Differently charged polypeptides and their impact on peritoneal and pleural postoperative adhesion formation

Åkerberg, Daniel

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Daniel Åkerberg

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Title and subtitle: Differently charged polypeptides and their impact on peritoneal and pleural postoperative adhesion formation

Abstract: Abdominal adhesions are formed after previous peritoneal traumas where previous surgery poses the most frequent cause. An increasing number of clinical complications due to adhesions have been detected such as small bowel obstructions, female infertility, and pain. Postoperative adhesions also form in pleura and pericardium after thoracic surgery. Complications include risk of bleeding, organ perforation and prolonged surgery, both in the thorax and abdomen, during reoperations. Previous reports have shown increased healthcare expenditures due to complications of abdominal adhesions. Several prophylactic anti-adhesion devices exist on the market, but none of them are sufficient in every aspect, such as being able to be used during abdominal infections, bleeding and in case of an intestinal anastomosis. The use of two differently charged polypeptides covering the peritoneal wounds during surgery has, in previous studies, shown promising anti-adhesion effects. The aim of this study was to investigate whether the polypeptides in any way affected different important healing aspects of the peritoneum and if the polypeptides may be administered as a spray in an animal study (I). Furthermore, the aim was to elucidate if the administered polypeptides affected important aspects of the healing process during an extended time dynamic pattern (II). It was also investigated whether the polypeptides reduced adhesions after adhesiolysis in the abdomen (III) and pleura (IV), and if there was an impact on peritoneal/pleural healing. In order to investigate the impact of polypeptides, an in vitro cell model was set up (V). A significant decrease in adhesions was seen both in the abdomen and pleura using the polypeptides. A significant decrease in adhesion reformation was seen after adhesiolysis and polypeptide administration. Despite some variation, no significant impact on key parameters of peritoneal and pleural resolving processes were seen after administration of the polypeptides. It was feasible to administer the polypeptides with a spray atomizer. Cell proliferation was decreased when higher concentrations of the polypeptides were administered, indicating a dose response relationship relying on the configuration and amount of charges of the polypeptides.

In conclusion, the use of the two differently charged polypeptides to prevent abdominal and pleural adhesions was feasible, reducing adhesions after primary surgery and relaparotomy, without affecting key parameters of the resolving process investigated.

Key words: Abdominal adhesions, small bowel obstructions, pain, female infertility, pleural adhesions, peritoneal, pleural, resolving process and key substances.

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Differently charged polypeptides and their impact on peritoneal and pleural postoperative adhesion formation

Daniel Åkerberg

Supervisor:
Associate Professor Bobby Tingstedt

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List of publications

I. Åkerberg D, Grunditz C, Posaric- Bauden M, Isaksson K, Andersson R, Tingstedt B. The Influence on abdominal adhesions and inflammation in rabbits after exposure to differently charged polypeptides. JBiSE 2012; 5: 432-38.

II. Åkerberg D, Isaksson K, Posaric- Bauden M, Andersson R, Tingstedt B. Effects of poly-L-lysine and polyglutamate on inflammation and the normal process of peritoneal healing after surgery. J Tissue Sci Eng 2012; 3: 117.

III. Åkerberg D, Posaric Bauden M, Isaksson K, Andersson R and Tingstedt B. Prevention of Adhesions by PL/PG after Adhesiolysis. J Tissue Sci Eng 2012, 3:4

IV. Åkerberg D, Posaric-Bauden M, Isaksson K, Andersson R, Tingstedt B. Prevention of Pleural Adhesions by Bioactive Polypeptides - A Pilot Study Int J Med Sci 2013; 10:1720-1726. doi:10.7150/ijms.6651

V. Åkerberg D, Grunditz C, Isaksson K and Tingstedt B Effect of polypeptides on mesothelial cells proliferation. In manuscript.

Abbreviations

ACP	Auto-cross-linked polysaccaride
BW	Body Weight
CO2	Carbone Dioxide
ECM	Extracellular Matrix
FDP	Fibrin Degrading Products
FITC	Fluorescein Izo ThioCyanate
GAG	Glycosaminoglycan
HA	Hyaluronic Acid
ICU	Intensive Care Unit
IL-1β	Interleukin-1-Beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IVF	In Vitro Fertilization
kDa	Kilo Dalton
LMWH	Low Molecular Weight Heparin
MCP-1	Monocyte Chemoattractant Protein-1
MSO-1	Mesothelial Cell Growth Medium
NSAID	Non-Steroid Anti-Inflammatory Drug
OD	Optical Density
ORC	Oxidized Regenerated Cellulose
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate Buffered Saline
PEG	Poly Ethylene Glycol

PG	Poly-L-Glutamate
PL	Poly-L-Lysine
PTFE	PolyTetraFluorEthylene
PMN	PolyMorpho Nuclear cells
SBO	Small Bowel Obstruction
SCAR	Surgical and Clinical Adhesive Research group
SEM	Standard Error of Mean
TGFβ	Transforming Growth Factor Beta
TM	Trademark
TNFα	Tumor Necrosis Factor Alpha
tPa	Tissue-type Plasminogen Activator

Introduction

Peritoneal structure

The peritoneum is the largest serous organ in the human body with an area corresponding very well to the total body surface area (Pawlaczyk et al. 1996). Peritoneum is of mesenchymal origin and divided into a visceral and a parietal part that encircles most of the abdominal organs and abdominal cavity (Albanese et al. 2009). Pericardium, pleura and testicular sac are other membranes of the same embryological origin and have similar properties as the visceral and parietal peritoneum (Michailova 1996).

Peritoneum consists of a single layer of mesothelial cells, loosely attached to a basal membrane underneath. Mesothelial cells are easily dislodged from the basal membrane. The basal membrane separates the mesothelial cells from the underlying submesothelial tissues (Ettarh and Carr 1996). Located at the apical end of the mesothelial cells are numerous microvilli that increase the total surface area of the peritoneum. Mesothelial cells are covered by a thin biofilm containing glycosaminoglycan, hyaluronic acid and phospholipids. Mesothelial cells are mostly squamous but cuboidal shaped cells exists in some areas of the peritoneum e.g. at the greater omentum and were the lymphatic vessels enters the diaphragm, so-called milky spots. Mesothelial cells in these areas seem to be more metabolically active than in other areas, since they contain more cytoplasmic organelles (Mironov et al. 1979), (van der Wal and Jeekel 2007).

The connective tissue in the submesothelial area constists of collagen, fibronectin, elastin, capillary network, lymphatic network and dormant resident cells such as fibroblasts, monocytes and mast cells. The lymphatic network form ducts that run up between the mesothelial cells (Ohtani et al. 1993; Cotran and Karnovsky 1968).

Peritoneal fluid

Peritoneal fluid has similar the function as the pericardial and pleural fluid which is to aid, through smoothing friction, free movement between the organs. The peritoneal fluid is in constant movement and in close contact with the pleural fluid via the lymphatic system. Normally, the volume of peritoneal fluid is more or less constant in men (5ml), but varies in females with the menstrual cycle and may be elevated above 5ml in the middle of the menstrual cycle (Bouckaert et al. 1986). The content of peritoneal fluid is, under normal conditions, equal to a transudate of the blood plasma. Only molecules under 20kDa can pass freely from the plasma to the peritoneal fluid (Flessner et al. 1985). Resident cells in peritoneal fluid, such as monocytes, are low in numbers during normal conditions but are active during peritoneal injury (Weinberg et al. 1991).

Peritoneal response to injury

Historical background

In 1919 Hertzler observed that there were differences in terms of re-epithelialization between peritoneal and skin defects. The latter form centripetal epithelialization, whereas peritoneal defects seemed to get epithelialized rather uniformly at the same time. This was an important theorem in the research field of peritoneal adhesions.

Furthermore, experiments (Ellis 1962) showed that the source of mesothelial cells contributing to its healing process originated from the defect itself and not from the surrounding intact peritoneal area (Ellis et al. 1965).

Cellular events during peritoneal injury

Peritoneal defects induced by various injuries such as surgery, infections and inflammation result in the loss of mesothelial cell integrity, unraveling the submesothelial area and thereby causing hemorrhage and a serosanginous exudate from the submesothelial capillaries. One of the earliest cell types to appear after peritoneal damage is the polymorph nuclear cell (PMN cell) (Shimanuki et al. 1986). The process of PMN cells appearing at the wound site is a part of the local cellular defense mechanism through which proteases, highly reactive metabolites

and cells are recruited by chemokine. The important chemokine IL-8 which is produced by injured mesohtelial cells may attract PMN cells (Topley et al. 1993a).

At 24 to 36 hours after the peritoneal injury PMN cells decrease in numbers and monocytes differentiated to macrophages, recruited by the chemokines MCP-1 and RANTES, increase in numbers at the wound site. MCP-1 and RANTES are secreted by injured mesothelial cells (Jonjic et al. 1992). The vast majority of macrophages come from resident inactivated monocytes located at the submesothelial area during normal conditions (Mutsaers et al. 2002). Macrophages build up a granulation tissue and take part in the remodeling of the injured tissue through various collagenases. Macrophages secrete important cytokines, such as IL-1β, TNFα and IL-6, which further control several cell functions and cell recruitment in the area. The cytokines have both autocrine and paracrine effects and are capable of further stimulation of both macrophages and mesothelial cells. IL-1β and TNFα stimulates mesothelial cells to produce IL-6. IL-6 is considered to be an important marker of mesothelial trauma (Offner et al. 1995). The macrophages remain in the wound between 10 to 14 days after the peritoneal injury (Ellis 1978).

After 3 to 4 days there is a gradual appearance of primitive mesenchymal stem cells at the peritoneal wound site. The primitive mesenchymal stem cells have functions that contribute to the restoration of injured peritoneum. At the same time proliferating fibroblasts appear; these may have their origin from inactivated fibroblasts at the submesothelial space, but may potentially also originate from the mesenchymal stem cells. The macrophages stimulate fibroblasts via Transforming Growth Factor β (TGFβ) and other substances to produce extracellular matrix (Rout et al. 2002; Tsukamoto et al. 1981). Fibroblasts are an important factor in tissues remodeling and build up of extracellular matrix (Saed et al. 2001).

Gradually as the submesothelial matrix is rebuilt, mesothelial cells begin to appear scattered on the wound surface and form a continuous layer between 5 to 7 days after the initial injury. Until now, there is no unifying theory regarding the source of mesothelial regeneration after peritoneal injury. However, studies have suggested that possible sources of mesothelial cells may be transformation from cells in the peritoneal fluid, transformation of mesenchymal stem cells lying at the wound base and/or transformation of blood cells into mesothelial cells (Foley-Comer et al. 2002).

Peritoneal fluid response to peritoneal injury

The peritoneum is in immediate and constant contact with peritoneal fluid throughout the entire process of acute injury and later on during the recovery process. Therefore, the peritoneal fluid corresponds very well to cellular and

interactive events that take place at the wound surface of the injured peritoneum and peritoneal fluid have an active part in peritoneal wound healing (Shimanuki et al. 1986) .

PMN cells are the first type of cells that appear in peritoneal fluid after a peritoneal injury. PMN cells are thereafter gradually replaced by macrophages as the process of inflammation proceeds (Vural et al. 1999; Haney 2000). As the peritoneal restoration continues, the peritoneal fluid might be a source of free floating mesenchymal stem cells that may contribute to the re-epithelialization at the wound surface by mesothelial cells (Mutsaers 2002).

Various cytokines have been detected in peritoneal fluid as a result of peritoneal injury. Some of those of major importance are IL1β, TNFα and IL-6. They have all been detected in peritoneal fluid during the first hours after peritoneal trauma (Kaidi et al. 1995; Tsukada et al. 1993). They are considered to be secreted actively to the peritoneal fluid by injured mesothelial cells, PMN cells and macrophages. TGFβ have also been detected in peritoneal fluid after various types of peritoneal trauma and reaches a maximum level at day 4 to 7 after the injury, reflecting the matrix remodeling during the recovery phase of peritoneum (Rong et al. 1997; Cheong et al. 2003)

The plasmin systems response to peritoneal injury

An essential aspect of the defense mechanisms of various types of human biological traumas, such as abdominal surgery, inflammation, infection and others is its ability to resolve the damaged tissue and regain its original structure and functions.

In order to resolve peritoneal tissue after trauma, certain tissue proteases are pivotal to degrade extracellular matrix such as the basal lamina and the submesothelial tissues. In this aspect, plasmin and other serine proteases are very important being capable of tissue remodeling (Martin et al. 2000; van Hinsbergh et al. 1990).

In the process of tissue restoration after injury fibrinogen, along with collagen and fibronectin, is one of the most important extracellular matrix proteins. It is released from capillaries during tissue trauma. Fibrinogen is polymerized to fibrin via thrombin, forming a semi rigid structure with fluid sealant properties. After peritoneal trauma fibrin is rapidly formed, merging adjoining injured peritoneal sites. Previous data indicate that firbin plays a crucial role in adhesion development (Ryan et al. 1971)

The inactive proenzyme plasminogen is converted to the active enzyme plasmin by tissue plasminogen activator (tPA). Plasmin is thereafter, via tPA, capable of

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degrading fibrin (fibrinolysis) into fibrin degrading products (FDP). TPA has a high affinity for fibrin thereby promoting the fibrinolytic effect of plasmin where its mostly needed (Mosesson et al. 2001) This is a crucial step in the remodeling process (resolving) of peritoneal trauma (Holmdahl and Ivarsson 1999). Plasminogen activator inhibitor type 1 (PAI-1) is an important inhibitor of tPA and thereby retards/terminates fibrinolysis (Kruithof et al. 1984).

tPA is, during normal conditions, stored in great quantities in endothelial cells in the submesothelial area and have been shown to be released from endothelial cells, macrophages and mesothelial cells during surgery. Thereby, it is believed that these cells take an active part in the fibrinolysis process during peritoneal trauma (Emeis et al. 1997).

Immunohistochemistry has revealed that PAI-1 is stored both intracellularly and extracellularly (associated with extracellular matrix) in peritoneum during normal conditions and rapidly released during peritoneal injury thereby inhibiting local fibrinolysis during peritoneal injury (Rougier et al. 1998).

The plasmin system in peritoneal biopsies and peritoneal fluid during and after peritoneal injury

During the 20th century a vast body of evidence has revealed fibrinolytic activity to be reduced during and after surgery, which has led to the theory of *the classical concept of adhesion formation* (Lehman and Boys 1940), (Raftery 1979) .

 A substantial amount of studies have shown that tPA is reduced and PAI-1 is increased in peritoneal biopsies during and after various abdominal surgery (Ivarsson et al. 1998; Brokelman et al. 2009; Bergstrom et al. 2002). According to this, studies have shown reduced fibrinolytic capacity up to 24 hours postoperatively, but interestingly also a rebound increase in the fibrinolytic capacity in the subsequent period (Hellebrekers et al. 2009).

Measurements from peritoneal fluid during the postoperative face have, on the other hand, revealed high tPA levels, implying an active released of tPA from the peritoneal wound site and possibly from "remote" intact peritoneum (Hellebrekers et al. 2009; Ivarsson et al. 2001; Bakkum et al. 1996). However, both studies from biopsies and peritoneal fluid point in the direction of increasing FDPs which has led to the idea that there is an ongoing high rate of fibrin turn over at the wound site of the peritoneum (Edelstam et al. 1998).

The results between different studies regarding fibrinolysis in peritoneal biopsies and fluid during the postoperative phase are rather diverse when it comes to measuring tPA and PAI-1 in a time dynamic scale. Therefore, another way of estimating the reduction in fibrinolytic capacity is to measure tPA/PAI-1 complex

which shows the inhibition of tPA by PAI-1 in a 1:1 manner, thereby demonstrating the functionally active PAI-1 (Sancho et al. 1995)

The plasmin system and formation of peritoneal adhesions

Peritoneal adhesions are considered to be developed when the fibrin polymer is not completely dissolved by fibrinolysis. This combined with fibroblasts invading the fibrin scaffold eventually replacing the fibrin with a more stable permanent protein structure, i.e. the collagen. This process generally takes 5 to 7 days to resolve (Rout and Diamond 2003). Therefore, there is no difference when it comes to the time dynamicity in terms of healing or adhesion development.

Furthermore, a crucial part contributing to the diminished fibrinolysis is local peritoneal tissue ischemia that increases PAI-1 and diminishes tPA activity both in vitro and in vivo in peritoneal fibroblasts (Saed and Diamond 2003; Diamond et al. 2004). Biopsies from adhesions have shown higher levels of PAI-1 indicating lower fibrinolytic capacity in the adhesions (Hellebrekers et al. 2005).

Macrophages entangled in the fibrin scaffold express both IL-1β, TNF-α and IL-6 that may induce PAI-1 and down regulate tPA and thereby increase the risk of formation of adhesions (Cheong et al. 2002b; Saba et al. 1996; Binnebosel et al. 2008).

TGFβ is mainly secreted by fibroblasts but also from mesothelial cells and macrophages. TGFβ have been shown to increase PAI-1 activity (Tietze et al. 1998; Dou et al. 1997; Chegini et al. 1994). Even though all these cytokines in combination with tissue ischemia points to a diminished fibrinolysis and increased rate of adhesions development there is no unifying theory that fully can explain the mechanism of adhesion formation.

Adhesions, a clinical issue

Prevalence

An important but difficult task is to determine the frequency of postoperative abdominal adhesions in humans since there is no possibility to check for adhesions once the patient is operated upon unless the patient is subjected to further surgery.

The majority of adhesions causing clinical complications are formed due to previous surgery. Abdominal adhesions may also be formed due to other causes than surgery such as infections or previous radiation therapy. They may also be congenital.

In an important study from 1973, 752 consecutive autopsies were investigated regarding the prevalence of abdominal adhesions (Weibel and Majno 1973). In the group of patients that had undergone previous multiple operations, 93 % had abdominal adhesions. The prevalence of postoperative abdominal adhesions caused by repeated laparotomies has, in other studies, been considered to be around 93-95 % (Parker et al. 2007; Liakakos et al. 2001) (Menzies and Ellis 1990; Attard and MacLean 2007).

Other identified risk factors for having abdominal adhesions on the autopsy were overweight and previous inflammations such as appendicitis, diverticulitis and cholesystitis without prior surgery. Interestingly, there was no difference in the prevalence of postoperative adhesions between the different age groups.

The distribution of adhesions involving different organs in the abdomen also differs (Weibel and Majno 1973). The most common organ to be involved in adhesions is the greater omentum followed by the small intestine and the colon.

Clinical aspects of abdominal adhesions

Clinical complications of abdominal adhesions include small bowel obstruction, female infertility, pelvic and abdominal pain. Furthermore future operations are prolonged and carry a higher risk of inadvertent enterotomies. A considerable amount of healthcare expenditures and hospital costs are related to abdominal adhesions. Moreover there is an onging debate on laparoscopy and adhesions whether the stated less traumatic surgery is valid.

Small bowel obstructions (SBO)

A total of 0.9 % of all readmissions were adhesion related SBO and 3.3 % of all laparotomies were due to adhesions related SBO (Menzies and Ellis 1990). Approximately 60-70 % of the SBO are due to abdominal adhesions (Ellis 1997). Women with adhesion related SBO had undergone hysterectomy in around 40 % of the cases (Al-Took et al. 1999) and 1 to 2 % of open appendectomy patients develop SBO (Tingstedt et al. 2004; Andersson 2001). The most common surgical procedure to produce adhesions is surgery performed below the transverse colon, i.e. surgery performed to the sigmoid colon, rectum, appendix and gynecological surgery (Menzies 1993). In developing countries the causes of intestinal obstruction differ hugely and here the most common cause is strangulated hernias

(Ellis 1997). This is probably due to the lower rate of abdominal surgery performed in these countries combined with demographic carachetistics. Interestingly data from 1930 in Europe reflects the same relationship (Weibel and Majno 1973).

Every organ in the abdomen has a different frequency of involvement of adhesions. For instance postoperative adhesions spanning from a scar to the omentum or from the intestines to the omentum are the most common to occur but this rarely lead to small bowel obstruction. On the other hand small bowel adhesions (not involving the greater omentum) are less common, but these are the most common ones involved in small bowel obstruction. This important finding tells us that adhesions have different clinical impact depending on the location and is not solely a result of how frequent they are but may hypothetically reflect different biological mechanisms (Menzies and Ellis 1990).

Attempts have been made to assess which type of adhesions that causes what type of complications. It was shown that 29 % of bowel obstructions were caused of adhesions spanning from bowel to bowel and in 42% of the cases spanning from bowel to another structure, the rest were combined (Maetani et al. 1984).

Adhesion related readmissions

The burden of healthcare due to complication of adhesions is not insignificant. When measuring admittance to hospital due to complication of previous formed adhesions it has been calculated that 3.8 % of all emergency surgical admissions leading to laparotomy were due to adhesive intestinal obstructions (Ellis et al. 1999).

Futhermore this large data base study reported that 5.7 % of the readmissions were directly related to adhesions and 23.7 % possibly connected to abdominal adhesion. The mean readmission rate was 2.1 times per patient (Ellis et al. 1999). One in three patients experienced at least one adhesion related readmission in the following ten years after surgery. Fiftyfour percent of these patients had one readmission but 41 % had more than two readmissions (Parker et al. 2001).

Readmission distribution varied and patients with adhesion related readmissions within the first year included around 25 %, although a huge part of the readmissions were scattered over the following ten years (Parker et al. 2001). Thus making it hard to predict when the clinical complications of previous abdominal surgery might occur. The overall adhesion related readmission risk due to previous lower abdominal surgery (appendectomies excluded) was estimated to 5 % the following five postoperative years (Parker et al. 2005)

Female infertility

Another field where abdominal adhesions pose a huge clinical challenge is gynecology. Gynecologists have been studying adhesions for a long time (mostly through laparoscopy) since adhesions are considered a major cause of female infertility and pelvic pain.

The adhesions that cause infertility may originate from previous surgery in the area, pelvic inflammatory disorders, appendicitis, endometriosis, tuberculosis and other infectious or inflammatory conditions. The cause of infertility may emanate from a distortion of the normal tubo ovarian relationship preventing the ovum capture of the Fallopian tube. There have also been some suggestions that adhesions may hinder the maturation and development of the oocyte (Nagata et al. 1998).

In one study, adhesions were found in 37 % of the patients having infertility problems and adhesions were considered to be the sole factor causing infertility in 15 % of the patients (Milingos et al. 2000). Some data indicate that pelvic adhesions might cause infertility in up to 30 % (Singhal et al. 1991).

Laparoscopic adhesiolysis and/or microsurgery on the tuba and ovaries may be performed to reduce the adhesions and increase the chance of pregnancy. However, these procedures have a varying degree of success, ranging from 30 up to at most 50 %, leaving the rest of the patient no other option than to try in vitro fertilization (IVF) (Oelsner et al. 1994). The IVF is not itself any warrant for pregnancies and it has been shown that periovarian adhesions diminishes the success rate of IVF (Nagata et al. 1998).

Another concern when it comes to reproduction is the risk of ectopic pregnancies that is most often increased by previous infections, endometriosis and high age. However, one cannot rule out that there also is a correlation between pelvic adhesions and ectopic pregnancy (Bouyer et al. 2003).

Pelvic and abdominal pain

It has been debated whether adhesions may cause abdominal pain or not and this issue is still not fully elucidated. It has been shown that adhesions may contain sensory nerve fibers (Sulaiman et al. 2001) but for obvious reasons it is impossible to fully investigate whether these produce pain or not. Nevertheless, laparoscopic neurolysis (through adhesiolysis) have been shown to reduce abdominal pain when evaluated long term postoperatively, though not in all of the patients (Nezhat et al. 2000). In one randomized double blinded controlled study where patients with adhesions and chronic abdominal pain were randomized to laparoscopic adhesiolysis or diagnostic laparoscopy no difference were seen comparing base line characteristics between the groups (Swank et al. 2003)

In clinical settings, reports have varied whether adhesiolysis can decrease or relieve pain. There are blinded prospective reports that support the theory of adhesions as a cause of chronic pelvic pain in women (Steege and Stout 1991). The maximum pain site correlated well with operative findings of adhesions, but there have been difficulties in correlating the amount of adhesions with the amount of pain (Thornton et al. 1997). Furthermore, randomized blinded trials have failed to prove the correlation between pelvic pain and adhesions (Hammoud et al. 2004).

When dealing with pain and adhesions it is important to evaluate what pain modality the patient experience, since adhesions may cause colicky pain around hollow organs and more chronic distinct pain when located around parietal nerve fibers. Thus, it is also important to carefully select which patients should be attributed to surgery in order to have a high rate of success.

Laparoscopy

Laparoscopic surgery poses, from a theoretical point of view an attractive option compared to open surgery. Laparoscopy reduces the total length of incision in the peritoneum making the total area of injured peritoneum smaller which reduces the area that may be affected by adhesions. Foreign particles from swabs and gowns are most often not exposed to the abdomen during laparoscopy. Foreign particles are known to induce adhesion formation and these are reduced in laparoscopy compared to open surgery (Luijendijk et al. 1996; Kavic and Kavic 2002). Open surgery most often result in more tissue trauma than laparoscopic procedures also indicating less risk of adhesions through laparoscopy. In open surgery, the tissue handling is extensive since the operative site most often may not be reached without tissue packing implying the manipulation of large areas and risk of desiccation. Furthermore, retractors exert a huge pressure on the abdominal wall increasing the risk of mechanical damage on to the peritoneum. Finally the postoperative recovery most often is faster than after open surgery, which could be a result of faster bowel movement separating coalescent peritoneal surfaces, theoretically diminishing adhesion formation.

Despite this, some disadvantages of laparoscopic surgery regarding adhesion formation still exist. Concerns have been raised regarding the potential desiccation risk of peritoneum from the high pressured CO_2 that expose the mesothelial cells. It has been shown that the peritoneum might change morphology microscopically, disturbing the mesothelial integrity leaving parts of the underlying basal lamina bare, thus increasing the risk of adhesion formation. It has also been speculated that the increased CO_2 levels may locally acidify the peritoneum (Volz et al. 1999). Pneumoperitoneum by CO_2 also affect the mesothelium. It has been shown that transgenic mice lacking tPa had significantly more adhesions than its wild type counterparts. It was further shown that transgenic mice lacking PAI-1 genes

also had significantly fewer adhesions after laparoscopy than their wild type counterparts. This implies that essential mechanisms of adhesion development, in part, might be caused by depressed tPa and increased PAI-1 activity (Molinas et al. 2003; Elkelani et al. 2004).

For obvious reasons it is difficult to evaluate differences in adhesion formation between laparotomy and laparoscopy in humans, however, several animal studies have dealt with this issue. The result of animal studies on adhesion formation comparing laparoscopy and laparotomy are somewhat confusing. Some studies show less adhesions in favor of laparoscopy (Tittel et al. 2001; Garrard et al. 1999; Chen et al. 1998) and some show no differences in adhesion formation between the two procedures (Marana et al. 1994). The reason for these differences in results may be due to many factors, some of them being the way of creating adhesions prior to the evaluation, some being differences in specific sites where adhesions were measured and others may be differences in the use of classification of adhesions.

Human studies comparing adhesion development after laparoscopy and laparotomy are sparse and most of them favor laparoscopy (Audebert and Gomel 2000; Milingos et al. 2000; Polymeneas et al. 2001). A recent study, including more than 4000 patients, favors laparoscopic appendectomy over open appendectomy regarding SBO during long term follow up (Isaksson 2013). This study further shows that open appendectomy and high age were independent factors for adhesion related SBO. Other studies have failed to show significant fewer adhesions after laparoscopic appendectomies compared to open. One reason may have been that they were under powered (Swank et al. 2011; Kouhia et al. 2010).

Although fewer adhesions seem to form after laparoscopy, it would be difficult to advocate this method in all cases of abdominal surgery and there are several reasons for this. Laparoscopy is not suitable for all surgical procedures, and although fewer adhesions are formed, laparoscopic procedures are not adhesion free. The differences in studies of animals and humans are hard to interpret since they do not compare adhesions in the same way. When it comes to entering an abdomen with a high suspicion of adhesions it is rather hazardous to choose laparoscopy over laparotomy, making it unsuitable in this situation especially in the field of general surgery.

As previously stated laparoscopy is widely used in gynecology. Despite this, laparoscopy is not hazard free even in the field of gynecology and complications may in some cases, be more difficult to discover compared to open surgery (Leonard et al. 2000).

Overall laparoscopy seems to reduce postoperative adhesions. The evidence of significant clinical endpoints, such as reduction of small bowel obstructions and female infertility seem to favour laparoscopy, but further evidence is needed.

Peroperative complications

An important consequence of adhesions in the abdomen is an increased time span from skin incision to the entry of free abdominal cavity. Studies have shown significantly prolonged time entering the abdominal cavity in patients with adhesions compared to those without adhesions (Beck et al. 2000),(Coleman et al. 2000).

The burden of hard and sticky adhesions has merely just begun when entering the abdominal cavity. Most often surgeons are forced to divide the adhesions in order to get access to various organs. The dissection is rather hazardous and increases the risk of inadvertent enterotomy.

Data have shown that one out of five patients (19 %) was subjected to inadvertent enterotomy during relaparotomy and subsequent adhesiolysis (Van Der Krabben et al. 2000). These inadvertent enterotomies in turn increased the complications by far and resulted in higher frequencies of sepsis, anastomotic leakage, pneumonia, wound dehiscence, ICU admittance, urgent relaparotomy and a high rate of mortality.

A risk factor for inadvertent enterotomies was previous multiple surgery (Van Der Krabben et al. 2000). The long term risk of peroperative undetected damages to the intestines or other organs during surgery may result in the development of fistulas having a huge impact on the medical conditions and life quality for the patients (ten Broek et al. 2013; Martinez et al. 1998; Kaltiala et al. 1972).

Financial impact

The financial impact of abdominal adhesions is huge. In the study by Ray et. al. from 1988, the 54100 patients had complications directly related to adhesions. The total cost of hospital care accounted for $1180 million USD in 1988. This sum does not include the total cost for the society such as time lost from work, reduced productivity and lost wages (Ray et al. 1998).

In 1994 Ray repeated the same study as above rendering a total cost of $1 325,9 million USD which actually is a reduction compared to 1988 (which converted to 1994 value would be $ 1 437,1 million USD). However this lower amount is not attributed to lower expenditures of adhesions but rather shorter hospital stays (Ray et al. 1998).

In another study from Scotland the annual costs from 1988 to 2008 increased more than one hundred percent from $ 1.3 billion USD to $ 2.28 billion USD (Parker et al. 2005).

In a retrospective study by Ivarsson et.al it was extrapolated that the direct annual adhesion related cost in Sweden would be $ 13 million USD (Ivarsson et al. 1997).

Another Swedish study by Tingstedt et.al followed patients with documented adhesions at operative adhesiolysisfor 10 years and the combined financial impact was calculated. The annual adhesion related cost of inpatient care, loss of production and cost of sick leave was added up to 39.9–59.5 million Euros. The inpatient cost corresponded to that of gastric cancer (Tingstedt et al. 2007a). Colorectal surgery is known to be a high risk of inducing abdominal adhesion related complications such as small bowel obstructions.

In Finland the annual adhesion related costs caused by colorectal surgery alone is estimated to be $ 72 520 USD in a district serving 450000 inhabitants (Kossi et al. 2004).

Pleural and pericardial adhesions

The pleura and pericardium lines the thoracic cavity and mediastinum and has the same features as the peritoneum sharing the same embryological mesenchymal background. The lining is a simple layer of mesothelial cells resting on a basal membrane and a submesothelial tissue comprising connective tissue and supporting cells (Li and Li 2003). At regular points there are stomata placed between the mesothelial cells and these holes are in contact with the underlying submesothelial vessels and lymphatics.

During early pleural and pericardial injury, PMN cells are attraced to the site of injury through chemotactic agents and gradually adjoined by fibroblasts and other tissue supporting cells as the resolving process continues as in the peritoneum (Mutsaers 2002; Davila and Crouch 1993).

Thoracic surgery has evolved since the 1960s and is nowadays as common as general surgery (Yoshimura et al. 1999; Gupta et al. 2010; Bailey et al. 2005).

Pulmonary resections due to metastatic disease have become more frequent and patients are also operated on more than once. Adhesions in the thoracic cavity make repeated surgery hazardous since it increases the risk of inadvertent organ injury and bleeding. It also prolongs the operating time (O'Brien et al. 2002; Schaff et al. 1983).

Although numerous animal experiments have been conducted, throughout the past years very few anti-adhesion devices to prevent pleural adhesions are available (Takagi et al. 2013), (Bicer et al. 2008).

After the introduction of video assisted thoracoscopy (VATS) the adhesion problem is probably less, but still the risk of complications from pleural and pericardial adhesions may force the surgeon to convert to open thoracotomy.

Experiments have been made to try to predict these adhesions preoperatively with low success rate this far (Sasaki et al. 2005). Various barriers are currently used to prevent pleural and pericardial adhesions with varying results (van der Linden et al. 2001; Bennett et al. 2003), (Jacobs et al. 1996).

Adhesion prevention

Many attempts have been made during the past decades to try to prevent postoperative abdominal adhesions with various success rates. Anti-adhesion devices should exhibit some ideal properties that are listed below. Anti-adhesion devices may be subdivided according to their way of separating peritoneal injured tissues, shown in table 1 (page 31). The separation process seems to be the most important mode of action since adjoining injured peritoneal tissues are more prone to develop adhesions (Lucas et al. 1996).

Ideal anti-adhesion device

To come up with a product that exhibits an ideal anti-adhesion devicce is not easy and poses a great challenge considering several aspects in the field of surgery. Until today there is no available anti-adhesion device on the market, fulfiling the criteria, mandatory for these purposes.

The ideal anti-adhesion device should have the following features:

Inert. That is exhibiting bio film properties and should be able to separate the damaged peritoneal tissues and not be directly incorporated in the submesothelial area.

Non Immunogenic. It should not, in any way, affect the immune system, neither inhibit nor potentiate. This includes both the acquired and innate part of the immune system. The obvious reason for this is that the immune response poses

pivotal parts of the resolving mechanism after surgery. Neither should the cytokine system be involved. This includes both pro inflammatory as well as anti-inflammatory cytokines (e.g. (IL-1β, TNFα and IL-6).

Coagulation. Another important feature is not to interfere with the coagulation system. Thus future anti-adhesion devices may interfere with coagulation; because of increased fibrin formation (due to potentiation) may form large deposits on peritoneum thereby being adhesiogenic. Bleeding, on the other hand (as a result of inhibition) may form large areas of fibrin clots and erythrocytes, inducing local tissue hypoxia on peritoneum thereby increasing the potentiation of adhesion development. Furthermore, important initiators of anti-coagulation and coagulations should not be affected (tPA, PAI-1 etc).

Systemic effects. A future anti-adhesion device may not pose any systemic effects per- or postoperatively. These include, allergic reactions, bleeding from remote areas or impact on various remote organs like lung, heart, kidneys and liver.

Resolving process. In all processes of recovery after surgery the resolving process is pivotal as it involves tearing down the injured tissue as well as remodeling it. None of these two processes should be affected locally on the peritoneal site of injury. Remodeling of an injured tissue is a dynamic process spanning over several days, thereby putting high demands on the anti-adhesion device. It is important that anti-adhesion devices do not interfere with supporting cells and important cell signal systems locally in submesothelial area (e.g. fibroblasts and TGFβ).

Nontoxic. The dose response curve entails, for every device, upper and lower limits for its efficacy and in order to have a safe marginal the toxicity should, at least, be 100 times above efficacy levels.

Elimination. An anti-adhesion device should be biodegradable. A device that is metabolized and eliminated through peripheral organs (liver, lungs or kidneys) would be preferable (Tingstedt et al. 2008).

Easy to handle. It is pivotal that the anti-adhesion device is easy to handle in every aspect. This includes easy administration both through laparoscopy and laparotomy. It should preferably adhere firmly to the underlying injured peritoneal tissue not having to be stitched or having to be removed later on.

Anti-adhesion properties. Of course this is the most important aspect and should entail not only primary surgery but also secondary and tertiary. That is, reformation of adhesions after adhesiolys should be low. The anti-adhesion properties should preferably span the entire abdomen as well as thorax cavity and include all kinds of surgical procedures.

Economic aspects. It is important that the anti-adhesion device expenditures is reduced to a limit were it is feasible to use it to overcome the expenditure of

adhesion related complications. Wilson et al concludes that antiadhesive products that exceed £ 200 are unlikely to pay back their direct costs (Wilson et al. 2002).

Measuring adhesions

Adhesions were early observed as being a major postoperative complication. Already in the early 20th century, studies were performed to decrease postoperative adhesions. Therefore, there has been an early interest in measuring and quantifying postoperative adhesions. However there is no unifying system of classification of adhesions and reviewing the litterature reveals over 20 different adhesion classification systems. Despite this some general criteria of measuring adhesions are common in every one of the classification systems.

Type of adhesion. When dissecting adhesions during surgery it is known that the quality may vary from being easily dissected to being very hard and almost impossible to dissect. Based on this, adhesions have been categorized into being filmy (easy to dissect), thick (somewhat hard to dissect) and dense (hard to dissect) (Oncel et al. 2003).

Tenacity of adhesions. Sometimes it is valuable to further describe the type of adhesion and describe by which modality it was lysed (Naito et al. 2008).

Location of adhesions. Adhesions may be found in the whole abdominal cavity thus being somewhat hard to classify according to their location. Studies have focused on scoring adhesions by adding the different locations thereby making accumulated scores (Bothin et al. 1999).

Involvement of adhesions. Adhesions may be quantified according to their extent. This is expressed as the ratio between the length of adhesions and the length of total injured area (mostly the abdominal wall) expressed in percent (Holmdahl et al. 1994).

Previous and existing anti-adhesion devices

Anti-adhesion prevention may be subclassified according to mode of action. Outlined below are the most common anti-adhesion devices described by their anti-adhesion effect and healing properties. Table 1 (page 31) summarizes possible influence on the resolving process.

Mechanical separation through hydroflotation

Crystalloids

Ringer® solution and Saline have previously been used at the end of the surgical procedure to diminish postoperative abdominal adhesions. However the problem with crystalloids in terms of anti-adhesion device, is the rapid absorption from peritoneum (500ml within 24 hours) thereby being unable to exert their effect during the critical time (5-7 days) of adhesion formation (Krediet and Arisz 1989). Other drawbacks include risk of overload of volume with subsequent, pulmonary edema, ascites and diminished postoperative resolving capacity.

Concerns have been raised against patient's defense against infections. Leaving a large volume of fluid in the peritoneal cavity may increase the risk of contamination through diminished opsonic capacity as a result of the dilution effect. Crystalloids may thereby increase postopoperative infections making them unsuitible for the use in abdominal surgery in this aspect (Dunn et al. 1984) . It also seems like intravenously given crystalloids may render longer bleeding time, probably through a dilution effect of the coagulation factors (Ogawa et al. 2013). Whether this is the case when administered locally in the peritoneum is not yet proven but cannot be ruled out since a huge amount of crystalloids must be given on to peritoneum to have any anti-adhesion effect.

The anti-adhesion effect has been questioned and some data point to a very moderate effect on the adhesion reduction (Wiseman et al. 1998).

Dextran

A water soluble solution of the molecule dextran (Hyskon®) has been used efficiently in abdominal adhesion prevention (ten Kate-Booij et al. 1985). The molecular weight is about 70 kDa which means that the molecule is not immediately absorbed from the peritoneal surface. The dextran is thereby located on the peritoneum acting as a hydro flotation (through osmosis) device separating injured peritoneal tissues.

The fact that dextran is not absorbed from peritoneum has its side effects and studies have noted ascites and the well-known anaphylactic shock problems using dextrane makes it questionable for this purpose (Markman et al. 1984). In some cases even coagulopathy have been discovered (Mangar et al. 1989). This is probably, in part, due to the increase in tPa seen after administering dextran but also due to the reduction of coagulation factors (Vipond et al. 1994). Dextran may activate and induce the peritoneal release of IL-1β, triggering the activation of several immune cells in the area, and thereby modulating the immune response when applied in the abdominal cavity (Kwon et al. 2007). Furthermore, dextran may release TGFβ from a its plasma bound inactive form and thereby increasing its effect and theoretically increasing postoperative adhesions (Logeart-

Avramoglou et al. 2002). Dextran is not efficient when it comes to reducing adhesions postoperatively (Diamond et al. 1988)

Icodextrin

Icodextrin is a glucose polymer, used as an antiadhesive agent in a 4% solution, Adept®. This is a starch molecule which is rapidly metabolized to glucose by the α-amylase in the systemic circulation, but it is adsorbed slowly from the peritoneal cavity thereby acting as hydroflotation anti-adhesion device. Adept® may not be used in patients suspected to have corn or starch allergies. Studies have shown reduction in postoperative adhesions (diZerega et al. 2002; Brown et al. 2007; Collins and Mujais 2002). Adept® decreases adhesions significantly but has not been significantly tested versus end points such as small bowel obstructions and pregnancy (Menzies et al. 2006; Kossi et al. 2009; Brown et al. 2007; Trew et al. 2011). One smaller study including 181 patients show a reduction of small bowel obstruction in a 3 year follow up (Catena et al. 2012).

Whether Adept® has any biological effects is hard to say since most studies examining the effect of icodextrin are done on the higher concentration, 7.5 %, used in continous ambulant peritoneal dialysis (CAPD). Data must thereby be extrapolated to the clinical situation were it is used as anti-adhesion device. CAPD studies show activation of PMN cells, macrophages and mesothelial cells (Matsumoto et al. 2012). Thus, in order to investigate if Adept® influences any of these components given as a single dose and at a lower concentration, more studies are needed. However, it is known that icodextrin reduces the production of tPa and PAI-1 from human cultured mesothelial cells (Katsutani et al. 2007). Furthermore icodextrin has been shown to enhance the activity of peritoneal neutrophils (Liberek et al. 1993).

Mechanical separation through barriers

Endogenous barriers

Endogenous barriers are classified as naturally occurring material (allografts) applied on to an injured area of peritoneum.

From a theoretical point of view the best option to prevent adhesions would be a flap of an endogenous material e.g. placenta, omentum, peritoneum and others (Wallwiener et al. 1998).

It seems like the endogenous barriers in fact are efficient reducing adhesions but only to some extent and exogenous barriers seem to be even more efficient. The endogenous barriers raises concerns regarding the handling procedure since these should be performed as flaps (free or pedicle shaped) which is not always easy. The flaps would have to be stitched into place thereby increasing the amount of foreign material in the abdomen and increasing the tension (and possible hypoxia) locally on the peritoneum, which in some cases might lead to yet further adhesion related problems.

Hyaluronic Acid and derivatives

Hyaluronic acid (HA), a linear polysaccharide, poses promising anti-adhesion properties since it is a naturally occurring glycosaminoglycan normally included in peritoneal structure. HA also is an important component in tissue healing (Ogston and Stanier 1950).

The mechanism of action of HA and its derivatives has been investigated and HA seems to reduce mesothelial cell detachment from peritoneum (Tsai et al. 2005) and may also reduce the monocyte involvement in adhesion formation (Falabella and Chen 2009). HA also promotes peritoneal healing through mesothelial proliferation, thereby increasing the healing potential (Reijnen et al. 2000). In vitro studies have shown that HA significantly increases the fibrinolytic activity in mesothelial cells when these are exposed to TNFα. The increased fibrinolysis resides in decreased PAI-1and increased tPa. This is an indication that HA actively interferes with the biochemistry of peritoneal trauma (Reijnen et al. 2001). HA also seem to have an effect on fibroblasts and it has been shown that the metabolism of fibroblasts is decreased during peritoneal injury and similar exposition to HA. It has also been shown that HA may modulate the cellular communications between macrophages and fibroblasts during peritoneal injury (Klein et al. 1996).

Hyaluronic acid has been used in different ways and combinations to prevent adhesions.

Hyaluronic acid combined with carboxymethylcellulose. Another promising barrier used in anti-adhesion settings is composed of hyaluronic acid with carboxymethylcellulose, Seprafilm® (Becker et al. 1996). It turns into a hydrophilic gel 24 h after placement and provides a protective coat for traumatized tissue for up to 7 days. The device is cleared from peritoneum within 4 weeks (Oncel et al. 2003). Data has indicated that Seprafilm® reduces adhesions solely as a barrier preventing the injured tissues from adjoining. It does not interfere with factors in the plasmin system, tPa/PAI (Tarhan et al. 2005). Cells involved in the peritoneal healing process of peritoneum such as, PMN cells and fibroblasts, have

also been investigated according to the possible action of Seprafilm® but seems to be unaffected by Seprafilm® (Otake et al. 2008; Gago et al. 2003a)

In vitro studies have also shown that the release of TGFβ seems unaffected and the important tissue serine proteases that remodels the tissues after injury seems unaffected by Seprafilm®. Furthermore, PMN cells did not seem to be affected, however the setup in this study left some questions unanswered. The PMN cells were harvested from peripheral blood and not from peritoneum and it was taken from healthy individuals not undergoing any surgery (Otake et al. 2008; Gago et al. 2003a)

Seprafilm® efficiency and safety has been recorded in 9 clinical trials involving humans (Fazio et al. 2006; Cohen et al. 2005a; Hayashi et al. 2008; Inoue et al. 2005; Kusunoki et al. 2005; Bristow and Montz 2005; Tang et al. 2003; Vrijland et al. 2002; Beck 1997) the largest being a multicenter randomized controlled safety study with 1701 patients (Fazio et al. 2006). The other studies are prospective clinical trials with altogether 961 patients enrolled.

No foreign body reactions were reported in a large safety study (Beck et al. 2003) and the two main concerns regarding Seprafilm® has been a significant increase in abscesses and anastomosis leakage in case of bowel resection and bowel anastomosis (Beck et al. 2003; Cohen et al. 2005b). The latter risk is reduced with the advice not to wrap the anastomosis itself during application of the Seprafilm® sheets. Seprafilm® reduced severity and amount of adhesions but had no significant effect on future small bowel obstructions as compared controls (Zeng et al. 2007)

There are two meta-analyses published of the safety and efficacy of Seprafilm® (Kumar et al. 2009; Zeng et al. 2007). Regarding the increased frequency of abscesses when using Seprafilm®, a recent meta-analysis of 2094 patients did not find any significant changes versus placebo regarding overall morbidity, anastomotic leak or abscess formation (Kumar et al. 2009). Seprafilm® barrier sheets have a proven adhesion preventive effect in all of the above mentioned studies.

Liquefied HA with phosphate buffered saline Due to difficulties using Seprafilm® in the lapaoscopic setting a liquefied HA solution solved in phosphate buffered saline (PBS) has been developed, Sepracoat® (Burns et al. 1995). Speracoat® is more rapidly absorbed then Seprafilm and has only moderate efficacy (van 't Riet et al. 2003).

Cross linked HA. Autocrosslinked polysaccharide (ACP) gel is yet another example of HA as an antiadhesion device. This is mostly used in laparoscopic situations and has promising anti-adhesion and antibleeding properties (De Iaco et al. 2001).

Crosslinked HA with Iron. In order to diminish the rapid absorption of hyaluronic acid a gel solution has been developed based on cross linking sodium hyaluronate with ferric chloride (Intergel®) with fine results regarding reduction of postoperative adhesions (Lundorff et al. 2001). However concerns have been raised regarding the increased risk of postoperative anastomotic leakage and morbidity. Also reports of late onset postoperative pain, foreign body reactions and tissue adhesions have been issued (Tang et al. 2006). Furthermore, speculations were raised whether the iron act as a substrate for bacteria. This resulted in a withdrawal of Intergel® from the market in 2003.

Another HA-ferric crosslinked device is Lubricoat® but clinical evidence is lacking (Johns et al. 1997).

Polyethylene glycol-based (PEG) liquids.

Several products have used PEG as active anti-adhesive component. One is Spraygel® which consists of two polyethylene glycol-based liquids that when mixed together rapidly cross-link to form absorbable hydrogel in situ.

The gel has some positive clinical data and is possible to use both in open and laparoscopic surgery (Mettler et al. 2004). The gel that forms coats the injured peritoneal tissue preventing bridging and adhesion formation. Good experimental results have been obtained using PEG including, those of marketed products, Sprayshield® and Adhibit® but clinical data is sparsely reported (Ferland et al. 2001).

Expanded polytetrafluoroethylene (ePTFE)

Expanded polytetrafluoroethylene (ePTFE) is commonly used in vascular surgery as graft. The non-dissolvable membranes experimentally prevents adhesions (PRECLUDE®) (Hellebrekers et al. 2000b) and has also been used in cardiac surgery to prevent pericardial adhesions with great success (Naito et al. 2008). The ePTFE barriers are inert and peritoneum re-mesothelializes within 5 to 7 days on top of the ePTFE. Furthermore the barrier does not seem to influence the inflammatory or fibrotic cells during peritoneal damage (Matthews et al. 2005). Unfortunately, ePTFE is not absorbale and requires suturing to keep in place, making it undesirable for use as barrier. Another concern is the cost since large areas need to be covered in the abdomen in order for it to be effective. It is very difficult to apply during laparoscopy. Therefore it is rarely used in abdominal surgery today in terms of adhesion prevention.

Oxidized regenerated cellulose (ORC)

ORC, Interceed®, has been used in order to prevent adhesion development. When administered in the peritoneum it transforms in to a gelatinous mass. ORC has been shown in both animal and human studies to reduce adhesion formation by

forming a barrier. The gel formation takes place immediately and is complete eight hours after administration (Diamond et al. 1991).

Although this seems to be a feasible anti-adhesion barrier one study have pointed to less efficacy if Interceed® is contaminated with blood, and observations have been made revealing that adhesion formation may increase if Interceed® is placed in areas where blood may accumulate e.g. in the pelvis (Wiseman et al. 1999). Interceed® increases the tPa activity in peritoneal mesothelial cells thereby acting both as barrier and local fibrinolytic agent (Gago et al. 2006).

It has also been shown that Interceed® decreases the inflammatory response of macrophages (Reddy et al. 1997). Regarding fibrosis, Interceed® seems to induce the expression of fibroblasts and TGFβ, thereby potentially increasing the risk of fibrosis and adhesions (Gago et al. 2003b). A meta-analysis including 10 clinical studies concerning gynecological surgery concluded that reduction of adhesions is significant but lack of information of preferred clinical outcome is noted, in this case pregnancy (Haney et al. 1995; Mais et al. 1995b; Mais et al. 1995a; Saravelos and Li 1996; Keckstein et al. 1996; Wallwiener D et al. 1998; Azziz 1993; Li and Cooke 1994; Larsson et al. 1995; Sekiba 1992).

Intervening drugs

Steroids

An early studie indicated a possible anti-adhesion effect of locally administered steroids (Replogle et al. 1966) but other papers have indicated the opposite (Jansen 1985). Today there are no available trademarks containing steroids for peritoneal anti-adhesion purposes. Steroids may alter the function of innate immune response and should be carefully considered when given as anti-adhesion agent (Liakakos et al. 2001).

Non steroid anti-inflammatory drugs (NSAID)

NSAIDs are shown to reduce postoperative adhesions in some studies (Bulbuloglu et al. 2005; Ezberci et al. 2006) but are considered to be of high risk given intra peritoneally since they increase the risk of bleeding and impairs wound healing (Hawkey et al. 2006). NSAIDs decrease vascular permeability, platelet aggregation and coagulation, and enhance macrophage function. NSAIDs modulate a number of aspects of inflammation. NSAIDs have been conflicting in terms of adhesion prevention (Greene et al. 2005). Neutrophils have a potential capability of reducing adhesions in the postoperative setting. Since NSAID lowers neutrophils this could be detrimental (Ar'Rajab et al. 1996)

Low molecular weight heparins (LMWH)

LMWH has been shown to reduce risk of postoperative adhesions in animals (Kutlay et al. 2004). Studies have however shown that LMWH may prolong wound healing thus make it unsuitable to be administered throughout a large peritoneal wound surface. Another apparent sideeffect when using LMWH is the risk of bleeding that causes high risk of adhesion development at the surgical wound site (Ryan et al. 1971). Evidence now exists that LMWH may exert its anti-inflammatory effects based on modulation of inflammatory cells (Lever et al. 2010).

Locally administered tissue plasminogen activator (tPa)

tPa, as the primary initiator for fibrinolysis, poses a promising mode of action against peritoneal adhesions and has previously been tested (James et al. 1965). The substance have been tested in a gel form in an animal model and showed promising anti-adhesion results in abdomen (Doody et al. 1989). However tPA lower the amount of fibrin and thereby causes bleeding problems which is a promoter of adhesion formation (Wiseman et al. 1992).

Bioactive polymers

Positively charged polypeptides, here named polycations, with various shapes, charges and sizes have been useful in many fields in bio research, e.g. gene delivery, coating of cell and culture plates, food preservatives and tissue engineering (Pack et al. 2005; Gillard et al. 1979).

Although polycations have been shown to be feasible as vectors in the gene therapy industry some obstacles have been noted. These obstacles may occur during the transport of the vectored material (DNA or RNA) from the blood into the cells and from there into the cell nucleus. The polycations are used to coat the vectors and also to protect the DNA/RNA. In order to reach the cell nucleus the vectors need to exhibit certain properties; not binding to albumin in the blood, being able to condensate DNA/RNA, being able to interact with the cell surface both through electrostatic forces and trough receptors, being internalized into the cell cytoplasm, protecting the vectors from enzymes in the cytoplasm and finally being able to internalize and incorporate the DNA/RNA in the nucleus.

Polycations have been used in animal studies (below) in order to investigate a possible postoperative anti-adhesion effect. Using polycatios in anti-adhesion settings reduces the above described demands on the polycations since they can be applied on to an injured peritoneal tissue and thereby binds locally.

The polycations may interact with various negatively charged molecules on the mesothelial cells of the peritoneum and the submesothelial space (i.e. fibronectin, laminin, vimentin and bilipids in cell membrane) (Brynda et al. 2005).

In order to investigate a possible anti-adhesion effect related to polycations, several studies have been made. The first one investigated hyaluronic acid and various polycations possible anti-adhesion effect in mice (Nehez et al. 2005)It was discovered that a promising anti-adhesion polycation was α-poly-L-lysine (PL). This polycation was installed in the peritoneal cavity through a syringe, binding to the injured raw peritoneal defects. The anti-adhesion effect of PL was significantly better when combined with a polyanion, α-poly-glutamate (PG), forming a neutrally charged biofilm PLPG.

The PL and PG were dissolved in 2.54% glycerol water as a vehicle in order to make an osmotic balanced solution. The resulting PLPG complex significantly reduced postoperative adhesions. The vehicle (glycerol water) did not have any anti-adhesion effects on its own (data not shown). It was also shown that the PL neither did affect the phagocytic function of macrophages nor their production of peroxides (Nehez et al. 2005).

A second study showed that 0.5% PL combined with 0.5% PG significantly reduced postoperative adhesions and the combined PLPG complex was gradually incorporated throughout the peritoneum and subperitoneal tissues the following six days after administration. The study further showed remesothelialization of the PLPG matrix within days. Furthermore, PLPG was ingested in peritoneal macrophages without any impact on their functions (Nehez et al. 2006).

The purpose of local PLPG administration onto an injured peritoneal surface was to seal the injured area and thereby diminish the fibrin deposits. This would sustain the normal restitution process of the peritoneum and thereby decreasing adhesions.

In a subsequent study the molecular weight of the PL was altered using PL with low molecular weight (4-15 kDa) and PL with high molecular weight (60 kDa). Also polyarginine, PA (a strong polycation) was used. It was shown that both low molecular and high molecular weight PL combined with PG was sufficient to reduce adhesions, however PA failed to reduce adhesions and the reason for this was unknown (Nehez et al. 2007).

Other studies show positive experimental results of using ε-poly-L-lysine/dextrin powder and poly-L-lysine/lactoferrine/HA (Nilsson et al. 2009; Takagi et al. 2013).

One study showed significantly increased strength in ileocolic anastomosis at day 1 and 3 postoperatively administering PLPG onto the anastomosis. It also showed

significantly reduced adhesions during peritoneal septic conditions using 0.5 % PLPG (Tingstedt et al. 2006).

Administration of 0.5 % PLPG on to a "badly sewn" anastomosis significantly increased the bursting pressure compared to controls, indicating it might be safe to use PLPG as a prophylactic agent to diminish the risk of anastomos leakage. Furthermore it was investigated that PLPG also was albe to seal of an "accidental" spleen and liver bleeding due to a sharp trauma thereby decreasing bleeding significantly (Tingstedt et al. 2007c).

In order to investigate a dose response relationship a toxicity study on mice was performed showing that the LD50 dose was 40 mg/kg and the lowest effective adhesion reducing dose was 1.6mg/kg. The study also showed that it is possible to use 0.5 % PLPG as a standard concentration only altering the volumes installed and still get anti-adhesion effects (Isaksson et al. 2010).

The possible relationship between anti-adhesion effects and differently shaped polycations, linear and globular, was examined. It revealed that linear polycations had better effect than globular ones, possibly due to the globular polycations having their charges partly faced inward (Isaksson 2011).

The amount of positive charges of the polycations that may interact with the negatively charges on the cells/tissues are dependent on the concentration of the polycation. Higher concentrations of polycations yields higher amount of positively charges that may interact with an injured tissue.

Other factors important for the interactions between the polycations and the cells/tissues are their shapes. The polycations may have, dendritic, branched and spherical shapes, resulting in various possibilities for electrostatic interaction.

Yet another factor influencing the interactions is the nature of the shaped polycation. A flexible structured polycation has higher ability to interact with negatively charges than a semi rigid or rigid polycation (Singh et al. 1992).

When the interactions occur, conformational changes occur, for example a random coiled structure of the polycations turns into an alpha helical structure incorporated in the cell membrane (Hartmann and Galla 1978).

Due to results of the observed toxicity of the positively charged PL further studies are warranted on the possible cytotoxic effects of the ingoing substances and relationship to different amounts and concentrations (Fischer et al. 2003).

Table 1

Different anti-adhesion devices and their possible influence on tPa (tissue plasminogen activator), PAI-1 (plasminogen activator inhibitor), IL-6 (interleukin 6), TGFβ (transforming growth factor beta), PMN (Polymorpho nuclear cells) and MO (macrophages), Y=yes (have influence), N=no (no influence), U=unknown influence and P=possible influence.

			Influence on different components in the peritoneum				
			tPa	PAI-1	IL-6	TGFβ	Inflammatory cells (PMN and MO)
Barriers	Hydroflotation	Crystalloids: Ringer Acetat®, Normal Saline	Y	Y	Y	U	Y
		Dextran 70 (Hyskon®)	Y	Y	Y	Y	Y
		Icodextrin (ADEPT®)	P	P	U	U	Y
	Endogen	Placenta, omentum, peritoneum, pericardium	N	N	N	N	N
		Autocrosslinked polysaccharide, (ACP-gel)	U	U	U	U	U
		Hyaluronic acid (HA)	Y	Y	Y	Y	Y
		HA with phosphatebuffered saline (Sepracoat®)	P	P	P	U	P
		HA with iron, gel (Lubricoat®)	P	P	P	U	P
		HA + carboxymethylcellulos (Seprafilm®)	N	N	N	N	N
		Polyethylene glycol (Spraygel®)	U	U	U	U	U
		Expanded polytetrafluoroethylene (ePTFE) (GoreTex®, Preclude®)	P	P	N	N	N
		Oxidized-regenerated cellulose-ORC (Interceed®)	Y	Y	Y	P	Y
		α-poly-L-lysine (PL) + α-poly-glutamate (PG) (Bioative Polymers ®)	N	N	N	N	N
	Drugs	Steroids	Y	Y	Y	Y	Y
		Non steroid anti-inflammatory drugs (NSAID)	Y	Y	Y	Y	Y
		Low molecular weight heparin (LMWH)	Y	Y	U	P	Y
		Tissue plasminogen activator (tPa)	Y	Y	P	P	P

The present study and overall aims

An optimal anti-adhesion device should be easy to use, nontoxic, have no effects on any homeostatic function and be cheap. At present there is no optimal anti-adhesion device taking all of these aspects into account.

Previous studiesusing differently charged polypeptides focused on evaluating the complex of α- poly-L-lysine (PL) and α-poly-glutamate, (PG) as anti-adhesion barrier and showed that the complex is nontoxic, efficient as anti-adhesion barrier, biodegradable and with no impact on the macrophage function.

In this study we wanted to elongate the research of the possible impact that PLPG has on the resolving capability of peritoneum by investigating parts of the plasmin system, the immune cytokines, the fibrosis system and the cellular events.

Animal studies were performed by creating abdominal adhesions using well known and established methods thereafter measuring key parameters over time in peritoneal fluid, peritoneal biopsies and peritoneal adhesions. Fibrosis was also measured through histology evaluation. This was done to draw conclusions of the potential impact on postoperative adhesions after primary surgery.

Many available anti-adhesion devices are difficult to use and sometimes even impossible to use in laparoscopic surgery. Previous studies used abdominal installations when evaluating the anti-adhesive properties of PLPG. Administration using spray is therefore interesting.

Moreover, the potential impact of PLPG on postoperative adhesions after secondary surgery (adhesiolysis) was studied. This is a most relevant clinical aspect with patients being operated upon several times

Furthermore, we also investigated the antiadhesion effect that PLPG had on pleural adhesions and the possible impact on parts of the coagulation and fibrosis pathways

Finally, we also wanted to elucidate the possible in vitro cytotoxicity exerted by polycations since these are a mandatory part of the anti-adhesion device.

Aims specified by study

Paper I

A pilot study where PLPGs anti-adhesion effect was studied using a spray applicator on peritoneum. Evaluating of the possible effect on the parameters of coagulation, inflammation and fibrosis as described:

- Postoperative peritoneal adhesion evaluation according to established scores
- tPA/PAI-1 complex from peritoneal adhesions before and after adhesion formation
- IL-6 from peritoneal fluid before and after adhesion formation
- TGFb1 from peritoneal fluid before and after adhesion formation
- Histology score

Paper II

Evaluation of the effect of PLPG on key parameters of coagulation, inflammation and fibrosis in peritoneal fluid over time:

- Postoperative peritoneal adhesion evaluation according to established scores
- tPA from peritoneal fluid before and after adhesion formation
- PAI-1 from peritoneal fluid before and after adhesion formation
- IL-6 from peritoneal fluid before and after adhesion formation
- TGFb1 from peritoneal fluid before and after adhesion formation

Paper III

Evaluation of the effect of PLPG after adhesiolysis including key parameters on coagulation and fibrosis:

- Adhesion evaluation according to established scores
- tPA from normal peritoneal tissue and adhesions before and after adhesiolysis
- PAI-1 from normal peritoneal tissue and adhesions before and after adhesiolysis
- TGFb1 from normal peritoneal tissue and adhesions before and after adhesiolysis

Paper IV

A pilot study investigating the anti-adhesion effect and parameters of coagulation and fibrosis of PLPG on pleural adhesions after thoracotomy:

- Adhesion evaluation according to established scores
- PAI-1 from pleural adhesions after adhesion formation
- TGFb1 from pleural adhesions after adhesion formation

Paper V

Evaluation of the toxicity through proliferation rates exerted by polycations on mesothelial cells:

- Cultivation of mesothelial cells
- Different charged and sized polycations were used to examine their possible effect on proliferation

Materials and Methods

Animals

Female Rabbits (Swedish Lop) weighing approximately 2.6 - 3.3 kg (I) and Sprague-Dawley rats (Taconic Farms, Inc., DK) weighing about 250 g were used (II-IV). The animals were kept under standardized conditions and had free access to water and pellets. The local ethical committee at Lund University approved the adhesion Lund Sweden), and the animals received the best animal care, in compliance with the guidelines of the Swedish Government and Lund University,Sweden.

Chemicals, test substances

The chemicals α-poly-L-lysine MW > 30,000 kDa (PL) and α-poly-L-glutamate MW 15 - 50 kD (PG) (Sigma Aldrich™, St. Louis, Missouri, USA) were freshly mixed on the day of the experiment with 2.54% glycerol and water in an osmotic balanced solution to a final concentration of 0.5% (5 mg/ml) paper I and III and 0.05% (0.5mg/ml) paper II. Substances used in paper V were Lactoferrine, Poly-L-lysine 1-5 MW kDa , Poly-L-Lysine MW 4-15 kDa, Poly Arginine MW 55 kDa, Polyglutamate, Poly L Lysine, Poly L Lysine MW > 30,000 kDa, Lysosym MW 14,7kDa, Epsilon Poly L Lysine MW 4,8 kDa all were purchased from Sigma Aldrich™, St. Louis, Missouri, USA.

Equipment

Surgical instruments (scissors, scalpel, forceps, drapes and sponges) were used in all studies (I-IV).Sutures polypropylene 3-0 and 4-0, with curved needles (Ethicon, Somerville, NJ, USA) were used (I-IV).Syringes (Beckton Dickinson, Helsingborg Sweden) were used (I-IV).Phosphate Buffered Saline 3 mM EDTA and 25 U/ml Heparin (LEO Pharma AB™ Malmö, Sweden) was used to collect peritoneal fluid (I,II).NaH2PO4 buffer containing 0.1% Triton X 100 (Sigma Aldrich™ St. Louis,

Missouri, USA) was used to homogenize peritoneal biopsies (I).Acetat acid, 1% Triton X-100 and thiocyanate was used to homogenize peritoneal and pleural biopsies (III,IV)CAD program (Auo-Cad 2011 Autodesk AB, Göteborg, Sweden) to evaluate adhesions (I).Spray vaporizer (Adaptar Pharma) were used to administer PL and PG (I-IV).Hematoxylin and Eosin was used to stain for inflammatory cells and Massons-Trichrome was used to stain for collagen (I,III,IV).7025 Rodent Ventilator Ugo Basile Italy for rat respiration (IV)16 Gauge needle (Beckton Dickinson, Helsingborg Sweden)Cell culture Human mesothelial cells were purchased from ZenBio (Raleigh, NC,US) (V)Growth medium MSO-1 Medium 199, FBS, Human Epidermal Growth Factor, penicillin, streptomycin and amphotericin B (ZenBio) (V)96 12-well plates (V) Polypeptides Sigma Aldrich (St Louis, MO, US) (V)MTT assay (Roche, Mannheim, Germany) (V)(3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide).

Anesthesia

The animals were anesthetized with 50 mg/kg Ketalar (Parker Davis™ Detroit, Michigan, USA) and Xylazine 6 mg/kg (Rompun: Bayer Sverige AB Sweden) by an intramuscular injection (I-IV).

Surgical models

I

In brief, after a 10 cm midline incision, the cecal area (approx. 2 cm^2) was abraded to petechial bleeding using a dry sponge. Peritoneal adhesions were created using an established model (Oncel et al. 2005). On the right side of the abdominal wall a square section of 1×1 cm peritoneum was excised down to the right transverse abdominal muscle and the wound surfaces were gently dabbed with sterile gauze to remove excess blood. The abraded cecum was put back into the abdomen adjacent to the denuded lateral wall. Treatment was then applied. The abdomen was closed using a running suture (Prolene™ 4-0, Ethicon, Somerville, NJ, USA) in two layers. The animals received subcutaneous saline (0.9%, 10 ml) for resuscitation and buprenorphine for postoperative pain control. All animals were weighed in conjunction with every operative procedure.

II,III

Peritoneal adhesions were created with an established method (Holmdahl et al. 1994) via a sharp incision, 15 mm long, on the lateral abdominal wall. The incision was sutured with 4 interrupted sutures Prolene™ Ethicon, Somerville, NJ, USA) with one suture in each corner. The area was then treated with active substance or saline using a spray atomizer. The abdomen was closed using a running suture, (PDS™ 4-0, Ethicon Somerville, NJ, USA) in two layers. The animals received buprenorphine for postoperative pain control. All animals were weighed in conjunction with every operative procedure.

IV

A left anterolateral thoracothomy (2cm) between the fourth and fifth costal space was performed during sterile conditions and a standardized pleural adhesion creating model were performed previously described by Takagi et al. (Takagi et al. 2013). In brief, the pleural adhesions were created by electro-cauterized incision of the thorax wall. Animals were then treated with the active substance or saline using a spray atomizer with an immediate closure of the external thoracic muscles by running sutures of (5-0 Prolene™ Ethicon, Somerville, NJ, USA. Thereafter bupivacaine was injected at the wound site as local anestethic. The animals received subcutaneous saline (0.9%, 5 ml) for resuscitation and buprenorphine for postoperative pain control. All animals were weighed in conjunction with every operative procedure.

Cellular model

V

Human mesothelial cells were purchased from ZenBio(Raleigh, NC,US). The cells were maintained in mesothelial cell growth medium (cat # MSO-1) containing Medium 199, FBS, Human Epidermal Growth Factor, penicillin, streptomycin and amphotericin B (ZenBio) in a humidified 5 % CO_2 atmosphere at 37o C. Medium was changed every other day. The experiments were performed after 6-7 days when cells became confluent. Then the cells were seeded overnight in 12-well plates at a density of 10,000 cells/well in mesothelial cell growth medium and starved in Medium 199 (ZenBio) supplemented with 0.5 % FBS (Gibco, Invitrogen, Paisley PA4 9RF, UK)

Experimental design

I

The rabbits were randomly and blindly divided at the beginning of the experiment into four different groups according to table 2. Group A (n = 6) received a dose of PL and PG (0.5%, 1 ml of each corresponding to a dose of 2.5 mg/kg administered by spray; first the PL was sprayed and after 10 - 15 seconds the PG was given. The control group B (n = 6) received saline (0.9%) 2 ml administered locally by spraying after the surgical procedure but before closure of the abdomen. Group C (n = 8) received PL and PG in the same dose and mode as group A (0.5%, 1 ml of each corresponding to a dose of 2 mg/kg and group D (n = 8) received saline 0.9%, 2 ml after the abrasion procedure. Ten days later the macroscopic peritoneal adhesions in group C and D were noted and carefully dissected

*Peritoneal lavage and biopsies with active proteins.*In groups A and B peritoneal lavage and biopsies of tissue (size 1 - 2 mm2) in the operation area of the abdominal wall were taken on day 0 (prior to surgery) and days 1, 4 and 10 postoperatively on repeated laparotomies. Biopsies were taken from new areas of the operated area on each occasion. Lavage containing 37°C 20 ml Phosphate Buffered Saline 3 mM EDTA and 25 U/ml Heparin (LEO Pharma AB™ Malmö, Sweden) was collected in tubes by (Becton Dickingson™, San Jose, California, USA) and immediately centrifuged at 1000 G for 4 min at 4°C. The supernatant was allocated and snap frozen up to –80°C. Peritoneal biopsies were homogenized in NaH2PO4 buffer containing 0.1% Triton X 100 (Sigma Aldrich™ St. Louis, Missouri, USA) and there- after centrifuged at 1000 G for 10 minutes at 4°C.

Determination of active proteins in peritoneal fluid was carried out using commercially IL-6 (R & D Systems™, Abingdon, UK), TGF-b1 (Promega Biotech AB™ Nacka, Sweden). The active proteins were normalized to total protein (Bio-Rad Laboratories AB Malmö, Sweden).

The tPa/PAI-1 complex was analyzed in supernatant obtained from homogenized peritoneal biopsies using ELISA (t-Pa/PAI-1 Complex ELISA, Haemochrom Di-agnostica AB, Mölndal, Sweden,) according to the manu- facturer's instructions. Results were also normalized to total protein concentration.

Histology In groups A and B additional peritoneal biopsies from the same area were taken on day 10 in order to stain for inflammatory cells (Hematoxylin and Eosin) and collagen (Massons-Trichrome). Prior to staining, the biopsies were treated with acetal- dehyde, fixated in alcohol, paraffin embedded and cut with a microtome.

Photos Pictures of macroscopically appearance of peritoneal adhesions were taken at day10

II

A total of 84 rats were randomly selected based on the evaluation time i.e. after 2, 4, 6, 8,24h and 7d according to table 3. The treatment groups received 1ml of 0.05% (2mg/kg) PL and 10 seconds later1ml of 0.05% (2mg/kg) PG administered through spray atomizers. Peritoneal lavage with pre warmed PBS (2ml) were made 2, 4, 6, 8, 24h and 7d after the surgical model. The peritoneal lavages were immediately allocated to smaller volumes (400 µL) and snap frozen to -70°C. The peritoneal lavage was then analyzed with ELISA to measure tPA, PAI-1(Labinova AB, Upplands Väsby, Sweden), IL-6 and active TGFb1 (R & D Systems Europe, Abingdon, UK) concentration.

III

The rats were randomly and blindly divided at the beginning of the experiment into three different groups all according to table 4. The treatment groups received 0,5 ml of 0.5% (10 mg/kg) PL and 10 seconds later 0,5 ml of 0.5% (10 mg/kg) PG administered through spray atomizers. At day 0, biopsies were taken from peritoneum on contralateral (opposite) side to the adhesion creating surgical side. Thereafter, adhesion creating surgery was performed, described in detail above, followed by treatment with either NaCl or PL/PG. At day 7, adhesions were evaluated and scored, biopsies from the formed adhesions and from peritoneum not subjected to surgery (opposite site) were collected. Adhesiolysis were performed, and the groups were again treated, with either NaCl or PL/PG. At day 14, adhesions were evaluated and scored, biopsies from the formed adhesions and biopsies from peritoneum not subjected to surgery (opposite site) were collected.

Photos Pictures of macroscopically appearance of peritoneal adhesions were taken at day 14

Biopsies Biopsies from adhesions and peritoneum were taken, immediately allocated to tubes, and snap frozen to -80°C. Tissue samples were homogenized in acetate buffer, in order to extract the proteins efficiently. Active proteins were normalized to total protein content. TPA, PAI-1 and active TGF-β1 levels were determined using ELISA techniques, all according to the manufacturer's instructions.

Histology Peritoneal biopsies from the same area were taken on day14, in order to stain for inflammatory cells (Haematoxylin and Eosin), in order to visualize the complex embedded in the mesothelial/submesothelial space. Prior to staining, the biopsies were treated with acetaldehyde, fixated in alcohol, paraffin embedded, and cut with a microtome.

IV

The animals were randomly selected to either control or experimental group according to table 5. After the creation of pleural adhesions animals received 0,1 ml of 0.5% (2 mg/kg) PL and 0,1 ml of 0.5% (2 mg/kg) PG or saline. Seven days later adhesions were noted and scored and biopsies, histology and pictures were taken from pleural adhesions.

Histology Pleural biopsies were taken at day 7 and stained for collagen (Massons-Trichrome). Prior to staining, the biopsies were treated with acetal- dehyde, fixated in alcohol, paraffin embedded and cut with a microtome.

Biopsies Biopsies from the adhesions were taken at day 7, immediately allocated to tubes and snap frozen to -80°C. Tissue samples were homogenized in acetate buffer in order to efficiently extract the proteins. Active proteins were normalized to total protein content PAI-1 and active TGFb1 levels were determined using ELISA techniques, all according to the manufacturer's instructions.

Photos Pictures of macroscopically appearance of peritoneal adhesions were taken at day 7

V

Medium was removed from the wells. Polypeptides diluted in Medium199 to different concentrations (Molar) according to Table 6 were added to the wells.

An established MTT assay (Holgate et al.) was used to measure proliferation of mesothelial cells in the wells. 10μL of MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) was added to each well and incubated for 4 hours. Thereafter 100μL of solublization solution 10% SDS in 0.01 M HCl was added and incubated for 20 minutes. Optical density (OD, absorbance, mesothel cell proliferation) for each wells were measured colorimetrically with spectrophotometri. OD was calculated as 595-660nm in wavelength as instructed by the assay procedure

Table 2
Experimental design P(PLPG) C(control)

Groups	Animals	Day 0	Day 1	Day 4	Day 10
A P	6	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy, Histology
B C	6	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy	Peritoneal lavage, Peritoneal biopsy, Histology
C P	8				Adhesion evaluation, Digital photography
D C	8				Adhesion evaluation, Digital photography

Table 3
Experimental design (II)

Groups (time for control of tPA, PAI-1, TGFb1 and IL-6 in peritoneal lavage)	Control (Saline), number of animals	PLPG, number of animals
0 h (before adhesion procedure)	42 animals from all groups	42 animals from all groups
2 h	6	6
4 h	6	6
6 h	6	6
8 h	6	6
24 h	6	6
7days	6	6

tPA, PAI-1, TGFb1 and IL-6 were collected from peritoneal fluid before surgery at 0h and after surgery at 2, 4, 6, 8,24h and 7d.

Table 4
Experimental design (III)

Group	Animals	Day 0	Day 7	Day 14
		Adhesion model	Adhesiolysis	Evaluation
A	8	NaCl *	NaCl*/**/+	*/**/+
B	8	PLPG *	PLPG */**/+	*/**/+
C	8	NaCl *	PLPG */**/+	*/**/+

Peritoneal biopsy collecting tPA, PAI-1 and TGFb1*, adhesion biopsy collecting tPA, PAI-1 and TGFb1**, Adhesion evaluation +.

Table 5
Experimental design (IV)

	Day 0	Day 7
Experiment group/PLPG	Adhesion creative surgery	- Evaluation of adhesions Oncel et al and Takagi et al - Evaluation of TGFb and PAI-1 in biopsies of pleural adhesions - Histology of pleural adhesions
Controlgroup/NaCl	Adhesions creative surgery	- Evaluation of adhesions Oncel et al and Takagi et al - Evaluation of TGFb and PAI-1 in biopsies of pleural adhesions - Histology of pleural adhesions

Pleural adhesions created at day 0. At day 7 adhesions were evaluated and pleural biopsies harvested analysed for TGFb and PAI-1. Pictures and histology were also taken.

Table 6

Polypeptides	Conc (M)	MW (kDa)
Lactoferrine (LF) G	10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9}	90
Poly-L-lysine 1-5 L	10^{-4} ,10^{-6}	1-5
Poly-L-Lysine 4-15 L	10^{5} , 10^{-6}	4-15
Poly Arginine (PA) L	10^{-4} , 10^{5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9}	55
Polyglutamate (PG) L	10^{-5} , 10^{-6} , 10^{-7} , 10^{-8}	35
Poly L Lysine (PL>30) L	10^{-6} , 10^{-7} , 10^{-8} , 10^{-9}	>30
Poly L Lysine (PL1530) L	10^{-6} , 10^{-7} , 10^{-8} , 10^{-9}	15-30
Lysosym (Lys) G	10^{-4} , 10^{5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9}	14,7
Epsilon Poly L Lysine (Replogle et al.) L	10^{-5} , 10^{-6} , 10^{-7} , 10^{-8}	4,8

Polypeptides, different concentrations and Molecular weights. Polycation conformation,globular (G) and linear (L). M molar

Evaluation methods

Adhesions

In study I the quality of the peritoneal adhesion was scored according to Lang *et al* (table 7) (Lang et al. 2009). Digital photo- graphs of the defined operated and colour marked adhe-sive areas were taken. Using a CAD program (Auo-Cad 2011 Autodesk AB, Göteborg, Sweden). ratios (percent) were calculated between the adhesion area and the total incised abdominal area and analyzed for differences.

Table 7

Evaluation of severity of adhesion according **according to Lang*et al* (Lang et al. 2009)**

Severity of adhesions	Filmy	Moderate	Severe

In study (II,III) at evaluation, the abdomen was opened with an inverted U incision with its base to the right and adhesions were measured according to Holmdahl et. al. (Holmdahl et al. 1994). In brief the lengths of the incisions as well as the adhesions covering the wound were measured with a calliper up to one-tenth of a millimetre. Data were expressed as the percentage of the wound covered by adhesions. The distances were measured at the peritoneal level. Other adhesions between intra-abdominal organs were also noted. Adhesions were also graded in study III according to a cumulative score developed by Bothin et al (Bothin et al.

1999) (table 8). In study IV pleural adhesions the severity of adhesion were graded according to Oncel et al (Oncel et al. 2003) (table 9) and the length of the adhesions according to Takagi et al (Takagi et al. 2013)(table 10).

Evaluation of mesothelial cell proliferation

In study V the mesothelial cell Optical density (OD, absorbance, mesothel cell proliferation) for each wells were measured colorimetrically with spectrophotometri. OD was calculated as 595-660nm in wavelength as instructed by the assay procedure.

Time evaluation

In study I-IV the time interval of evaluating adhesions after 7 days were chosen based on evidence that most adhesions are formed within 7 days (diZerega and Campeau 2001). In study II, the analysed peritoneal fluid at 2, 4, 6, 8, 24h and 7d were based on previous studies (Hellebrekers et al. 2000a). These intervals for adhesion related active substances in peritoneal fluid were determined most appropriate. In study III the time interval of analysing peritoneal biopsies at day 7 and 14 postoperative were chosen based on previous data (Prushik et al. 2010). In study IV the time interval of evaluating pleural adhesions after 7 days were chosen based on evidence that most pleural adhesions are formed within 7 days (Takagi et al. 2013).

Histology

Histology slides were evaluated in study I,III and IV and fibrosis was scored based on collagen deposition (table11) according to Hooker et. al.(Hooker et al. 1999) Histology evaluation was performed separately by two examiners and in a blinded fashion.

Statistics

In order to see the range of distribution of the values, all results were given in median. Mann Whitney U- test was used to analyze possible significant differences in adhesion score (study I-IV) from digital photos and histology slides (inflammation and fibrosis). Mann Whitney U test was used to analyze possible

significant differences active protein levels of peritoneal fluid, peritoneal biopsy and pleural biopsies between control and experiment group (study I-IV). Statistic differences was set to $p<0.05$. (SPSS v17.0 SPSS Inc, Chicago, Ill, US).

Results were shown as mean ± Standard Error (SE). Mann Whitney/ U test were used to determine differences between the groups, both regarding adhesions, as well as tPA, PAI-1 and active TGFb1. $P<0.05$ were considered significantly. The statistical analyses were performed with SPSS used for analysis (SPSS v17.0, SPSS Inc., Chicago, Ill., US).

Table 8
Cumulative adhesion scoring scale (developed by
Bothin (Bothin et al. 1999)) 1 + 1 = 2 points:

+ 1 No adhesions.
+ 1 One adhesive band from the omentum to the
target organ.
+ 1 One adhesive band from the omentum to the
abdominal scar.
+ 1 One adhesive band from the omentum to another
place.
+ 1 One adhesive band from the adnexa/epididymal
fat bodies to the target organ.
+ 1 One adhesive band from the adnexa/epididymal
fat bodies to the abdominal scar.
+ 1 One adhesive band from the adnexa/epididymal
fat bodies to another place.
+ 1 Any adhesive band other than already described
(e.g., liver to scar).
+ 1 Target organ adherent to the abdominal wall.
+ 1 Target organ adherent to bowel.
+ 1 Target organ adherent to the abdominal scar.
+ 1 Target organ adherent to the liver or the
spleen.
+ 1 Any other organ adherent.

Table 9
Evaluation of severity of adhesion according to Oncel et al (Oncel et al. 2003)

Grade 0	**No adhesions**
Grade 1	**Loose filmy adhesions that can be separated by traction**
Grade 2	**Adhesions requiring blunt dissection for separation**
Grade 3	**Adhesions requiring sharp dissection for separation**
Grade 4	**Serosal injury**
Grade 5	**Tissue injury**

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Table 10
Evaluation of the length of the adhesions according to Takagi et al (Takagi et al. 2013)

Grade 0	No adhesions
Grade 1	≤25% of intercostal incision line
Grade 2	25%–50% of intercostal incision line
Grade 3	50%–75% of intercostal incision line
Grade 4	75%–100% of intercostals line

Table 11
Fibrosis score according to Hooker et al (Hooker et al. 1999)

Score	Fibrosis/inflammation
0	None
1	Mild
2	Moderate
3	Severe

Results

Paper I

Adhesions

All operated animals survived the postoperative period. A significant difference in abdominal adhesions could be seen between the experimental and control (group C and D) groups, p < 0.005 (**Figure 1**). In the control group, 100% (8/8) of the rabbits had severe adhesions vs. only 9% (1/8) in the PLPG group. In the PLPG group 63% (5/8) had no adhesions and 25% (2/8) had flimsy adhesions. The digital photo (**Figure 2**) showed that the peritoneal surface was smooth and similar to the rest of the non-traumatized peritoneal surface.
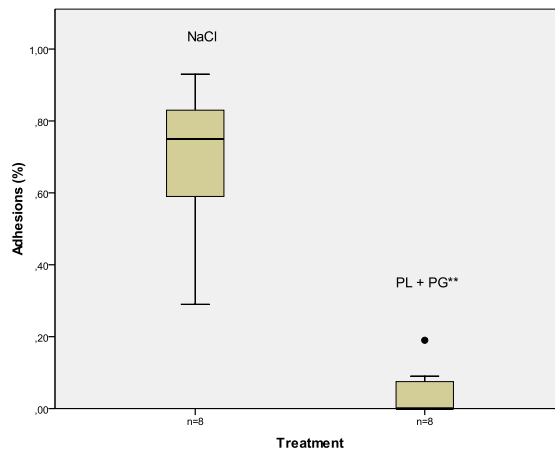
Figure 1.
Results of adhesion reduction in animals receiving PLPG. Adhesions in percent of traumatized area in animals tretead with saline and PLPG
** Significant reduction; p<0,005. • depict outlier.
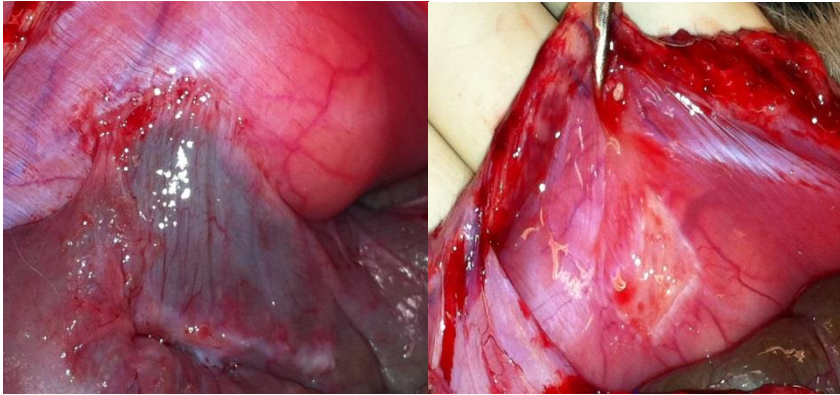
Figure 2.
Photographies of treatment effects after 10 days. Non treated animal with 100 % severe adhesions (left picture) and PLPG treated animal with no adhesions and smooth surface (right picture).

IL-6, TGFb1 and tPa/PAI-1

No significant changes were seen in active protein levels of peritoneal biopsies (**Figure 3**) or in peritoneal fluid (**Figure 4**) and (**Figure 5**)
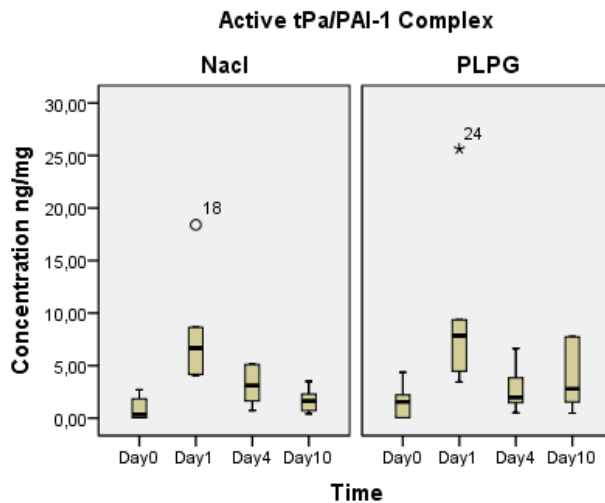

Figure 3**.**
No significant changes in levels of tPa/PAI-complex from peritoneal biopsies were seen between the groups receiving Nacl or PLPG after peritoneal surgery° and* depict outliers. .
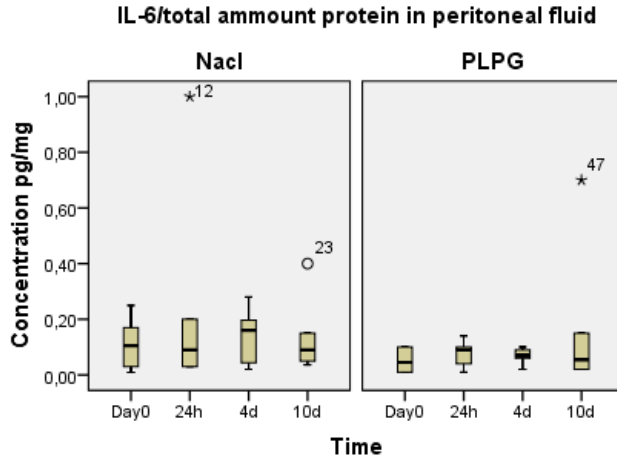
Figure 4 .
No significant changes in levels of IL-6 in peritoneal fluid were seen between the groups receiving Nacl or PLPG after peritoneal surgery, ° and* depict outliers.
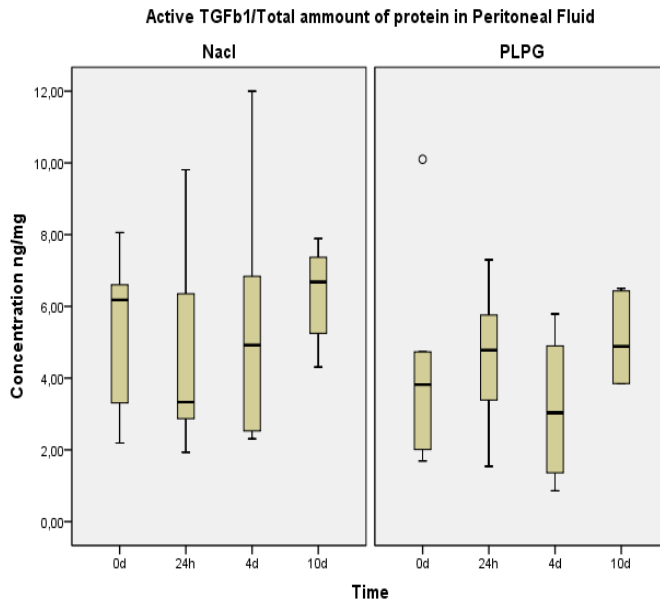
Figure 5 .
No significant changes in levels of TGFb1 in peritoneal fluid were seen between the groups receiving Nacl or PLPG after peritoneal surgery. ° Depict outlier.

Fibrosis

Ten days after surgery, an increase incollagen deposition on peritoneum was seen on histology in the control group compared to the experimental group p<0.05 (**Figure 6**) and (**Figure 7**)
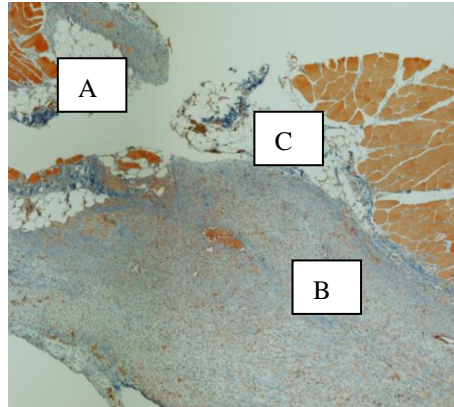
Figure 6. Histology specimen after 10 days. Differences in the fibrosis reaction between **A** peritoneum treated with PLPG) and **B** (adhesions formation not treated with PLPG) after surgery. **C** is the submesothelial area.
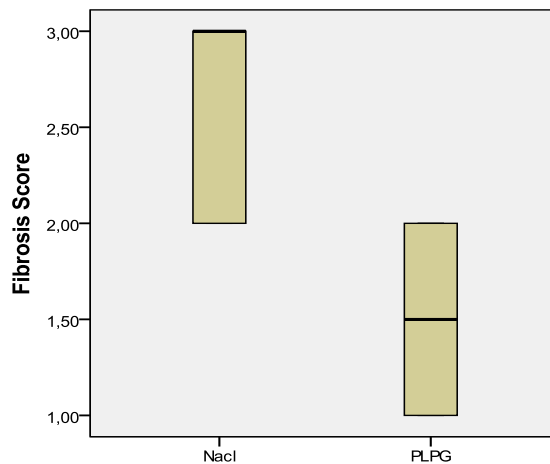

Figure 7. Result of collagen evaluation between treated and control groups. Differences in collagen deposition on peritoneum in the saline group as compared to the PLPG group; p<0.05 according to Hooker et. al(Hooker et al. 1999)

Paper II

Adhesions

Adhesions were significantly reduced on day seven, measured as a percentage of the inflicted wound, p=0.002,

tPA

After the adhesion procedure the tPA levels increased at 2 and 4 h similarly for both the control and experiment groups ((**Figure 8**). A significantly lower tPA concentration in the experimental group compared to the controls was seen at 6 h (p=0.002). Decreasing levels(for both the experimental and control groups) were seen at 6 h, followed by increasing levels at all further times up to 7d (**Figure 8**)The tPA values were all significantly raised compared to time 0 h (p<0.05) (Figure 1).

PAI-1

PAI-1 levels were significantly raised (p=0.019) at 4 h in controls as compared to the experimental group (**Figure 9**) At 7d, PAI-1 levels were significantly lower in controls than in the experimental group (p=0.026). Levels of PAI-1 elevated above physiological range were seen at 4 h for the control and at 6 h for the PL/PG. Thereafter, the reduction in concentration displayed similar patterns in both groups (**Figure 9**).

IL-6

IL-6 levels were steeply elevated after the adhesion procedure (in both the control and experimental groups) at 4 h (**Figure 10**).Thereafter a gradual decline in concentrations could be seen in both groups to 7d (Figure 3). A minor significant difference in IL-6 concentration was seen between the groups at 7d (p=0.041) (**Figure 10**)).

TGF-b

A gradual incline of the active TGFb1 concentration (in both experimental and control groups) could be seen after the adhesion procedure (**Figure 11**) Elevated levels (in both groups) were seen at 7d
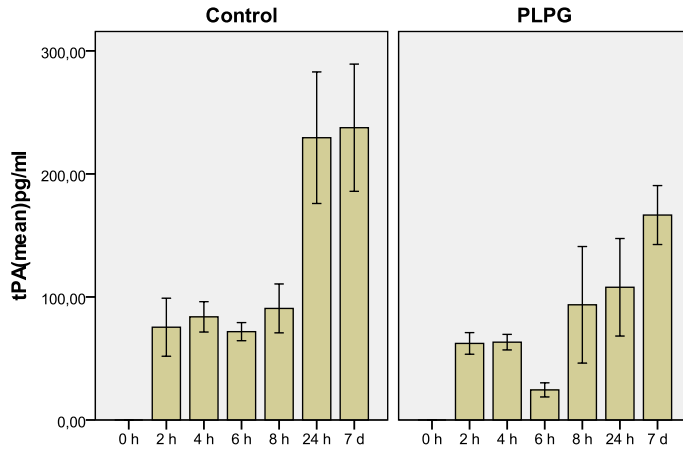
Figure 8.
Peritoneal concentrations of tPA (pg/ml) in control (NaCl) and experiment group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and control as a total. A significant difference in tPA concentration between PL/PG and control were seen at 6h (p=0.002). Graphs show tPA concentration ±SE

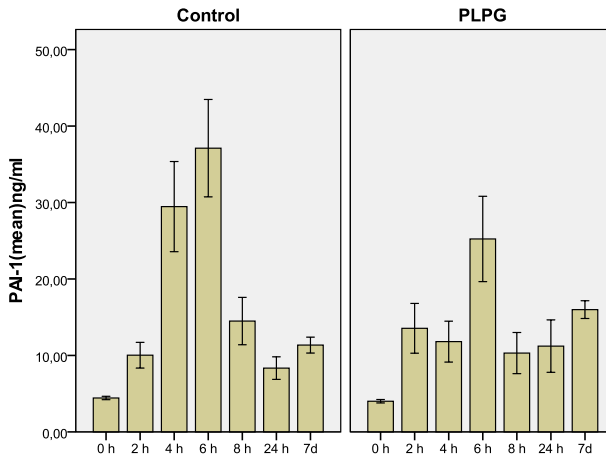**Figure 9.**
Peritoneal concentrations of PAI-1 (ng/ml) in control (NaCl) and experiment group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and control as a total. A significant difference in PAI-1 concentration between PL/PG and control were seen at 4h (p=0.019) and 7d (0.026). Graphs show PAI-1 concentration ±SE
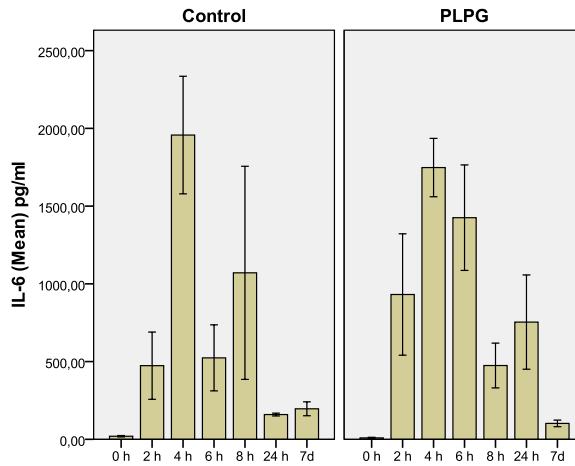
Figure 10.
Peritoneal concentations of IL-6 (Pg/ml) in control (NaCl) and experiment group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and control as a total. A small significant difference in IL-6 concentration between PL/PG and control were seen at 7d (p=0.041). Graphs show IL-6 concentration ±SE
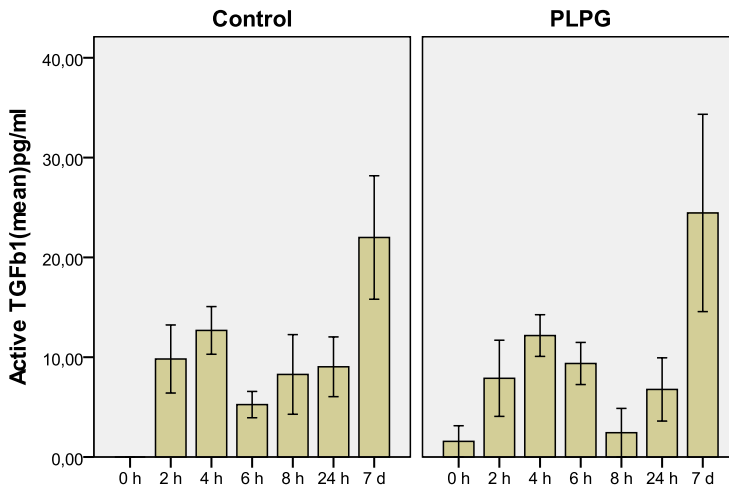

Figure 11. Peritoneal concentrations of Active TGFb1 in control (NaCl) and experiment group (PL/PG) before (0h) and after surgery (2h-7d) No significant difference in concentrations were seen between the PL/PG group and control as a total. Graphs show Active TGFb1 concentration ±SE

Paper III

Adhesions

Adhesions were significantly lower at day 7, p=0.001 and day 14, p=0.007 in group B compared to group A (**Figure 12**). Significantly less adhesions were observed at day 14 in group C compared to group A, p=0.003 (**Figure 12).**

tPA in biopsies of normal peritoneal

Differences of tPA levels in biopsies from normal peritoneum were seen at day 0, 7 and 14 between group A, B and C although none of these were significant (**Figure 13)**

Biopsies from adhesions

Significantly higher levels of tPa from adhesion biopsies were seen at day 7 in group B compared to group A, p<0.05and in group C compared to group A and B at day 14, p<0.05 (**Figure 14)**

PAI-1in biopsies of normal peritoneum

Significantly higher levels of PAI-1 in biopsies from normal peritoneum were seen in group A and C compared to group B at day 7, p<0.05 (Figure 4). Significantly lower levels of PAI-1 were seen in group B at day 14 compared to group A and C, p<0.05 (**Figure 15)**

Biopsies from adhesions

Significantly higher levels of PAI-1 from adhesion biopsies were seen at day 7 in group A and C compared to group B, p<0.05 (Figure 5). Significantly higher levels of PAI-1 were seen in group C compared to group B and A p<0.05 at day 14 (**Figure 16)**

TGFb1in biopsies of normal peritoneal

Levels of active from normal peritoneum were significantly higher at day 7 in group A and group C compared to group B p<0.05. At day 14 group A and C had significant higher levels of active TGFb1 compared to group B p<0.05 (**Figure 17)**

Biopsies from adhesions

Active TGFb1 from biopsies of adhesions in group B were significantly higher than in group A and C at day 14, p<0.05 (**Figure 18)**

Adhesion score

Significant lower adhesion score were seen at day 7 in group B compared to group A and C p<0.05 Significant lower adhesion score were seen in group C compared to group A at day 14 (**Figure 19)**

Histology

Histology shows less inflammatory cells surrounding the PL/PG complex (**Figure 20)**. Macroscopical appearance is also disclosed (**Figure 21)**
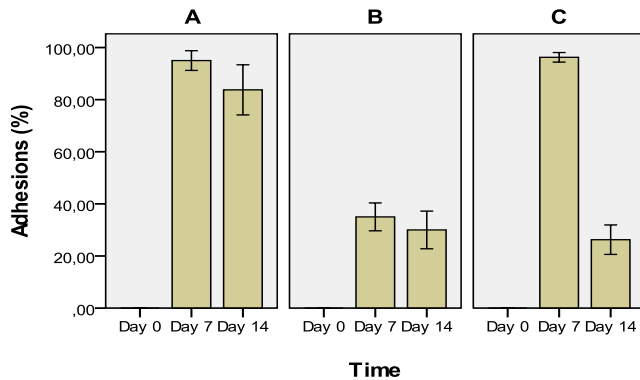

Figure 12. Adhesions (%) evaluated at day 7 after surgery and day 14 after adhesiolysis in group A receiving NaCl before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Significantly less adhesions at day 7 and 14 in group B compared to group A ($p<0.05$). There were significantly less adhesions at day 7 and 14 in group C ($p<0.05$). Graphs show adhesions (mean) ±SE
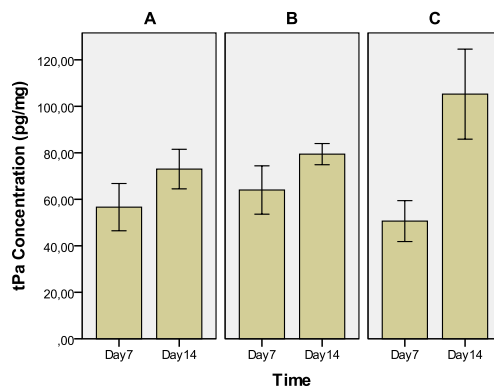

Figure 13. Concentrations of tPA (pg/mg) from biopsies of normal peritoneum (contralateral to the surgery site) taken at day 7(after surgery) and day 14 (after adhesiolysis) in group A receiving NaCl

before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Graphs show concentrations (mean)±SE.

Figure 14. Concentrations of tPA (pg/mg) from biopsies of adhesions at day 7 (after surgery) and at day 14 (after adhesiolysis) in group A receiving NaCl before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Graphs show concentrations (mean) ±SE

Figure 15. Concentrations of PAI-1 (pg/mg) from biopsies of normal peritoneum (contralateral to the surgery site) taken at day 7(after surgery) and day 14 (after adhesiolysis) in group A receiving NaCl before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Graphs show concentrations (mean) ±SE
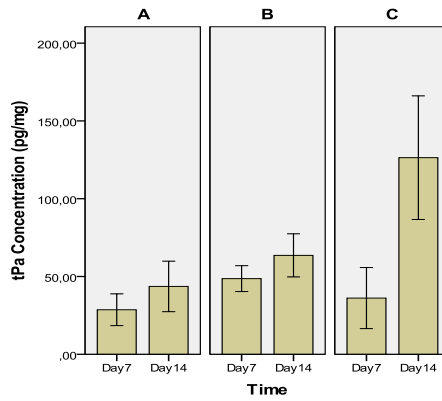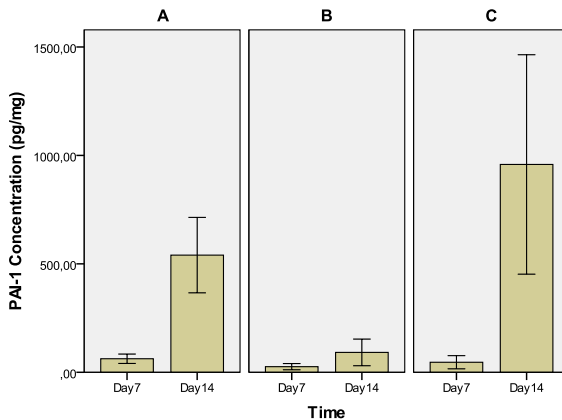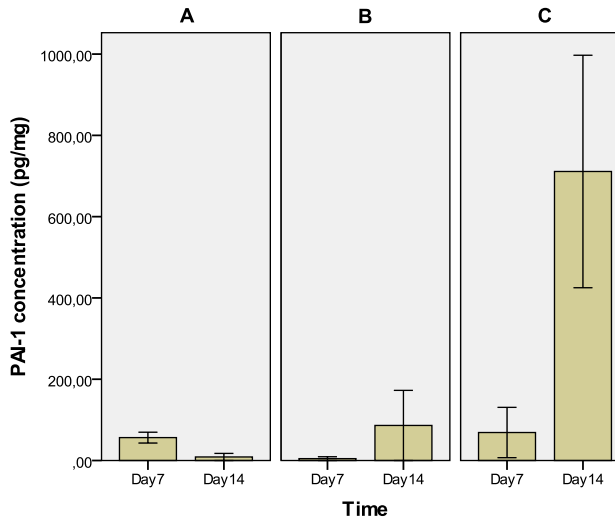
Figure 16. Concentrations of PAI-1 (pg/mg) from biopsies of adhesions at day 7 (after surgery) and at day 14 (after adhesiolysis) in group A receiving NaCl before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Graphs show concentrations±SE

Figure 17. Concentrations of active TGFb1 (pg/mg) from biopsies of normal peritoneum (contralateral to the surgery site) taken at day 0 before (Catena et al.), at day 7(after surgery) and day 14 (after adhesiolysis) in group A receiving NaCl before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Graphs show concentrations (mean) ±SE.
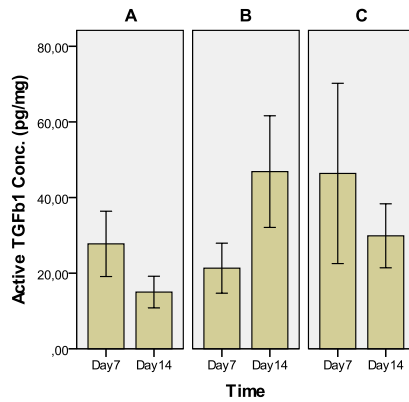
Figure 18. Concentrations of active TGFb1 (pg/mg) from biopsies of adhesions at day 7 (after surgery) and at day 14 (after adhesiolysis) in group A receiving NaCl before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Graphs show concentrations (mean) ±SE
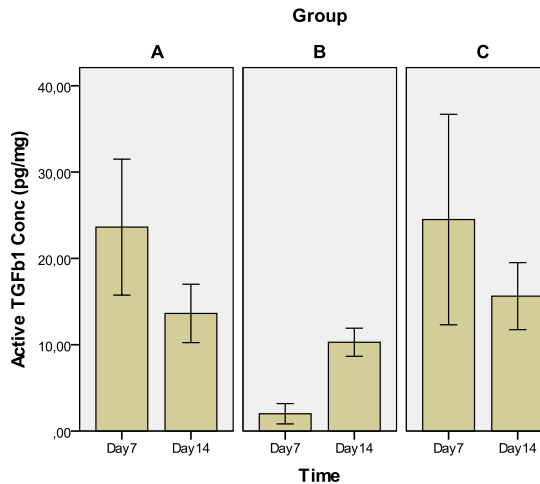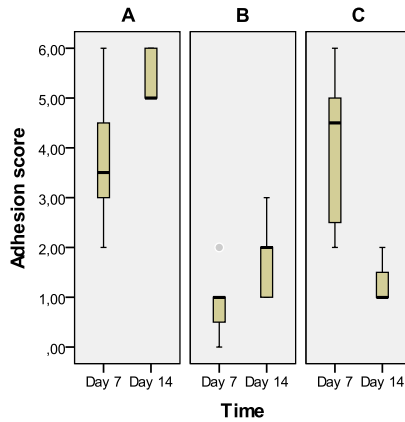

Figure 19. Adhesion score graded according to Bothin et al(Bothin et al. 1999) in group A receiving NaCl before and after adhesiolysis, group B receiving PL/PG before and after adhesiolysis and group C receiving NaCl before and PL/PG after adhesiolysis. Graphs show (median) ±interquartile range
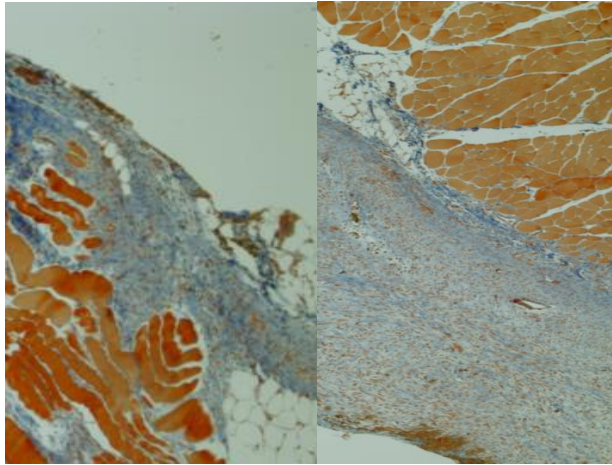
A   B

Figure 20. Histology of adhesion area with and without PL/PG complex. A shows less fibrosis (treatment with PL/PG) than B.
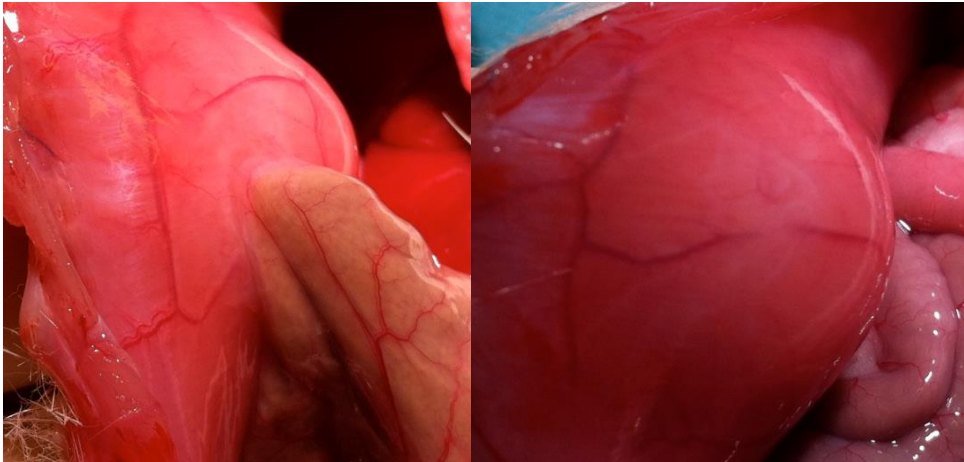
A   B

Figure 21. Animal treated with saline (A) and PLPG treated animal (B) at day. In the control the cecum is totally adhered to the lateral abdominal wall whereas in the treated animal a smooth and healed peritoneum is noted (white/transparent area in picture).

Paper IV

Adhesions

Almost all animals in the control group showed severe and long adhesions on gross examination (**Figure 22).** No animals exhibited the worst severity In the PLPG treatment group, adhesions were less severe and shorter on macroscopic examination, but no animals in the treated group had no adhesions. The median length of adhesions according to Takagi et al(Takagi et al. 2013) was 2 (+/- 1) in the treated animals and 4(+/-2) in the control group at day 7. This reached statistical significance (p<0.05; **Figure 24**). A significant lower severity in quality of the adhesions according to Oncel et al(Oncel et al. 2003) was also detected at day 7 (p<0.05; (**Figure 24).**

The weight of the animals did not differ between groups before surgery (day 0) or before evaluation procedure (day 7).
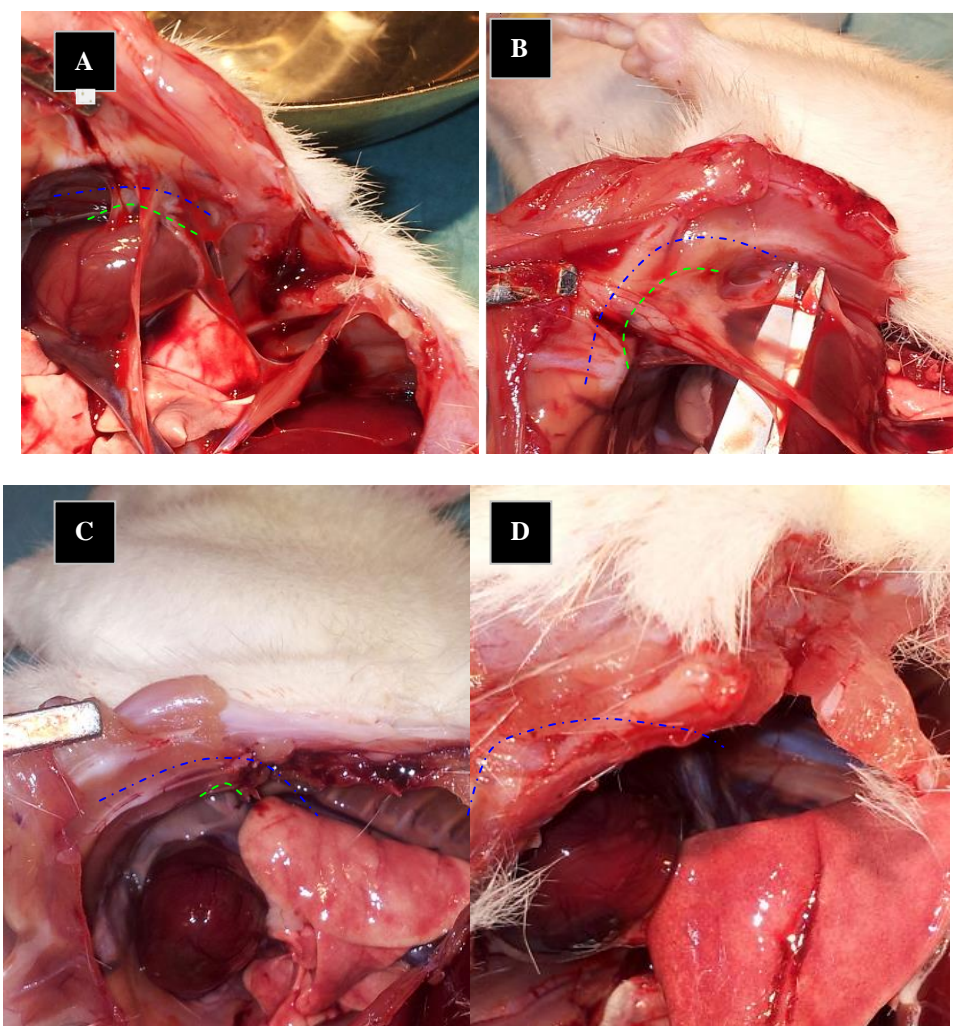
Figure 22 Adhesions lengh marked as green dotted line when evaluated at day 7 and blue dotted line mark the total lenght of pleural cauterized incison at day 0. Animals in A and B recieved Nacl after pleural adhesions were created and animals from picture C and D recieved PLPG. Dotted lines depicts thoracotomy incision.

PAI-1 and TGFb1

No significant differences were seen in PAI-1 (p=0.322) or TGFb1 levels (p= 0.378) between PLPG and control animals at day 7 (**Figure 25**and **26**

Histology

Significant reduction in collagen were seen in experiment group (**Figure 27)**.Histology slides showed macroscopically less fibrosis in the pleura of animals

treated with PLPG than seen in the control group (**Figure 27**), but this was not quantified.)
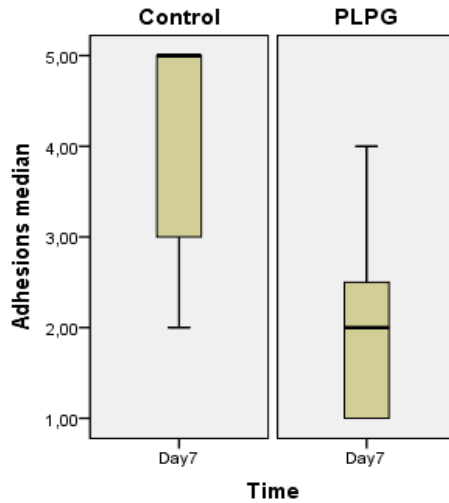
Figure 23. Severity of adhesions
Severity of adhesions according to Oncel et al (Ref 19) expressed as median +/- interquartile range measured in the control and experimental groups. A significant decrease in the quality of adhesions was seen in the PLPG ($p < 0.05$) group at day 7 as compared to the controls.
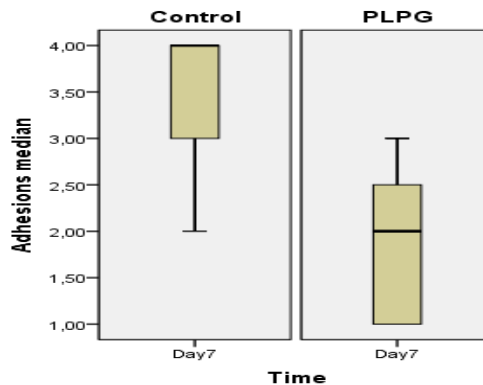
Figure 24. Length of adhesions.The length of adhesions was graded according to Takagi et al (Ref 11) and expressed as median+/- interquartile range measured in the control and experimental groups. Significantly less adhesion were seen in the PLPG ($p < 0.05$) group at day 7 as compared to the controls.
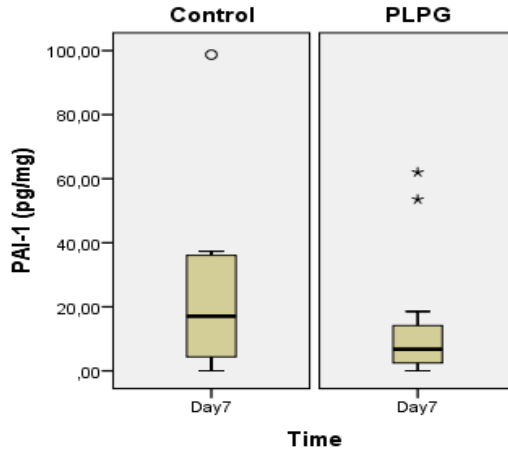
Figure 25**.** Results of PAI-1 in biopsies Levels of PAI-1 (pg/mg) in pleural adhesions at day 7 in the control group as compared to the PLPG group expressed as median +/- interquartile range. No significant difference was seen between the groups, (p=0.322). º and * show outliers.
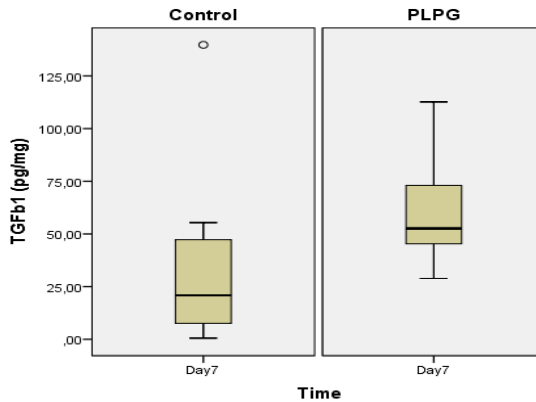
Figure 26. **Results of TGFb1 in biopsiesLevels of TGFb1 (pg/mg) in pleural adhesions at day 7 in the control group as compared to the PLPG group expressed as median +/- interquartile range. No significant difference was seen between the groups, (p= 0.378). º show outliers.**
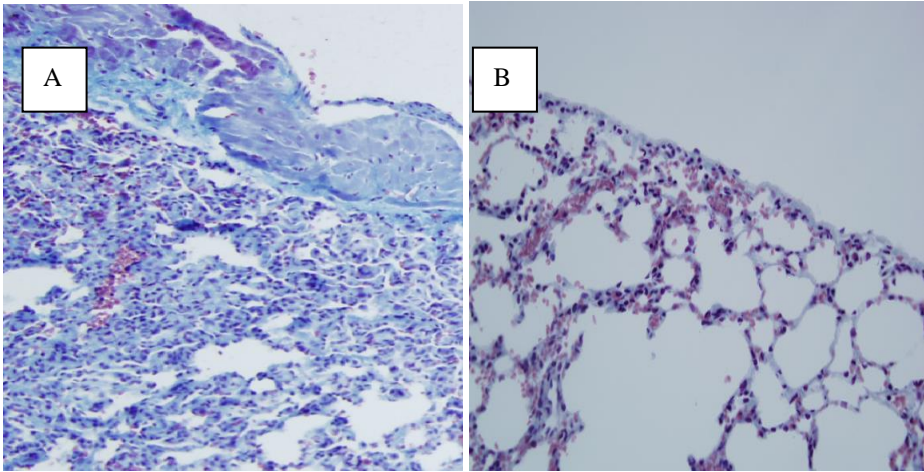
Figure 27. Appearance of pleural fibrosis on lung side with and without treatment. Histology slides stained for collagen with Massons-Trichrome showing more pronounced fibrosis in the control (A) group as was seen in the PLPG group (B)

Paper V

Proliferation

Higher concentrations and molecular weight had higher impact on mesothelial cell proliferation rendering low proliferation. This was seen both for Poly-L-lysine (**Figure 28**) and other polycations (**Figure 29)**
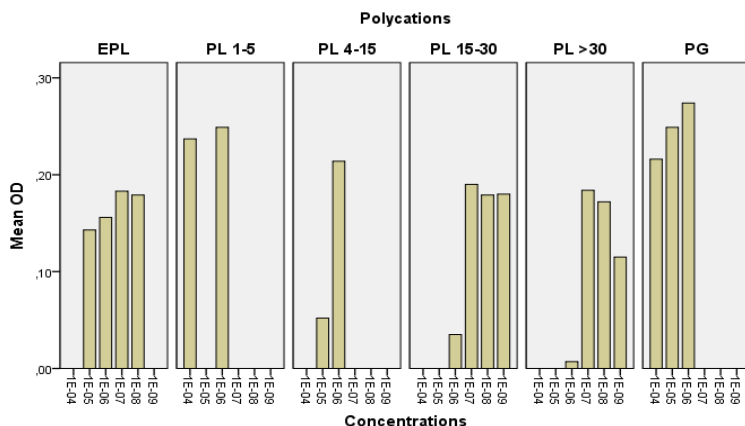
Figure 28. The graph depicts polycations (on top), highest molecular weight to the right, with various impact on mesothelial cell proliferation (left, OD, optical density) in vitro. Higher concentration and molecular weight results in lower mesohelial cell proliferation rate
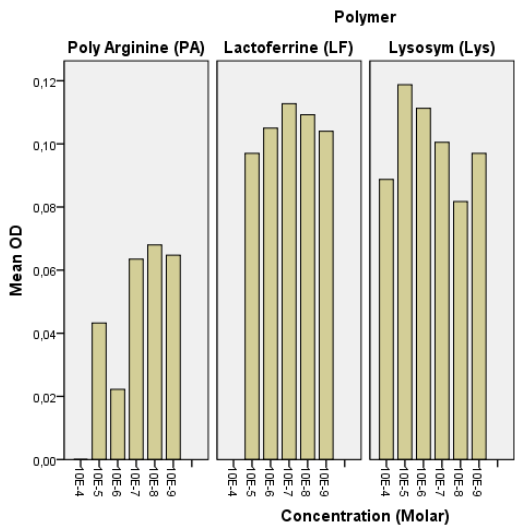
Figure 29. The graph depicts polycations (on top), highest molecular weight to the right, with various impact on mesothelial cell proliferation (left, OD, optical density) in vitro. Higher concentration and molecular weight results in lower mesohelial cell proliferation rate

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Discussion

PL is known for its electrostatic interactions with the bilipid membrane of the cells (Richert et al. 2002; Fischer et al. 2003; Moreau et al. 2002; Jan and Chien 1973). It has previously been shown that PL may form a non charged complex with PG being feasible for gene delivery entering bilipid membrane (Khalil et al. 2006). Earlier studies have shown promising postoperative peritoneal anti-adhesion properties when administering PL on to peritoneal wounds combining it with PG. The anti-adhesion effect is considered to be restricted to electrostatic interactions between the PLPG complex and the harmed peritoneum causing the PLPG to form a biodegradable complex (Tingstedt et al. 2007b; Tingstedt et al. 2007c). These studies have further shown that PLPG does not affect the normal function of local peritoneal macrophages and PLPG has also been shown to be incorporated in the subperitoneal tissue (Nehez et al. 2005).This thesis aims to investigate if the PLPG in any way affect the peritoneal secretion of components involved in the peritoneal healing process such as tPa, PAI-1, TGFb, IL-6 and including histology evaluation of fibrosis/collagen deposition. Furthermore it investigates whether a spraying atomizer is feasible to use when administering PLPG on peritoneal or pleural postoperative defects. Finally, in vitro experiments were carried out to evaluate if PLPG might have any impact on mesothelial cell proliferation (not yet published data in press)In the pilot adhesion study (I), a crude and validated adhesion creating model was used, where cecum was abraded and placed nearby a lateral defect of the peritoneum of the same size. The controls were given NaCl and the experiment PLPG, both through a spray bottle not used previously for this purpose. The spray bottle were easy to use and feasible because it sprayed aerosols of PLPG on to a large wounded area leaving significant less adhesions at the day of evaluation after 10 days. Furthermore we investigated if PLPG had any impact on the major serine protease initiator tPa or its inhibitor PAI-1. tPa is known to initiate fibrinolysis and degrading fibrin to fibrin degrading products and PAI-1 counteracts this process (Menzies and Ellis 1989, 1991; Dunn and Mohler 1993; Kayoglu et al. 2005). Fibrin is considered a template for abdominal adhesions since it may (if not degraded) are replaced by tha stable collagen within 5 to 7 days after its formation (Rajfer 2005; Lindenberg and Lauritsen 1984). Hypoxia, the major initiator of peritoneal adhesions, has been considered to decrease tPa and increase PAI-1(Brokelman et al. 2011; Neuhaus and Watson 2004). Previous studies have shown that PAI-1 may inhibit tPa in a one to one fashion and that

investigating PAI-1 alone may be somewhat hard to interpret (Sancho et al. 1995) and therefore we used the tPa/PAI-complex. The PLPG did not have any significant impact on the tPa/PAI-1 secretion during peritoneal trauma and PLPG was therefore interpreted as having no affection on the ability of peritoneum to secrete tPa and PAI during and after surgical trauma.

All the experiments were started at the same time point of the days in order not to get to much impact on the circadian rhythm which have shown to be an important factor considering the secretion of substances such as tPa or PAI-1 (Johansen et al. 1989; Andreotti and Kluft 1991; Sancho et al. 1995)An important cytokines involved in the initial peritoneal trauma is IL-6 that is released from several local cells (mainly supportive and inflammatory) in the injured peritoneal area (Cheong et al. 2002a; Rapkin et al. 2000; Topley et al. 1993b). The IL-6 was investigated in the peritoneal fluid after surgery with no significant changes between the PLPG and the control group. These findings are hard to interpret and directly relate to any specific findings but important since IL-6 is a cytokine considered to be directly relating to peritoneal trauma and the release of inflammatory cells in the area. This implies no impact on the secretion of IL-6 when using PLPG. TGFb is an important cytokine secreted from various fibroblasts and other supporting cells in the wounded peritoneal area. It is stored intracellular as an inactive precursor and released upon different stimuli, peritoneal trauma being one of them (Karaca et al. 2013) It is also an important factor when it comes to the tissue remodeling during wound healing. Studies have shown that hypoxia is a great inducer of local tissue TGFb especially on peritoneum (Saed et al. 2000, 1999). TGFb is a major initiator for the production of collagen by supporting cells such as fibroblasts and other extracellular matrix cells (Saed and Diamond 2002) .PLPG in our study did not affect the levels of this cytokine in the peritoneal fluid after trauma and it was interpreted that the potential to sustain the wound remodeling was not affected in this aspect. However in the control group there were significantly more local peritoneal adhesions implying that the collagen formed in this group was less/absent in the PLPG group (supported by the histology, below). The results suggest that the ability to secrete the TGFb is sustained but the late result of collagen formation is in the PLPG group.

Histology evaluation of the late product in a double blinded fashion correlated to the macroscopic picture, the PLPG group showed significant less collagen staining at day 10 thereby strengthening the picture that PLPG reduces one of the most important promoters of adhesion formation.

The levels of tPa/PAI-1 complex, TGFb and IL-6 were all tested over time, at 24h, 4d and 10d postoperatively. These time points were chosen in conjunction with previous literature (Hellebrekers et al. 2005) but were somewhat questioned since we did not depict any changes in the levels of tPa/PAI-1 complex, TGFb and IL-6.

Hence it was hypothesized that the time points should be narrowed down in order not to miss out on changes of the tested substances that might exist in the study.

In the second study, we used another method to induce adhesions than in the first study. The peritoneum was incised and stitched it with interrupted non absorbable sutures. This is an established method and very crude since the sutures exert tension in the peritoneal tissue inducing local ischemia and peritoneal adhesions. Using this adhesion creating method we could depict, again, significant less adhesions in the PLPG. Spray atomizers were used to administer PLPG. We choose to measure tPa PAI-1 TGFb and IL-6 from peritoneal fluid in order to be able to compare the results with the first study and narrowed the measuring time points down to 2, 4, 6, 8, 24 h and 7d after peritoneal surgery. Despite this we could not detect any significant changes in the levels of the tested substances between the control and the PLPG groups. Levels of tPa was raised in both PLPG group and NaCl group at 2-6h which is in line with previous data (Hellebrekers et al. 2000a). It implies that the peritoneum sustains it fibrinolytic capacity despite deploying PLPG. It even may imply that peritoneum may have a rebound capacity of secreting tPa and inducing hyperfibrinolysis despite the PLPG. We noted a rather high incline in tPa amount at day 7 postoperatively implying a rebound secretion of tPa which is in line with previous findings (Edelstam et al. 1998, (Hellebrekers, 2005 #1970). This was seen in both groups and implies that the raise in tPa is due to a high consumption of tPa in the area due to fibrin. Interestingly the raise of tPa in the PLPG group was not as high, implying lower amount of fibrin in this group, supported in literature PAI-1 had in general lower levels in peritoneal fluid in the PLPG group compared to the NaCl group. It could be speculated that this might be due to the known sealant effect that the PLPG matrix exerts because PAI-1 is mainly located and secreted from the submesothelial area. TGFb was somewhat lower the 2 first hours in the PLPG group compared to the NaCl. This implies that the PLPG exerts its sealing effect from start and that TGFb is released into the peritoneal fluid to a lesser extent. Despite this, we have no indication that this had any clinical impact and the lower TGFb is not considered to be related to any later fibrotic effects. Il-6 showed similar levels between the NaCl and PLPG group and it was concluded that the Il-6 release was not affected during the healing process of peritoneum using PLPG.

The third study aimed to investigate if PLPG might have an anti-adhesion effect after relaparotomy and adhesiolysis. This is important, since general surgeons often face a reality with already formed adhesions that have to be lysed during relaparotomy and (hopefully) not reformed again. Unfortunately, most adhesions lysed during surgery will reform again. (Chapron et al. 2002; Swank et al. 2003; Ray et al. 1998; Luciano et al. 2008).In the third study, biopsies were taken both at the site of adhesions and contra lateral peritoneum and analyzed for tPa, PAI-1 and TGFb. This was done in order to detect if the unharmed peritoneum had the ability

to secrete these substances and were systemically unaffected by the adhesions created on the other side and furthermore to detect if adhesions had any changes of the substances. Another reason for measuring proteins from biopsies instead of peritoneal fluid, as in the second study, was to eliminate the risk that the peritoneal lavage may dilute the peritoneal fluid samples, even if corrections were made for the dilution effect. Since adhesions, in this study, were evaluated twice we considered an accumulated score to be appropriate for evaluation in order to efficiently catch the adhesion development over time (Bothin et al. 1999). Interestingly the PLPG group showed anti-adhesion properties both after primary and secondary surgery (adhesiolysis). Worth noting is the possibility of PLPG, used within 7 days after primary surgery, might have a synergistic anti-adhesion effect (not significant). The ability to secrete tPa in the normal peritoneum seemed unaffected and correlated to the secretion of PAI-1 implying that the unharmed peritoneum is unaffected by the administration of PLPG on the contra lateral side. The TGFb, PAI-1 and tPa differed between the groups measured from the biopsies of adhesions. This is largely unknown but might be due to the local application of PLPG. One explanation might be the adhesions that differed in quality (varied from hard to smooth and filmy), which is known to affect the levels in the biopsies (Holmdahl et al. 2001; Ivarsson et al. 1998). Proteases in the area are known to activate tPa, PAI-1 and TGFb. The different quality of adhesions might theoretically induce proteases to degrade adhesions to a different extent, thereby showing different levels of tPa, PAI-1 and TGFb in biopsies.

The impact on adhesions formation after pleural surgery was examined in a pilot study. The rats were cannulated through trachea and connected to a ventilator in order not to create any pneumothorax (Morris et al. 2013). The tracheotomy was chosen since an ordinary blind intubation most often resulted in perforation of the posterior tracheal wall previously described (Stark et al. 1981; Sun et al. 2009; Rennard et al. 1984; Davila and Crouch 1993). This pilot study showed that PLPG significantly reduced pleural adhesion formation and collagen formation. The levels of PAI-1 and TGFb were similar between the groups implying no impact of the production of these factors after PLPG administration. Interestingly we managed to administer PLPG through a spray device and hypothesized that the PLPG easily might be administered also through thoracoscopy.

Previous results (data not shown) have shown in vivo toxicity of high levels of poly-L-lysine when administered alone. In vitro experiments were thus carried out in order to detect possible impact on mesothelial cell proliferations by various sized PL molecules as well as other polycations. The results were in conjunction with previous observations that there is a correlation between lower cell proliferation and higher amount of charges on the PL (Moreau et al. 2002). This correlation is also dependent on the conformation of the polycation (Yamagata et al. 2007).

Summary and conclusion

Abdominal and pleural adhesions mainly form due to previous surgery. The overall mechanism of adhesion formation is clear although some pieces remains to be elucidated. The number of patients as well as the health care expenditures related to complications of postoperative adhesions has been reported to grow. This is most certainly due to increasing number of patients subjected to surgery. Many anti-adhesion devices exists on the market at present, however, no anti adhesive device is sufficient in every aspect reducing adhesion complication.

Polypeptides, positively charged poly-L-lysine and negatively charged poly-L-glutamate (PLPG), have previously shown to be effective reducing postoperative adhesions. Therefore, this thesis aimed to investigate PLPGs possible impact on key parameters of peritoneal and pleural healing process after surgery.

From the experimental settings some general cocnlusions, listed below, could be drawn:

I.   Positively charged poly-L-lysine and negatively charged poly-L-glutamate (PLPG) reduces abdominal adhesion significantly when administered as a spray on to peritoneum. Key parameters of the peritoneal healing process, measured from 1 to 7 days, are not influenced by the administration of PLPG postoperatively. PLPG reduces the formation of the primary adhesion template collagen on peritoneum.

II.   Key parameters of the peritoneal healing process, measured during 24 hours postopertively, are not influenced by the administration of PLPG.

III. PLPG applied as a spray reduces adhesions significantly after adhesiolysis when administered on the peritoneum. Furthermore, some variations in key parameters of the peritoneal healing process are seen when PLPG is administered. However, the variations do not imply any impact on the peritoneal healing process.

IV.   Pleural adhesions are reduced after surgery when PLPG is applied as a spray. Key parameters of the healing process are not affected.

Furthermore, PLPG significantly reduces the collagen depositions on pleura.

V. Due to electrostatic interactions between the cell membrane and various positively charged polycations mesothelial cell viability was affected in a dose concentration and size of polycations response. Higher concentrations and bigger sized polycations imply a more pronounced impact on cell viability than vice versa.

Future aspects

Since there is no available anti-adhesion devices that truly reduces the abdominal and pleural postoperative adhesions the research must perservere. Today high demands are put on such a device since it should reduce the possible future complications and at the same time be traceless, leaving no footprints on the resolving process. It should be easy to handle and applicable both in open and minimal invasive surgery for abdomen as well as thorax. Furthermore the cost must be reduced to a minimum.

The concept of applying poly-L-lysine (PL) and poly-L-glutamate (PG) seems feasible regarding some of the above mentioned aspects since it reduces adhesions in animal experiments, does not seem to affect key parameters of peritoneal or pleural healing process and may be used both in abdomen and thorax. However some important steps in order to be able to use PLPG as anti-adhesion device must be investigated:

The possible dose related toxic effect must be examined extensively. It has previously been reported that the LD50 dose is 40mg/kg (Isaksson et al. 2010) and the lowest effective dose is 1.6mg/kg. The toxic effect of poly-L-lysine has been attributed to the electrostatic interaction to the cell membranes (negatively charged).

A smaller pilot study in humans evaluating the PLPG concept in open abdominal surgery ought to be done. A feasible group of patients would be those surely destined for relaparotomy e.g. subtotal colectomy due to ulcerative colitis and diverting loop ileo stomas later converted after primary surgery. Since colorectal surgery causes a high amount of adhesions colectomy patients would be optimal for studying adhesions in such a setting (Parker et al. 2004).

Further clinical studies that would be relevant in the early evaluation of PLPG as an anti-adhesive concept are pilot studies in thorax surgery, both in open and minimal invasive surgery, as well as treatment after ahesiolysis because of small bowel obstructions.

Furthermore, cost benefit analysis must be simultaneously performed when a commercial product has evolved.

Populärvetenskaplig sammanfattning

Sammanväxningar/ärrbildningar i buken och bröstkorgen efter kirurgiska ingrepp är mycket vanligt. Bland de patienter som opereras kommer cirka 95 % att få någon form av sammanväxningar. Nästan 1 procent av alla inläggningar på kirurgiska avdelningar och tre procent av alla bukoperationer anses i grunden vara orsakade av sammanväxningar i buken. Detta är ett stort antal patienter som årligen drabbas och kostnaden för samhället är betydande. Den årliga kostnaden i Sverige för sammanväxningar har beräknats till mellan 40 och 60 miljoner Euro.

 Dessutom finns indikationer på att kostnaden ökar årligen i takt med att allt fler patienter genomgår operation. För den enskilde patienten varierar panoramat av komplikationer.

En av de vanligaste komplikationerna som kan tillstöta, på grund av sammanväxningar efter tidigare kirurgi i buken, är tarmvred. Det som händer då är att tarmen har växt samman med sig själv eller ett annat organ och hamnat i ett så pass ogynnsamt läge att dess innehåll inte kan passera. Symtom som uppstår är smärta, illamående och kräkningar. Tillståndet kräver vård på sjukhus och en del av dessa patienter måste akut opereras. Operationen är inte helt riskfri och kan i värsta fall innebära skada på tarmen med åtföljande livslångt lidande. En annan konsekvens av sammanväxningar efter tidigare operation är att en eventuell ny opertion ofta blir mer komplicerad och utdragen eftersom sammanväxningar gör det svårare för kirurgen att komma in i bukhålan och nå fram till det aktuella organet.

Ytterligare komplikationer är kvinnlig infertilitet, där sammanväxningarna gör att äggstockar, äggledare och ägg kan fastna mot omkringliggande vävnader. Därmed ökar risken för att ägglossningen inte fortgår som normalt alternativt helt uteblir. Man tror numera att upp till 40 % av kvinnors infertilitet kan bero på sammanväxningar.

Sammanväxningar efter kirurgi kan även ge upphov till smärttillstånd i buken. Tillståndet är dock inte helt belagt och sannolikt ett område som är svårt att utforska, delvis på grund av att smärta är en subjektiv upplevelse och att buksmärta kan bero på många orsaker. Därmed ökar risken att man vid dylika studier råkar få med patienter med smärtor av andra orsaker. Trots detta vet man

idag att smärta till följd av sammanväxningar efter kirurgi är en realitet och behöver utredas mer.

Kirurgi i bröstkorgens organ har blivit alltmer vanlig, exempelvis kranskärlskirurgi, borttagande av lungvävnad vid spridd men botbar cancer och hjärtklaffskirurgi hos barn och vuxna. Därmed ökar andelen patienter som opereras mer än en gång i bröstkorgen varpå risken att få komplikationer på grund av sammanväxningar efter kirurgi också ökar. Komplikationer som kan tillstöta är framför allt blödningar, organskada och förlängd operationstid vid ny opertion.

Under flera decennier har det forskats kring sammanväxningar efter kirurgi och idag vet vi, både på mikro och makroskopisk nivå, till stor del varför dessa uppkommer. Problemen man framför allt brottas med är hur dessa skall förebyggas för att förhindra framtida komplikationer.

Det finns ett flertal produkter på marknaden som förebygger sammanväxningar men ingen av dessa har visat sig vara utan biverkningar eller helt reducera mängden sammanväxningar. Några har visat sig ha skadliga effekter och dragits in från marknaden.

Tidigare lovande djurförsök har visat att om man applicerar både positivt laddade proteiner och negativt laddade proteiner på skadad bukhinna bildar dessa en matta av en biofilm som hindrar inläkning av sammanväxningar i det skadade området. Tidigare studier har visat att biofilmen inte påverkar immunförsvaret och fungerar trots pågående infektion och blödning i buken.

Denna avhandlings syfte var att ytterligare utreda konceptet med biofilm som profylax mot sammanväxningar. Avhandlingen visar att sammanväxningarna kan minskas efter kirurgi både i bröstkorg och buk. Det har också visats att viktiga mekanismer som har med den normala läkningen efter kirurgi att göra inte påverkas, vare sig i buk eller bröstkorg, vid användandet av biofilmen. Dessutom konstateras att biofilmen kan sprayas på den skadade ytan, vilket ökar möjligheten till användning vid både öppen och titthålskirurgi.

I framtiden kommer studier på människa krävas för att undersöka biofilmens kliniska värde avseende att förebygga utvecklingen av sammanväxningar efter kirurgi.

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APPENDICES

Paper I-V