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If your experiment needs statistics, then you ought to have done a better experiment.

Ernest Rutherford (1871- 1937)

To my sister and inspiration for life, Eva

Acknowledgements

This thesis is respectfully presented to the Katholieke Universiteit Leuven through its rector prof. dr. M. Waer and its vice-rector and chairman of Biomedical Science prof. dr. M. Casteels, to the Faculty of Medicine through its dean prof. dr. B. Himpens, and to the Department Women and Child through its chairman prof. dr. J. Deprest.

I am grateful to the members of the jury, prof. dr. F. Penninckx, prof. dr. P. Moerman, prof. dr. H. Van Goor (Nijmegen) and prof. dr. A. Wattiez (Strasbourg,) for their most valuable remarks and critical reading of the manuscript and to the chairman of the jury prof. dr. F. Amant. Thanks to prof. dr. D. Deridder who chaired the reading committee and for guiding me through the final mazes of the doctoral net.

I want to thank my promoter, prof. dr. Philippe R. Koninckx, the engine that kept me going throughout my doctoral thesis and thought me there is more in life than gynecology. He guided me on every investigational road I took and made me take control off my own goals. His enthusiasm, perseverance and most of all strive for quality made me a better scientist.

I would like to thank my co-promoter prof. dr. Ignace Vergote for supporting me in my first steps in gynecology now some twelve years ago. He improved my surgical skills in gynecology which will be mine for the rest of my career.

I am grateful to all my colleagues, prof. dr. Myriam Hanssens, prof. dr. Jan Deprest, prof dr. Dirk Timmerman, prof. dr. Willy Poppe and dr. Anne Pexsters who relieved me of some of my clinical and other duties to be able to work on my thesis or gave me advise which I could use all the same. Thanks to Marleen Craessaerts and Diane Wolput for being there when I needed you to answer practical and unpractical questions. They are (probably) the best trial team in the world.

I would also like to thank my sister Mieke Verguts for helping me preparing this manuscript. Great job!

A special thanks to prof. dr. Bernard Vanacker, Roberta Corona, An Coosemans, Mercedes Binda, Carlo De Cicco, Karina Mailova, and Tom Bourne for substantial help in our trials and sharing thoughts on design and results which cleared the way for even more trials. A perpetuum mobile which I am grateful to be part of. Many thanks to all working in the lab for guiding me and making the experiments possible. You've opened a new world to me.

Thanks to Sandra Meeus and her team from the A-cluster in the operating theater for making the impossible possible, Chris Verheyden and Mark Declerck from the technical department, Eugeen Steurs from Storz®, and Thomas Koninckx and Bob Koninckx from eSaturnus®.

Thanks to my dearest friends Wim Vandoninck and Gert Vangeel for supporting me through some difficult years. Thanks to all I ever met, this thesis is the result of a number of events you where definitely part of.

Where it all comes down to are you, my family and my loving and caring wife Sandra and our four most precious gifts to this world: Lisa, Anne, Marit and Andreas. All this means nothing compared to what you mean to me.

Leuven, June 2011

Chapter 1

Introduction: post-operative adhesion formation

Adhesions are fibrous bands between tissues and organs and are one of the most underestimated problems which may occur following surgery. The term "*adhesions*" comes from the Latin "*addere*" which means "*to add something*". Adhesions are therefore not restricted to one type of organ or tissue, but can involve any kind of tissue or even foreign material.

There are two different classifications of adhesions that are commonly used. First there is the classification by Pouly et al.[1] which recognizes three types of adhesions. Adhesions occurring between injured areas are classified as "*adhesions*". "*De novo adhesions*" occur at an area outside the surgical trauma site where no previous adhesions occurred. Adhesions at a site where previous adhesions were lysed are classified as "*adhesion reformation*". The classification of Diamond et al. recognizes two types of adhesions: "*de novo adhesions*" and "*adhesion reformation*" as Pouly et al., but sub classifies them depending on whether adhesions occur at or outside the surgical site[2].

Until recently adhesions were documented to be an almost unavoidable consequence of abdominal and pelvic surgery, as they occurred in as much as 93% of all patients undergoing abdominal surgery[3]. It was shown during autopsy that in up to 28% of patients who never underwent surgery also developed adhesions, as they may be caused for example by pelvic inflammatory disease or endometriosis[4].

As adhesions may cause pain, infertility and/or bowel obstruction, treatment can be necessary. Treatment of adhesions may fail since adhesions can be difficult to remove or, when removed, tend to reform. Therefore prevention (primary and secondary) is of utmost importance. Primary prevention consists in preventing tissue damage. Secondary prevention of adhesions consists in treating already inflicted tissue damage. Gentle tissue handling is thought to be a form of primary prevention. The use of barriers to seal off the damaged area is thought to be a form of secondary prevention. The focus on primary or secondary prevention only gained interest with the understanding of the mechanisms that cause adhesions, and with the ability to prevent adhesions efficiently with some basic surgical principles (e.g. gentle tissue handling, avoiding desiccation). These principles were only later

evaluated in animal and clinical trials, but served well for the time being. Laparoscopy, when introduced in the eighties, was also thought to be beneficial in the prevention of adhesions as it is considered minimal invasive. However, the positive effect of this new approach on adhesion prevention was not as good as expected.

A. Clinical significance of post-operative adhesions

A study published in Digestive Surgery showed that adhesions developed in more than 90% of patients who underwent open abdominal surgery and in 55%–100% of women who underwent pelvic surgery[5]. Adhesions from prior abdominal or pelvic surgery can decrease visibility and access at subsequent abdominal or pelvic surgery. In a very large study (29,790 participants) published in The Lancet, 35% of patients who underwent open abdominal or pelvic surgery were readmitted to the hospital on an average of two times after their surgery due to adhesion-related or adhesion-suspected complications[6]. Over 22% of all readmissions occurred in the first year after the initial surgery and was linear over time. In the SCAR trial it was demonstrated that the risk of readmission due to adhesions was 5% over a ten year period following an initial open surgical procedure for a gynecological condition[7]. Of the readmissions about 40% was readmitted between two and five times. This suggests that a great number of adhesions formed after surgery occur without symptoms.

In 2001 Parker et al. published a study based on the same population group as Lower et al., but they concentrated their analysis on those patients who had lower gastro-intestinal surgery[8]. In this cohort (32.6%) patients were readmitted on an average of 2.2 times in the subsequent 10 years for a potential adhesion-related problem (twice as high as in the first study). Although 25.4% of readmissions occurred in the first postoperative year, they steadily increased throughout the study period. After open lower abdominal surgery 7.3% of readmissions were directly related to adhesions. The overall average rate of readmissions was 70.4 per 100 initial operations, with 5.1 directly related to adhesions.

Major complications of adhesions will depend on localization of the adhesion, causing chronic pelvic pain (*cfr. infra*), bowel obstruction or infertility[9;10]. Adhesion-related complexity at re-operation added significant risk to subsequent surgical procedures[11].

The high clinical significance of adhesions however will not always prompt practitioners to discuss the matter with their patients.

B. Pathophysiology of adhesion formation

I. The classic model: a local phenomenon between opposing lesions

Adhesion formation is mediated through different mechanisms. (Figure 1) Damage to peritoneal surfaces induces a response starting with an acute inflammatory reaction, and a process involving mesothelial cells, macrophages, exudate with cytokines and coagulation factors, neutrophils and leukocytes[12]. The inflammatory response directly results in fibrin deposition at the lesion within the first hours after the peritoneal trauma with a peak on postoperative day four to five. Mesothelial cells obviously play an important role in peritoneal repair. Within hours a peritoneal defect (i.e. caused by a trauma during surgery) is covered with macrophages and mesothelial cells, previously described as tissue repair cells[13].

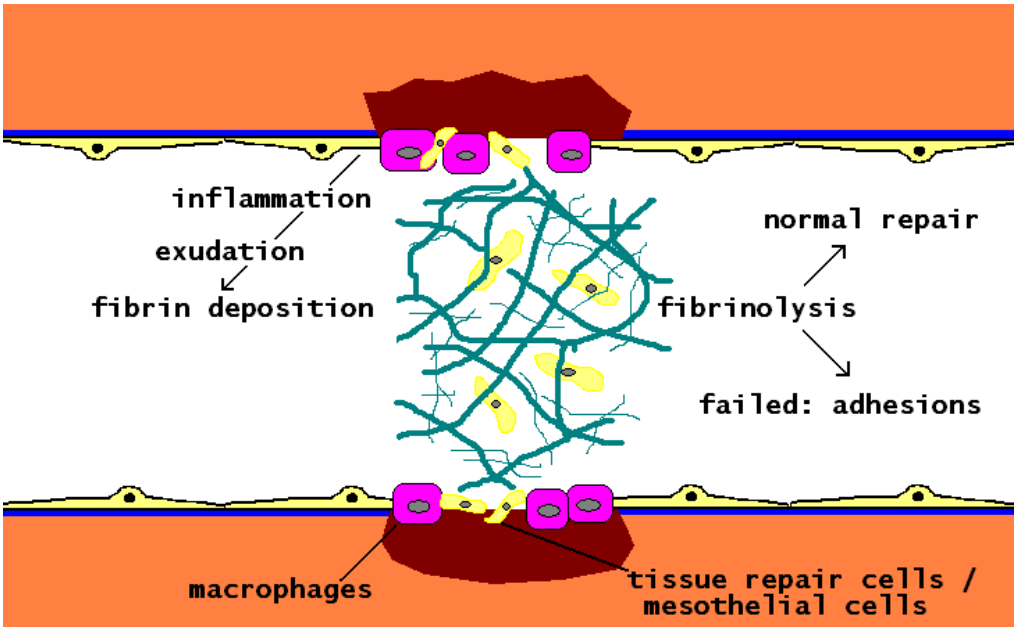


Figure 1: the classic model

The healing process starts from multiple islands over the defect from where mesothelial cells proliferate. Hence small and large defects heal in the same short time from seven to ten days[14]. Mesothelial cells at the edge of a lesion, and mesothelial cells of the opposing mesothelial surface develop an increased dividing capacity, a phenomenon being maximal within two days, which suggest locally secreted factors[15]. If mesothelial cells are capable in covering the lesion, then fibrinolysis will complete within a few days with resorption of degradation products and reepithelialization will result in a smooth healed tissue surface.

If the normal rapid repair of peritoneal lesions fails or when repair is delayed, other processes which were activated may become dominant. Within 4 to 6 days fibroblasts invading the fibrin scaffold start to proliferate, and angiogenesis starts, leading invariably to adhesion formation. The importance of the fibrin scaffold between 2 injured surfaces was elegantly demonstrated since by separating these areas by silastic membranes for as little as 30 hours abolished adhesion formation[16]. This type of experiments reinforced the belief that adhesion formation is a local process and that prevention should aim at separating the surfaces for at least 2 days. Medical treatment given intravenously or intraperitoneally has thus been considered less important since this type of treatment would have difficulties to reach the injured zone because of local ischemia after coagulation and since it is shielded by the fibrin plug. The pathophysiology of this local process has been considered an inflammatory reaction, with players and mechanisms as fibrinolysis, plasmin activation and PAI's, local macrophages and their secretion products and the overall oxygenation of the area or the absence thereof, driving angiogenesis, fibroblast proliferation and mesothelial repair. This concept of a local process is widely supported by reduced adhesions with administration of antibodies against PAI-1[16] or fibrinolytic agents[17], neutralizing antiserum against VEGF, antibodies against PlGF and against VEGFR-1[18].

II. The updated model: the important role of the peritoneal cavity

Traditionally the peritoneum is considered a tissue, reducing friction between organs. It's the largest 'organ' of the body lining the peritoneal cavity with visceral and parietal surfaces covering the internal organs and body wall, respectively. It is considered as the epithelium of mesodermal cavities and it has mesenchymal and epithelial properties.

Two types of cells cover the peritoneal surface: squamous cells and cuboïdal cells. Squamous cells are the predominant type and have some degree of active fluid and molecule transport. Cuboïdal cells are predominant at areas of injury, at milky spots of the omentum and at the peritoneal side of the diaphragm overlaying the lymphatic lacunae. They are specialized in leucocyte transport, synthesis of cytokines, growth factor and CA-125 and control of coagulation and fibrinolysis by secretion of plasminogen activator inhibitor (PAI)-1 and -2 and plasminogen activators (PA) urokinase PA (uPA) and tissue PA (tPA).

Transport of proteins over this mesothelial membrane is low for larger proteins, explaining low concentrations of albumin and fibrinogen in the peritoneal fluid and low plasma concentrations of CA 125[19;20].

Following serosal injury and the subsequent inflammation with production of exudate, there is a fine balance between the secreted proteins, such as fibrinogen and plasminogen, which, if disrupted may result in the formation of adhesions. This exudate has similar concentrations of proteins as the plasma.

The origin of the mesothelial cells involved in the repair of a serosal injury (*cfr. supra*) remains somehow unclear. Possible origins are macrophages which could transform into mesothelial cells when in contact with a defect, subserosal mesothelial cells which could migrate into the wound or free-floating mesothelial which cells could implant. These new mesothelial cells could eventually originate from bone marrow precursors[21]. Indeed, subserosal mesothelial cells are pluripotent, since they can differentiate to mesenchymal and epithelial cells. After peritoneal injury they differentiate and start expressing cytokeratin while losing expression of vimentin[22;23]. The exudate after injury contains a high number of fibroblast and epitheloid like cells. These cells have low proliferative activity for at least 48 hours[24]. That free floating mesothelial cells are important, is attractive since they are present at all times and since their number increases twelve times within in two to five days after injury[25], since these cells were demonstrated to implant at and since extensive lavage with removal of these free floating cells slows down peritoneal healing[26]. That mesothelial cells might originate from bone marrow precursors in blood is probably less important since total body radiation has no effect upon peritoneal wound healing[27].

Intraperitoneally injected exogenous mesothelial cells can implant into the injured area and reduce adhesion formation in the human by injection of cultured mesothelial cells obtained from omentum[28]. This confirms the observations in a staphylococcal peritonitis rabbit

model[29;30]. In a rat abrasion model the injection of cultured mesothelial cells or mesenchymal stem cells derived from skeletal muscles of newborn rats decreased adhesion formation[28;31]. Recently intraperitoneal implantation of an artificial peritoneum with collagen gel, fibroblasts and overlying mesothelial cells on an injured site significantly reduced adhesions[32]. Mesothelial cells grown from adhesions moreover are different form fibroblast derived from normal peritoneum[33].

The entire peritoneal cavity is exposed to the laparoscopic gas and to air during laparotomy and the mesothelial cells are thus influenced as homeostasis is disrupted. The peritoneal pH seemed to immediately decrease during insufflation of CO_2 into the peritoneal cavity, a response that might influence biological events. This peritoneal effect also seemed to influence systemic acid-base balance, probably due to trans-peritoneal absorption[34;35].This acidification of the peritoneal cavity whether by abdominal insufflation or by experimental peritoneal acid lavage increased serum IL-10 and decreased serum TNFalpha levels in response to a systemic lipopolysaccharide challenge mimicking a septic status. The degree of peritoneal acidification correlated with the degree of inflammatory response reduction and these results supported the hypothesis that pneumoperitoneum-mediated attenuation of the inflammatory response after laparoscopic surgery occurs via a mechanism of peritoneal cell acidification[36]. The direct relation between CO_2 insufflation, acidification of the peritoneum and a decreased immunoprotection might thus result in an altered adhesion formation. The reduction of a systemic immune response was already shown by Vittimberga et al. who conducted a systemic review on CRP and IL-6 after laparotomy and laparoscopy[37].

During the last decade the entire peritoneal cavity has been shown to be a cofactor in adhesion formation thus intervening with the local phenomena of peritoneal repair. Identified so far are hypoxia of the mesothelial cells due to CO_2 pneumoperitoneum, desiccation of cells or tissue manipulation[38-40]. CO_2 Pneumoperitoneum has been demonstrated to increase adhesions and this increase is time- and pressure-dependent[41]. It is thought that these factors damage mesothelial cells altering mesothelial cell morphology[42;43] and general structure of the mesothelial layer: trauma to the large and flat mesothelial cells will induce them to retract as a defense mechanism leading to mechanisms through which adhesion formation is further modulated. We can only speculate that macrophages and their secretion products, blood constituents or other inflammatory products affect directly the repair process or the differentiation of mesenchymal cells at the

injured area. Peritoneal hypoxia was suggested as a driving mechanism[41;44;45] since pneumoperitoneum-enhanced adhesions could be reduced by addition of 2-4% of O_2 to the CO_2 pneumoperitoneum in various animal models (rabbits, mice). It was shown that the mesothelial layer then stains hypoxic and since the increase in adhesions is prevented by the addition of 3-4% of oxygen (restoring the physiologic intraperitoneal partial oxygen pressure of 30 to 40 mm Hg), and is absent in mice deficient for HIF1a or HIF2a, hypoxemia response factor being the first to be activated by hypoxia. This concept was supported by the observed decrease in adhesions in mice deficient for the hypoxia-inducable factors (HIF) 1alfa and 2alfa, VEGF A and B and PlGF[40;46]. Hypothermia also decreases adhesion formation[43;47] and this was demonstrated for cooling and cooling associated with desiccation[39].

Another aspect of adhesion formation during laparoscopy is an increase in Reactive Oxygen Species (ROS) as a result of an ischemia-reperfusion process during insufflation and deflation[48;49]. Adhesions can be reduced by the use of ROS scavengers[50]. Induction of ROS by laparoscopy and the subsequent acidosis is comparable to the ischemic preconditioning which has been studied over the last two decades in coronary occlusion models where ischemia and reperfusion may activate a cascade of events leading to the death of myocardial cells. There is agreement that reactive oxygen species production by the mitochondria is an essential part in the protective mechanism of ischemic preconditioning[51]. Even brief ischemic preconditioning puts the cardiac cells in a protective phenotype for several hours, but this mechanism is not well understood today. This mechanism of protection is only exerted in the reperfusion phase, not in the ischemic phase. We noted that ROS scavengers protected against adhesion formation in a laparoscopic mouse model exposed to a carbon dioxide pneumoperitoneum[52]. I believe that the ischemic condition of a carbon dioxide pneumoperitoneum may stimulate ROS production and only demonstrate a protective effect in the reperfusion phase as was demonstrated by Kece et al. who noted that adhesions reduced when laparotomy was preceded by laparoscopy[53]. A continuous ischemic phase will not display any protective effects from the ROS formed.

C. Prevention of adhesion formation

Adhesion prevention would suggest a comprehension of adhesion formation, but recognition of the principles of good surgical practice started with the introduction of microsurgery formation without proper studies supporting gentle tissue handling, comprising moistening of tissues by continuous irrigation, moistening of abdominal packs, glass or plastic rods for mobilization of tissues, and precise micro-instruments. it is only recently that the importance of prevention of desiccation and of gentle tissue handling have been proven, emphasizing how important and accurate clinical observation can be[38].

Comprehension of adhesion formation by formal animal experiments the former two decades made introduction of further steps in the prevention of adhesions possible. Extrapolation of data from animal research to the human implies however the believe that the detrimental effect of a CO_2 pneumoperitoneum, the duration dependent increased CO_2 resorbtion as observed in mice and in rabbits, also occurs in human.

Considering primary prevention of adhesions with prevention of peritoneal damage, animal models suggest reduction of mesothelial hypoxia and dessication by addition of 3-4% of oxygen en maximal humidification of the insufflation gas[39]. Moreover, cooling of the peritoneal cavity is important since cells become more resistant to metabolic damage at lower temperatures[54]. Other important considerations are a reduction in operation time, reduction in blood loss and the extent of tissue manipulation.

Generally when foreign material comes into contact with the peritoneum an inflammatory reaction takes place provoking adhesion formation. This is observed in cases of infections due to trocar insertion through an infected umbilicus of when opening the vagina during laparoscopic surgery. This is even more likely with entry into the bowel. Whether extensive lavage following surgery might reduce adhesion formation or the risk of some minor infection is unknown. Following deep endometriosis surgery with full thickness resection and a bowel suture, extensive lavage with 8 liters clearly decreased the postoperative inflammation as judged by CRP concentrations while preventing late bowel perforations (De Cicco et al. submitted)

Taken together these principles of good surgical practice with conditioning and cooling of the pneumoperitoneum and prevention of inflammation a reduction in adhesion formation by 60% could be obtained.

Secondary prevention of adhesion formation focuses on restoring already damaged mesothelial tissue. This can be achieved by using gels, sheets or by instilling fluids keeping the organs separated until complete healing of the peritoneum or by medication altering the inflammation pathway leading to adhesion formation. A wide range of products has been shown to be effective in animal models. Efficacy of all products described so far has been extensively investigated in our laparoscopic mouse model. It should be realized that in this model all criteria of good surgical practice as described are fulfilled, with standardized lesions, controlled duration of surgery, and strict control of temperature and absence of desiccation. It should moreover be realized that the laparoscopic mouse model, is a model for three distinct pneumoperitoneum conditions: normoxia, hypoxia and hyperoxia. The first normoxia model with addition of 4% oxygen intends to minimize any peritoneal damage except for the lesions inflicted to induce adhesions on uterus and abdominal walls. Thus, adhesions would form according to the classic model, with little or no effect on the peritoneal cavity. The second model is the hypoxia model, since adhesions are enhanced by CO_2 pneumoperitoneum induced mesothelial hypoxia. In the third model, called the hyperoxia model, 12% of oxygen was added to the CO_2 pneumoperitoneum, a concentration known to enhance adhesions probably by cell damage by reactive oxygen species.

The use of dexamethasone decreased adhesions by some 30% in the hypoxia model[52], by 60% in the hyperoxia model, and by some 76% in the normoxia model when it is combined with low temperature. ROS scavengers decreased adhesions by 10 to 15% in the hypoxia and hyperoxia model, an effect too small to be demonstrated in the normoxia model, with fewer adhesions to start with. Calcium channel blockers decrease adhesion formation by some 35% of inhibition in both hypoxia and hyperoxia models, and around 58% in the normoxia model when is combined with low temperature; recombinant Plasminogen Activator (rPA) decrease adhesion formation by 40% in the hypoxia and normoxia models whereas less inhibition, around 17%, was observed in the hyperoxia model. Ringers lactate as a flotation agent was marginally but significantly effective[56]. The effects of other flotation agents as carboxymethylcellulose (CMC) and Hyskon were marginal (and surfactants as phospholipids gave some 35% of inhibition in the hypoxia and hyperoxia models and 58% in the normoxia

model when is combined with low temperature[52].Icodextrin, (Adept, a 4% α 1-4 glucose polymer) unfortunately could not be evaluated since it degrades enzymatically in mice. Barriers as Hyalobarrier gel, spraygel and Intercoat were highly effective in all models with a reduction of adhesions between 58 and 90%.

Prevention of angiogenesis also reduces adhesion formation, as demonstrated in PlGF knockout mice and by the administration of anti VEGF and anti PlGF monoclonal antibodies[40;55].

The transplantation of cultured mesothelial cells into the peritoneal cavity also is effective in decreasing adhesion formation and in remodeling the area of mesothelial denudation as described earlier (*cfr. supra*).

Translation of these findings to the human is not evident, as mechanisms may alter between species. All the principles of good surgical practice were implemented in microsurgery, but not in laparoscopic surgery and even then are adjuvant measurements necessary to further reduce adhesion formation. The last decade many products have been tested in the prevention of adhesions in human.

Flotation agents as Ringers lactate are marginally effective and its efficacy has not been proven. Adept® (Icodextrin, Baxter, Deerfield, Illinois, USA) a macromolecular sugar with a high retention time in the peritoneal cavity has shown some evidence of adhesion prevention in a clinical trial versus ringers lactate[56]. There was a significant difference of 9.8% between Adept (49%) and ringers lactate (38%) in prevention of *de novo* and adhesion reformation. A major advantage is the safety and absence of side effects, which were well established since extensively used for peritoneal dialysis. The strength of the available evidence demonstrating efficacy was considered not very solid in a Cochrane review[57].

Interceed® (Johnson & Johnson, Gynecare, Somerville, New Jersey) is a biodegradable oxidized regenerated cellulose sheet to be used in open and laparoscopic surgery. The use in laparoscopy however is not practical and complete hemostasis is necessary to have the desired effect in adhesion formation reduction. A significant effect of 32% reduction in incidence of adhesions was shown compared to good surgical practice alone[58].

Seprafilm® (Genzyme Corporation, Cambridge, Massachusetts) is a hyaluronic acid film degraded over a 2 tot7 day period. It does not require complete hemostasis, but is also difficult to use during laparoscopic surgery. It was used in only one trial for laparoscopic myomectomy to assess the incidence, severity, extent, and area of uterine adhesions after

myomectomy[59]. Seprafilm significantly reduced the incidence postoperative uterine adhesions: mean number of sites adherent tot the uterus = 4.98 vs. 7.88 ($p<0.0001$). Additionally, Seprafilm treatment was not associated with an increase in postoperative complications.

Since Intergel (0.5% ferric hyaluronate gel) has been withdrawn from the market, only Hyalobarrier gel (Auto-cross linked hyaluronic acid gel), Spraygel (Polyethylenglycol) and Intercoat/Oxiplex remain available as gels for clinical use. Overall efficacy appears to be similar for all 3 products. A comparison between these 3 gels can unfortunately not be made since comparative trials do not exist. Also the strength of the available evidence varies and a Cochrane review of hyaluronic acid and Spraygel concluded that only for hyaluronic acid the evidence was solid[57]. Most important is that for none of these products efficacy has been proven for endpoints that really matter, i.e. pain, infertility, and bowel obstruction or reoperation rate. We moreover should realize that large RCT trials were needed because of the high intra-individual variability, and that in these trials the surgical interventions were limited to rather simple and straightforward interventions as cystectomy and myomectomy. In addition, these trials have been performed during interventions performed by laparotomy or by laparoscopy under conditions of CO_2 pneumoperitoneum enhanced adhesion formation and slight desiccation. It therefore is still unclear to what extent the available results of efficacy can be extrapolated to more severe or other types of surgery, whether highly variable efficacy in adhesions and in prevention, is patient or intervention or surgeon dependent and whether in the human the effect will be additive to good surgical practice and conditioning of the peritoneal cavity.

Studies on anti-adhesion products should always include second-look laparoscopy to assess incidence, extent and severity of adhesions as this may represent the true effect of the method used. In order to address and compare adhesions a reliable and validated scale should be implemented.

D. Important clinical factors related to the pneumoperitoneum

I. CO_2 absorption during laparoscopy

Cardiovascular stimulation with tachycardia, arrhythmias and hypertension occur when intra-abdominal pressure (IAP) exceeds 12-14 mm Hg; these effects are not clinically relevant in ASA I and II patients but can compromise ASA III-IV patients[60;61]. The venous blood return is reduced by the elevated IAP and intermittent pneumatic compression is recommended to reduce the venous stasis. IAP must be kept low to limit impaired organ perfusion. A sudden drop in blood pressure and in end-tidal CO_2 concentration during gas insufflation is a sign of gas embolism.

CO_2 pneumoperitoneum also causes hypercapnia and respiratory acidosis. During laparoscopy, monitoring of end-tidal CO_2 concentration is mandatory. Normocapnia should be maintained by increasing minute volume and frequency of ventilation and by changing ventilatory pattern (Intermittent positive pressure ventilation (IPPV) to pressure controlled ventilation (PCV)). IAP must be kept as low as possible to reduce CO_2 absorption and to protect the microcirculation of the peritoneal surface. Acidosis may cause the following effects: O2Hb dissociation curve shift to the right, depression of myocardial contractility, decreased sensitivity to catecholamine, venoconstriction of peripheral veins, vasoconstriction of pulmonary arteries, and hyperkalemia by extracellular shift of potassium. In patients with limited pulmonary reserves, CO_2 PP carries an increased risk of CO_2 retention, also after the operation.

When ventilation is kept constant in rabbits, as was shown by Mynbaev et al.[62], CO_2-pneumoperitoneum profoundly affected blood gases and acid base homeostasis i.e. increasing pCO_2, HCO_3 ($P < 0.001$) and lactate concentrations ($P < 0.05$) and decreasing pH, actual base excess and standard bicarbonate ($P < 0.001$), resulting in metabolic hypoxemia with desaturation, lower pO2 ($P < 0.001$) and O2Hb ($P < 0.05$). These effects were more pronounced with higher insufflation pressure and suboptimal ventilation

CO_2 absorption from the pneumoperitoneum increases over time. Rabbits eventually died as acidosis increased.

Acidosis in rabbits was prevented by adding 4% of oxygen to the CO_2 pneumoperitoneum[63]. This increase in CO_2 absorption was equally decreased by adding oxygen and it is reasonable to assume that the mesothelial hypoxia causes a trauma exposing directly extra cellular matrix (ECM). Adding 4% of oxygen results in a partial oxygen pressure of 30 mmHg (4% of 760 atmospheric pressure + 15 mmHg insufflation pressure) which is within the normal physiologic range of 20-40 mmHg for peripheral tissues. This should prevent this mesothelial hypoxia, thus preventing the exposure of ECM, thus preventing the increased CO_2 absorption.

II. Nitrous oxide pneumoperitoneum in laparoscopy

Considering the cardiovascular and other side effects of CO_2, other gases have been explored for pneumoperitoneum especially, helium and N_2O. The former is an inert gas but has a low solubility in water. The latter has a solubility and lung exchange capacity similar to CO_2, and thus is inherently safe.

Nitrous oxide (N_2O) has long been used in laparoscopy for sterilizations or diagnostic procedures as it had beneficial effects on pain during and after surgery compared to carbon dioxide. The use of this gas was always debated as it displays a risk of explosion with concentrations above 30% in combination with a combustible gas such as methane, i.e. released during colonic surgery and an electrical charge.

Nitrous oxide shares several advantageous properties compared to CO_2: it is an inexpensive gas, it is rapidly eliminated, and it has diffusion and solubility quotients similar to CO_2. It also has anesthetic and analgesic properties, without the cardiopulmonary side effects of CO_2[64]. Several clinical and experimental studies demonstrated the safety and the advantages of N_2O pneumoperitoneum[65].

In a clinical trial Tsereteli et al. demonstrated that the use of nitrous oxide compared to carbon dioxide for pneumoperitoneum significantly decreased post-operative pain and reduced the end-tidal CO_2 during laparoscopy[66]. As investigated by Wont et al. carbon dioxide caused a slight worsening of the parietal peritoneal acidosis at higher intra-abdominal pressure (12-15 mmHg), whereas, nitrous oxide, helium and lifting of the abdomen did not cause parietal peritoneal acidosis[67]. Again the reduction in acidification of

the peritoneum by the use of nitrous oxide can be the cause for the beneficial effects as was demonstrated by Tsereteli et al. but is still unknown.

III. Tumor implantation in laparoscopy

Laparoscopic surgery in patients with intra-abdominal cancer is controversial since in animal experiments, laparoscopy had a stimulating effect on tumor growth compared to laparotomy[68]. Survival in women undergoing laparoscopy for ovarian cancer, however, was not impaired[69].

The positive effect of the CO_2 pneumoperitoneum in animal studies on tumor implantation was mainly attributed to the influence of the CO_2 pneumoperitoneum on the dispersion of free floating tumor cells. The effect of the carbon dioxide on tumor implantation and growth was higher than other gasses (helium, nitrous oxide, xenon or gasless laparoscopy)[70;71]. This detrimental effect therefore was attributed to the CO_2 pneumoperitoneum, either as a direct effect on the peritoneum or as an indirect effect on macrophage function[72].

As homeostasis is the single most important factor to maintain the integrity of the mesothelial lining, we demonstrated in previous experiments that the addition of 3% oxygen to the CO_2 pneumoperitoneum did indeed decrease adhesions[73]. Carbon dioxide damages the mesothelial cell layer with direct exposure of the basal lamina as evidenced by scanning electron microscopy. This exposure of extracellular matrix proteins such as laminin, fibronectin and vitronectin to the tumor cell surface was suggested to increase tumor cell adherence[74]. Since the cascade of events following pneumoperitoneum (i.e. mesothelial damage and peritoneal healing leading to adhesion formation) might also be important in implantation and growth of tumor cells[75], one can anticipate the effect of a pneumoperitoneum with pure CO_2 and of adding oxygen to the CO_2 pneumoperitoneum upon tumor implantation in our laparoscopic mouse model for adhesion formation. If any effect in the human by the addition of 4% of oxygen to the CO_2 pneumoperitoneum on incidence of port site metastases, by preventing tumor cell hypoxia caused by CO_2 pneumoperitoneum remains to be answered.

Port-site metastases (PSM) are a frequent, but clinically not very important complication of laparoscopy for malignant conditions[76]. PSM especially occur after laparoscopy in advanced

ovarian cancer with an incidence of 17% PSM. The risk of clinical PSM was independent of the type of tumor, the tumor grade and the presence of ascites. Different mechanisms have been suggested, such as direct wound contamination during extraction of the malignant specimen through the laparoscopic port, aerosolization of exfoliated tumor cells, gas leaks (known as the chimney effect) and a lowered immune response due to the carbon dioxide[70;77;78]. Anyway, the concern remains that laparoscopic surgery, and more specifically the CO_2 pneumoperitoneum, might be a predisposing factor for PSM.

IV. Post-operative pain and inflammation in laparoscopy

Postoperative somatic pain is widely believed to be caused by the surgical injury of nerve fibers and by the subsequent inflammatory reaction, a necessary mediator for healing of tissues. Pain after laparoscopy is believed to be caused for 50% by the incision of the surgical wounds, for about 30% by the pneumoperitoneum (PP) and for another 20% by the operation wound[79;80]. This concept anyway has lead to the relatively widespread use of local anesthesia of the skin before making an incision for laparoscopy. Visceral pain is known to be very different from somatic pain. Visceral pain is indeed more mediated by stretching than by injury, while over 80% of the visceral nociceptors are sleeping. Following inflammation, the dormant nociceptors are recruited and the threshold is decreased leading to a much increased firing activity to any stimulus including bowel movements[81]. The speed of recruitment and activation of dormant nociceptors, however, has not been investigated in detail. It equally is unclear what the exact time courses of pain are over the first 48 hours after surgery. Given the difficulty to measure pain and the fact that most estimations are indirect, this is not surprising.

It is not clear what the exact mechanism of pain is after laparoscopic surgery and why pain is less when a similar type of surgery is performed by laparoscopy than performed by laparotomy, as demonstrated for hysterectomy[82] or cholecystectomy[83;84]. It is unclear whether the abdominal incision itself is sufficient as an explanation, while the minimal invasiveness of surgery is more a concept than a proven model to reduce pain. Anyway, after laparoscopic surgery the systemic immune response is less pronounced in comparison with open surgery as estimated by levels and duration of postoperative C-reactive protein (CRP)

and interleukin-6[37]. The exact contribution of each component to pain after laparoscopic surgery and how this fits with the known mechanisms of visceral pain mediation remains unclear.

Without a surgical injury adhesions are not likely to form as we already demonstrated in our laparoscopic mouse model[41]. Quantitatively however, the effects of acute inflammation of the entire pelvic cavity were some 20 times more important. This acute inflammation of the entire pelvic cavity was shown to be caused by duration and severity of mesothelial hypoxia during CO_2 pneumoperitoneum, by reactive oxygen species (ROS) and by even gentle manipulation of bowels[38]. Each of these factors results in a retraction and bulging of the mesothelial cells in the entire peritoneal cavity and an acute inflammatory reaction. We in addition demonstrated that this peritoneal trauma could be decreased/prevented by adding 3% of oxygen to the CO_2 pneumoperitoneum, i.e. a local physiologic partial oxygen pressure of some 22 mm Hg (0.03*760), thus resulting in a decreased postoperative adhesion formation[73].

We therefore investigated whether the addition of 4% of oxygen to the CO_2 pneumoperitoneum could decrease the peritoneal acute inflammatory reaction and postoperative pain in women undergoing a promontofixation.

E. Aims of the thesis

To understand the aims of this thesis it is important to know the historical perspective in which they were formed. From 1996 up to now, the concept of full conditioning was developed through different steps. Roger Molinas and Mercedes Binda developed a laparoscopic mouse model and looked at different factors influencing adhesion formation. Hypoxia was recognized as an important factor and addition of 4% of oxygen was proven to be the optimal concentration during laparoscopy. From this, the first translational trials in the human were designed. Further research investigated desiccation and temperature as important factors in the prevention of adhesion formation, but translation was only possible through a joint venture with eSaturnus[tm]. During the development of the equipment to control for desiccation and temperature, the addition of nitrous oxide was investigated upon by Roberta Corona and Karina Mailova in our laparoscopic mouse model. The concept of full

conditioning was further completed with the addition of dexamethasone and rinsing with heparinized solution. Full conditioning and consequences on inflammation and adhesions was investigated in parallel in our laparoscopic mouse model and in the human.
Additional research in mice on tumor implantation and transplantation of mesothelial cells was done.

More specific hypotheses and general aims of this thesis were:

1/ Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and prevent retraction of the mesothelial cells and exposure of the extracellular matrix

2/ Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and will decrease CO_2 absorption

3/ Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and will decrease tumor growth and tumor implantation in laparoscopic port sites

4/ Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and decrease post-operative inflammation and pain

5/ Full conditioning of the pneumoperitoneum will prevent mesothelial damage and will decrease CO_2 absorption

6/ Full conditioning of the pneumoperitoneum will prevent mesothelial damage and will decrease post-operative inflammation and pain

7/ Full conditioning of the pneumoperitoneum will prevent mesothelial damage and will decrease adhesion formation

8/ Full conditioning of the pneumoperitoneum will prevent mesothelial damage and decrease speed of absorption of fluid (Ringer lactate or Adept®)

9/ Autologous transplantation of mesothelial cells after laparoscopy will decrease adhesion formation

Chapter 2

General materials and methods

A. The laparoscopic mouse model

After having used rabbits for studying adhesion formation we progressively developed the mouse laparoscopic model. This model actually is strictly standardized. The following are important aspects: Balb-c mice are an inbred strain (and low variability) with genetically important adhesion formation; anaesthesia, ventilation, temperature, timing of events, surgical lesions and surgical experience. Adhesions can be scored after 7 days which facilitates planning. The model permits 6-8 mice to be operated / day.

Each animal model has advantages and limitations. The main disadvantage of mice is the size. In addition, the thickness of the entire abdominal wall is equivalent to the first muscle layer in rats and the same relationship exists between rats and rabbits. The ratio of peritoneal surface area / body weight is higher in smaller than in larger animals, being 0.195 m2/kg in mice, 0.115 m2/kg in rats, 0.084 m2/kg in rabbit and 0.026 m2/kg in humans. The volume of peritoneal fluid is disproportional higher in smaller than in larger animals being 18.1 ml/kg in mice, 10.7 ml/kg in rats, 7.7 ml/kg in rabbit and 2.4 ml/kg in humans[85].

The mouse model has advantages: they are inexpensive and easy to handle. Several well characterized types are available, such as inbred strains, genetically manipulated mice, e.g. mice non-expressing or over-expressing specific genes, mice with an altered immune system, e.g. nude and SCID mice. Moreover, many specific assays and monoclonal antibodies exist as is the case for Balb/c mice.

Also the genetic background has an influence in adhesion formation. Some mouse strains developed more adhesions than others and, in addition, outbred strains have more variability in the results than inbred strains[86], therefore, to have the maximum of adhesions with less variability 9-10 weeks-old female BALB/c mice inbred strain of 18 to 24g were used. As Balb/c mice are also not too expensive, it is was selected as the preferred animal for our experiments.

In our laboratory a mouse model was developed in which many parameters were modulated and/or monitored: conditions of the pneumoperitoneum (duration, pressure, type of gas, humidification and temperature), body temperature and ventilation. All these parameters can have an influence in adhesion development and should be controlled during the studies.

Exact timing was found to be crucial in order to standardize the slight hypothermia associated with anesthesia[54]. After injection of pentobarbital 0.08 mg/g pentobarbital i.p. (Nembutal, Sanofi Sante Animale, Brussels, Belgium) at time zero (T0) animals were put in a conditioned room at 37°C. Further animal preparation, i.e. shaving, placement on the table in the supine position, intubation and ventilation started exactly after 10 min (T10). Animals were intubated with a 20-gauge catheter and ventilated with a mechanical ventilator (Mouse Ventilator MiniVent, Type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) using humidified air with a tidal volume of 250 µl at 160 strokes/min. Ventilation finished at T80, i.e. after a pneumoperitoneum of 60 minutes.

At exactly T20, CO_2 pneumoperitoneum was induced using the Thermoflator plus® (Karl Storz, Tüttlingen, Germany) through a 2 mm endoscope with a 3.3 mm external sheath for insufflation (Karl Storz, Tüttlingen Germany) introduced into the abdominal cavity through a midline incision caudal to the xyphoid process appendix. The incision was closed gas tight around the endoscope in order to avoid leakage and thus higher flow rates of insufflation gas. The insufflation pressure was set at 15 mmHg as insufflation pressure increased adhesions as demonstrated in rabbits[45;87] and in mice[41;73]. We decided to use this maximum pressure in order to observe a maximum effect of ischemia/hypoxia and since adhesions are pressure dependent. In addition, a pneumoperitoneum with a pressure of 15 mm Hg could be relevant since it can mimic the conditions that happen in surgery on humans. For gas humidification, the Storz Humidifier 204320 33 (Karl Storz, Tuttlingen, Germany) was used, because when non-humidified gas is used to induce the pneumoperitoneum, desiccation occurs and adhesions increase[39]. In addition, desiccation is associated with cooling. The CO_2 pneumoperitoneum was maintained for 60 minutes until T80 .

Temperature of the mice was strictly controlled at 37°C using a closed chamber maintained at 37°C (heated air, WarmTouch, Patient Warming System model 5700, Mallinckrodt Medical, Hazelwood, MO). All equipment and mice were placed in this chamber, thus keeping them at a constant 37°C throughout the experiment. The temperature in the chamber was measured with a Testo 645 device (Testo N.V./S.A., Lenzkirch, Germany),

whereas the temperature of the animal was measured via the rectum with a Hewlett Packard 78353A device (Hewlett Packard, Böblingen, Germany).

After the establishment of the pneumoperitoneum, two 14-gauge catheters (Insyte-W, Vialon, Becton Dickinson, Madrid, Spain) were inserted under laparoscopic vision. Standardized 10 mm x 1.6 mm lesions were performed in the antimesenteric border of both right and left uterine horns and in both the right and left pelvic side walls with bipolar coagulation (20W, standard coagulation mode, Autocon 350, Karl Storz, Tüttlingen, Germany). (Figure 2)

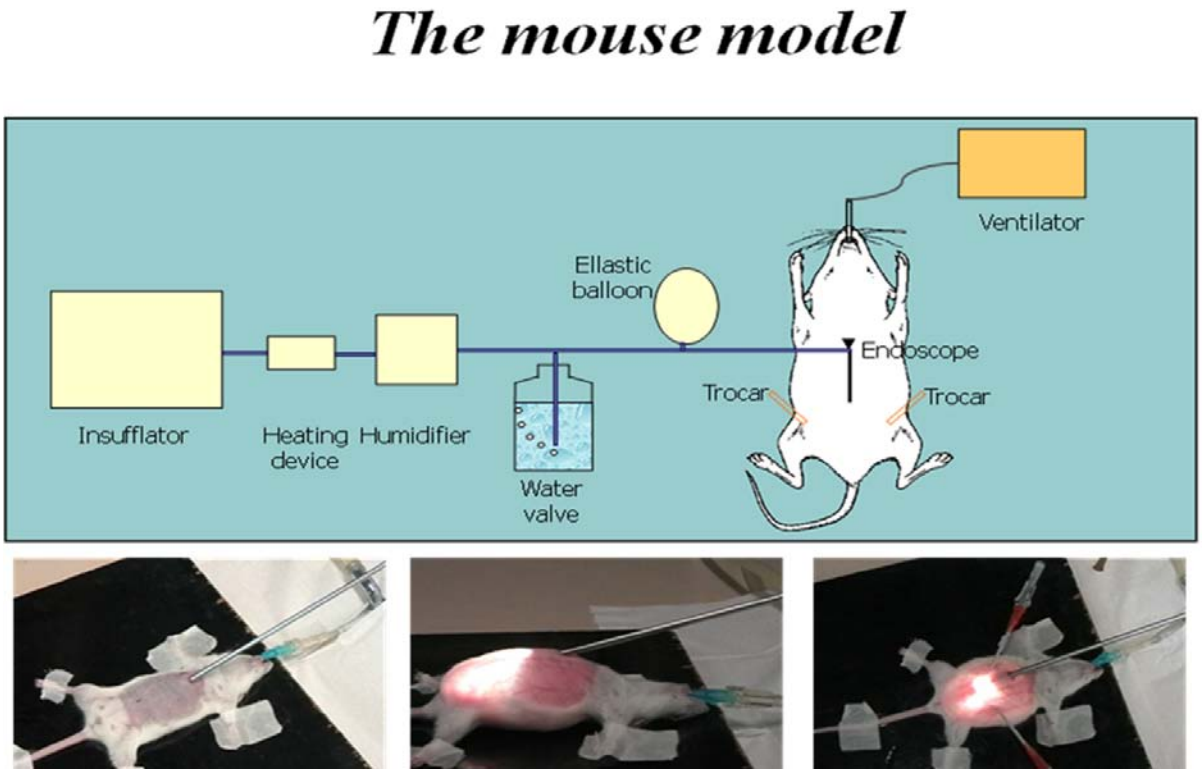


Figure 2: the laparoscopic mouse model

Since adhesion scores on day 7 were equal to adhesion scores on day 14 or later, scores on day 7 were taken. Adhesions were scored quantitatively (proportion and total) and qualitatively (extent, type, tenacity) blindly by laparotomy under a stereomicroscope. The qualitative scoring system assessed: extent (0, no adhesions; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% of the injured surface involved, respectively), type (0, no adhesions; 1, filmy; 2, dense; 3, capillaries present), tenacity (0, no adhesions; 1, easily fall apart; 2, require traction; 3, require sharp dissection) and total (extent + type + tenacity). The quantitative scoring system assessed the proportion of the lesions covered by adhesions

using the following formula: (sum of the length of the individual attachments/length of the lesion) ×100. The results are presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum), which were scored individually. The entire abdominal cavity was visualized using a xyphopubic midline and a bilateral subcostal incision. After the evaluation of ports sites and viscera (omentum, large and small bowels) for de novo adhesions, the fat tissue surrounding the uterus was carefully removed. The length of the visceral and parietal lesions and adhesions were measured. Adhesions, when present, were lysed to evaluate their type and tenacity. The terminology of Pouly was used[1], describing de novo adhesion formation for the adhesions formed at non-surgical sites, adhesion formation for adhesions formed at the surgical site and adhesion reformation for adhesions formed after the lysis of previous adhesions.

B. Clinical trials

All patients for the clinical trials were recruited at the University Hospital Leuven, campus Gasthuisberg. Written and informed consent was obtained for all.

Inclusion criteria always consisted of women undergoing gynaecologic laparoscopic surgery for at least 60 minutes of planned laparoscopy time and signed informed consent.

Exclusion criteria always mentioned: pregnancy and women with a pre-existing condition increasing the risk of surgery such as clotting disorders, heart or lung impairment. All conditions that might interfere with inflammatory parameters such as immunodeficiency, chronic inflammatory disease as Crohn's disease were also exclusion criteria except for the trial on port-site metastasis and the first trial on CO_2 absorption.

Approval of the ethical committee from the University Hospital Leuven was obtained for all clinical trials.

Clinical trials were registered through clinicaltrials.gov:

NCT00678366: Evaluation of Adding Small Amounts of Oxygen to the CO2 Pneumoperitoneum Upon Pain and Inflammation

NCT01344499: Clearance Rate of Peritoneal Fluid After Full Conditioning Pneumoperitoneum

NCT01340989: CO2 Absorption During Laparoscopy

NCT01344486: Peritoneal Cavity Conditioning Decreases Pain, Inflammation and Adhesions

I. Adding 4% oxygen to the pneumoperitoneum

For the clinical trials where 4% of oxygen was added to the carbon dioxide pneumoperitoneum the Thermoflator plus® (Karl Storz AG, Tüttlingen, Germany) was used for insufflation. This insufflator allowed a manual regulation of the amount of oxygen added to the carbon dioxide pneumoperitoneum from 1 to 12%.
The mixture of gas is patented through KUL research and development.

II. Full conditioning of the peritoneal cavity

In the trials where full conditioning was used, a premixed bottle of 86% carbon dioxide + 10% nitrous oxide + 4% oxygen was used. All bottles were officially certified to have the correct mixture. The gas had the same composition at the beginning and at the end of the bottle, so no different composition of the gas occurred over time (Ijsfabriek Strombeek).
Full conditioning further existed in insufflation temperature regulation at 32°C and 100% humidification through a modified Fisher and Paykel (F&P) humidifying system (Type: MR860 AEA, SN: 070514000016, Fisher & Paykel Healthcare®, Auckland, New Zealand). The F&P humidifier blows gas over a heated water chamber. In order to avoid condensation, the tubing delivering the gas is heated to over 40°C with a heating wire. In order to permit higher flow rates, the luer lock limiting flow rates to 7.8 L/min at 15 mm Hg insufflation pressure, was removed for our experiments. In the F&P humidifier, modified by eSaturnus®, the heating of the chamber and the heating of the tubing was continuously adapted by an electronic feedback loop in order to maintain at the end of the tubing 100% relative humidity (RH) at a preset temperature between 25°C and 36°C and this at any flow rate between 0.5 and 30 liters/min. The flow rate, relative humidity and temperature of the gas at the end of the insufflation tubing and of the gas outflowing from the peritoneal cavity or from the box used for the experiments, were measured with a sensor for temperature and relative humidity (model SHT75, Sensirion, Switzerland).
Both composition of mixture of gas and cooling by third means are patented through KUL research and development.

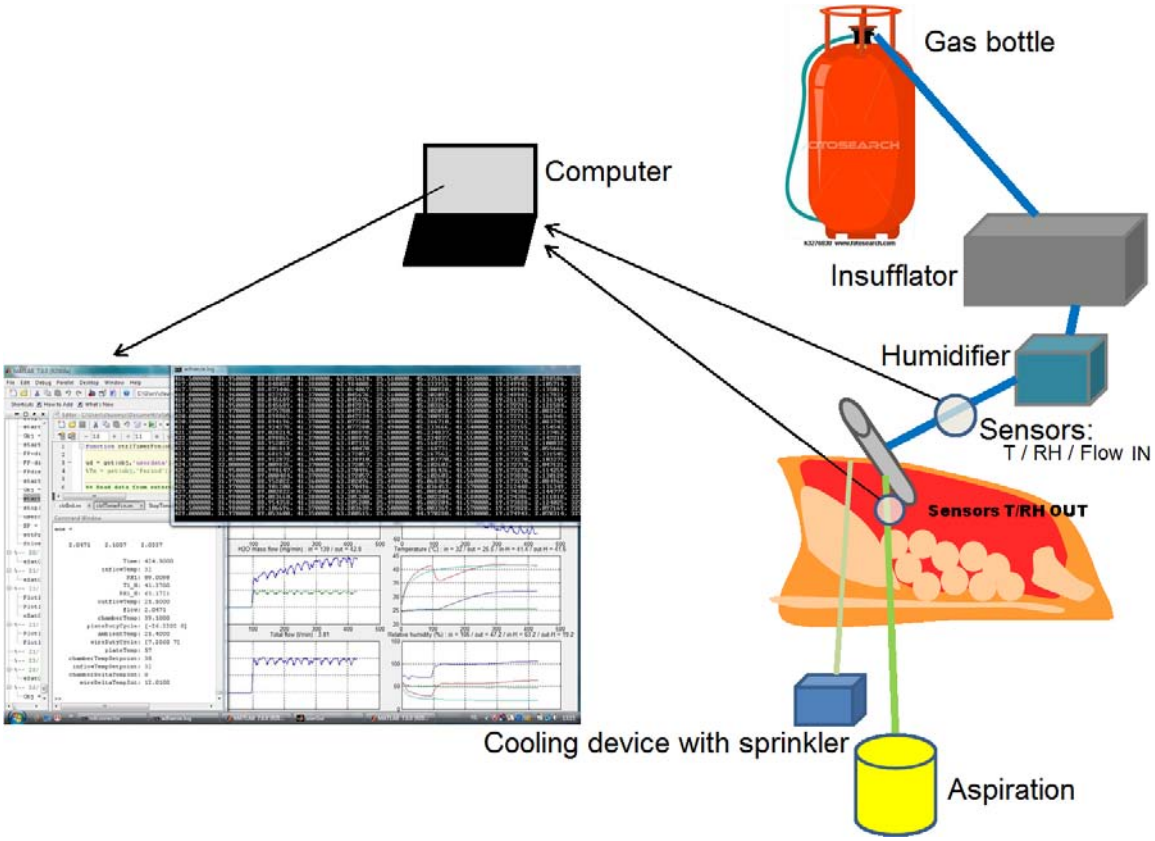


Figure 3: set-up for full conditioning

The set-up of the system was tested to ascertain the control of temperature and humidification. (Figure 3)

First in vitro experiments using a closed polystyrene box imitating a clinical setting by placing heated water at 37°C inside the box were carried out. From these experiments the setting for the steering of the humidification and heating in a clinical setting were determined.

Second we carried out an observational clinical trial with different flow rates (2.5, 5, 7.5, 10 and 15 liters per minute) and RH and temperature in and out were measured. (Figure 4)

These data permitted us to use the insufflator at different flow rates and to maintain 100% RH at insufflation with an inflow temperature set at 32°C. In vitro and in vivo data were similar.

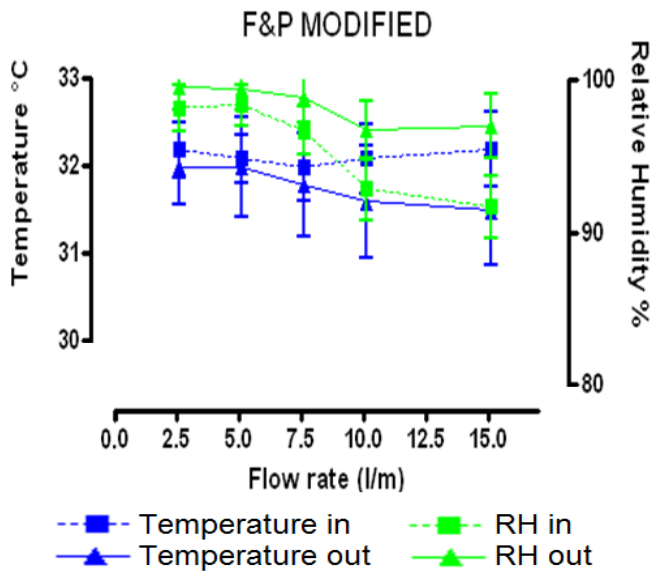


Figure 4: relative humidiy and temperature with different flow rates

The system could correct for any cooling inside the abdominal cavity as well as for heating by coagulation and maintain a 32°C inside the abdominal cavity. A sprinkler with normal saline was inserted into the abdominal cavity to correct for any loss of water and thus loss of energy by the body. (Figure 5)

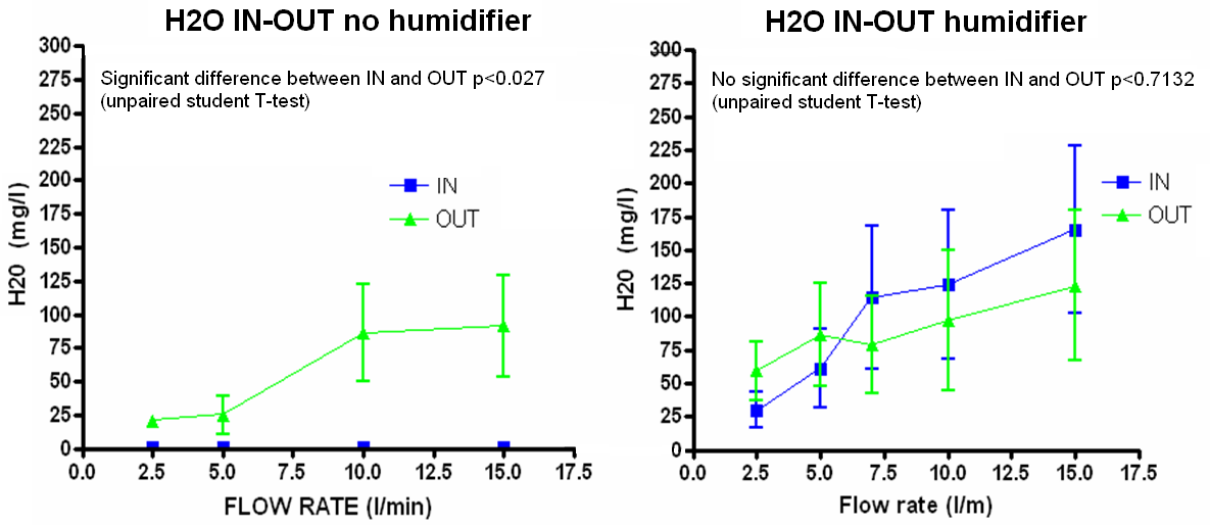


Figure 5: Loss of water without and with humidifier

The use of humidified, mixed (86% CO_2 + 4%O2 + 10% N_2O) gas at 32°C with the use of the sprinkler is referred to as "*full conditioning*".

An overview of the surgical procedures in the clinical trials using full conditioning is shown in table 1.

Type of laparoscopic surgery	CO_2 absorption	Pain - inflammation	Adhesions	Fluid absorption
Deep endometriosis	+	+	+	-
Total hysterectomy	+	+	-	-
Myomectomy	+	+	-	-
Colpopexia	+	+	-	-
Adhesiolysis	+	+	-	-
Subtotal hysterectomy	-	+	-	+

Table 1: overview of different trials using full conditioning

C. Statistics

Statistical evaluation was done with Prism or with the SAS system, using paired and unpaired t-tests for normally distributed populations or Wilcoxon, (proc n-par1way) for non normally distributed populations. Correlations were evaluable by the Spearman and or Pearson test. Two way analysis of variance was done with two way ANOVA or proc GLM, for normally and non normally distributed populations respectively.

For animal experiments, a factorial design was generally used, but also for the trial on fluid absorption. Indeed a strictly two x two factorial design (latin cross) generates almost similar significances and power for two variables with in addition interaction, for almost half the number of mice as would have been required when each factor would have been investigated separately.

Chapter 3

Results

A. Animal experiments

I. Establishing a learning curve

Corona R, Verguts J, Binda M, Molinas R, Schonman R, Koninckx P. The impact of the learning curve upon adhesion formation in a laparoscopic mouse model, Fertil Steril, accepted

AIM: to assess the effect of training assessed by consecutive surgeries, upon operating time and upon the extent and variability of post operative adhesion formation.

MATERIALS & METHODS: The operating time was measured from $T_{20,}$ i.e. from the beginning of surgery, exactly 20 minutes after induction of anesthesia, until the end of the procedure with the removal of catheters for instrumentation and port sites closure. Postoperative formation of adhesions was evaluated blindly by an independent experienced observer 7 days later. Moreover, the codes of intervention order were broken only at the end of the training experiment. The study was performed as a prospective randomized (for scoring) trial initially in Swiss mice and later in Balb/c mice. Each trainee performed sequentially 80 interventions to induce adhesion formations. All trainees were inexperienced for the laparoscopic procedure in a mouse model, but some of them were experienced gynecologists after their training in gynecology whereas others were inexperienced at the end of medical school.

All experiments were performed using block randomization by days. Therefore, a block of animals comprising one animal of each strain, one Balb/c and one Swiss mouse, were always operated in a single session the same day to avoid day-to-day variability. In addition, within a block, experiments were performed in random order. Before surgical procedure, initial temperature and weight were measured.

Statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software Inc., San Diego CA). Multifactorial analyses were achieved with GLM and Logistic procedures using the SAS System (SAS Institute, Cary, NC, USA).

RESULTS: Experienced (gynecologist) and inexperienced (undergraduate) surgeons had to operate each 80 mice (40 blocks of 1 Balb/c and 1 swiss mouse) before being permitted to do experiments. This was derived from clinical impression, showing that initially duration of surgery, adhesions and variability was much higher. With training, assessed by consecutive surgeries, duration of surgery decreased exponentially in both groups, demonstrating a clear learning curve that can be described as a two-phase exponential decay model as shown in figure 1. (non-experienced: R=0.72; experienced: R=0.73) (Figure 6). In Balb/c mice the real and calculated operating times for non-experienced surgeons decreased from 19 ± 6 min and 22 ± 4 min for the first procedures to 7 ± 1 min and 7 ± 1 min for the last procedures (P=0.0001; P=0.0001, respectively). For experienced surgeons time decreased from 8 ± 1 min and 10 ± 3 min for the first ten procedures to 4 ± 0.3 min and 4 ± 0.03 min for the last ten procedures.

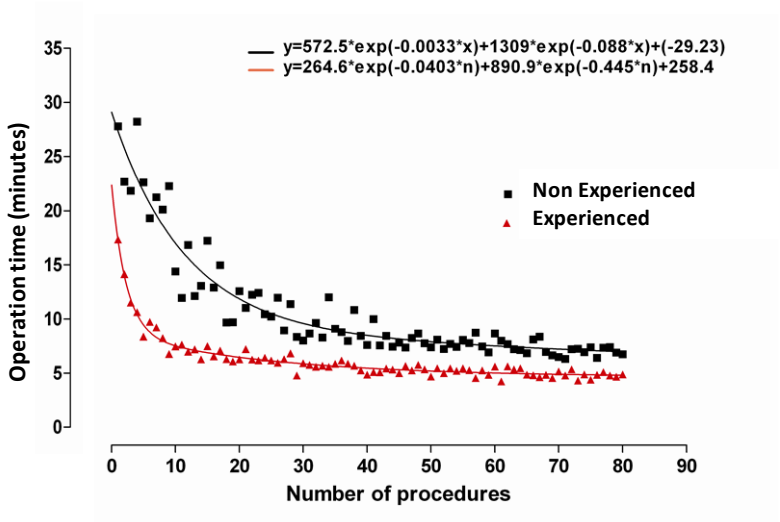


Figure 6: Duration of surgery during training: Learning curve expressed as duration of the surgery vs number of consecutive procedures

With experience also adhesion formation decreased (Figure 7) for both experienced and inexperienced surgeons. Adhesion formation was lower with the consecutive number of surgeries (proportion: $p<0.01$; total: $p<0.01$), for surgeries of shorter operating time

(proportion: p< 0.02; total: p< 0.04), and among experienced surgeons (proportion: p<0.0001; total: p< 0.04).

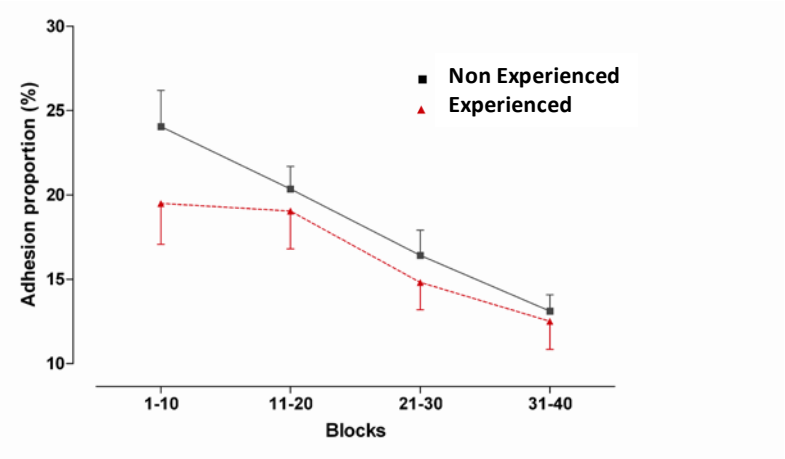


Figure 7: Adhesion formation during learning curve: Adhesion formation expressed as proportion vs number of consecutive blocks in both experienced and non experienced surgeons.

It was decided to arbitrarily group the blocks of animals in 4 groups of ten blocks each (G1, block 1-10; G2, block 11-20; G3, block 21-30; G4, block 31-40). With this grouping, the inter-animal variability for operating time and for adhesion formation was assessed by the coefficient of variation (CV). The CV of operating time among non-experienced surgeons was 41%, 35%, 25% and 20% for Balb/c mice for the first, second, third and fourth group, respectively; among experienced surgeons the CV was 45%, 24%, 20% and 22% for Balb/c mice for the first, second, third and fourth group. Taken all surgeons together the CV of the proportion of adhesions was 62%, 48%, 54% and 38% for Balb/c mice for the first, second, third and fourth group, respectively.

Both operating time and its CV decreased with the number of surgeries (p<0.0001 for both), whereas they were not affected by mouse strain (p=NS).

These results suggest that experience and thus decreased manipulation and operating time will decrease mesothelial damage as this damage may be inflicted by even minor manipulation of intra-abdominal organs.

II. Mesohelial cells in prevention of adhesions

Verguts J, Coosemans A, Corona R, Praet M, Mailova K and Koninckx P. Intraperitoneal injection of cultured mesothelial cells decrease CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. Gyn Surg. 2011(8)-addendum

AIM: Autologous transplantation of mesothelial cells after laparoscopy will decrease adhesion formation

MATERIALS & METHODS: Mesothelial cells were obtained from 9-16 week old inbred female Balb/c mice. Following anesthesia with intradermal pentobarbital (Nembutal, Sanofi Sante Animale, Brussels, Belgium; 0.06 mg/g) the abdomen was shaved, the animal was secured to the table in supine position and the skin disinfected with a 50% alcohol solution. A 14-gauge catheter (Insyte, Vialon, Becton Dickinson) was inserted into the abdomen at the umbilicus and fixed leak-proof with a Prolene 5/0 purse suture.

The cells were collected via a 2-step procedure per mouse. Firstly, 10ml phosphate buffered saline pH 7.3 (DPBS) was injected, of which at least 8 ml could be re-aspirated. This first set of 'free floating cells' was centrifuged at 200G for ten minutes and the pellet was then suspended in culture medium (RPMI 1640 (Lonza, BioWhittaker) supplemented with 15% fetal calf serum (FCS; CSL UK Ltd, Andover, UK), 4 mM L-glutamine (Gibco, Paisley, UK), 0.4 mg/ml hydrocortisone (Sigma Aldrich, Poole, UK) and antibiotics: penicillin, 120mg/l, amphothericine (2,5mg/l) and gentamicine (4mg/l) as used by Foley-Comer et al.85 Secondly, 10ml of DPBS with 0.25% Trypsin and 1 mM EDTA4Na at 37°C was injected through the same catheter and the mouse was softly shaken. After ten minutes the instilled fluid was re-aspirated. Next, the abdominal cavity was washed with 5ml culture medium; to finish, the abdominal cavity was opened and gently rinsed 8 times with 2 ml culture medium. All mesothelial cells obtained via this second procedure were collected together, centrifuged for 10 minutes at 200g and re-suspended in culture medium. Each time two mice had undergone the procedure, cells of those mice were pooled, keeping cells of step 1 and 2 separately. Cells were then put in an incubator at 37°C with 5% CO2. If cells were confluent, which was normally the case after 1 week, cells were split at a ratio 1:2, after having them

detached with Trypsin-EDTA. After 3 weeks, cells were detached and counted for further use (pilot: 400.000 cells, dose-response: 400.000 cells, 133.000 cells and 44.000 cells).

To ascertain the growth of mesothelial cells, cells from 3 weeks old cultures were seeded on slides. After 2 days of culture, slides were fixed and endogenous peroxidase activity was blocked by 0.5 % H_2O_2 in methanol. Cells were evaluated for intercellular connections and for cytokeratin staining.

All mice received 4 standard bipolar lesions and 60 minutes of CO_2 pneumoperitoneum as described above. After all laparoscopic ports had been closed, 1ml of DPBS with or without mesothelial cells, was injected intraperitoneally.

A first experiment was designed to prove that cultured mesothelial cells could prevent CO_2 pneumoperitoneum enhanced adhesions. Mesothelial cells were collected in 4 mice, and cultured in 8 flasks of 25cm². Two weeks later 2x106 cells were harvested. In order to investigate the effect in 5 mice 400.000 cells per mouse were used. The control group which only received DPBS consisted of 5 mice.

A second experiment was designed to obtain a dose response curve of adding different amounts of cultured mouse mesothelial cells in adhesion reduction. Considering the effect of 400.000 cells from the first experiment, and taking into account the number of cells available, we decided to inject per group of 5 mice 400.000 cells, 133.000 cells and 44.000 cells, i.e. a logarithmic decrease. The control group which only received DPBS consisted of 5 mice. The study was approved by the Institutional Review Animal Care Committee.

A third experiment was performed with 144.000 cells remaining from the second experiment to evaluate, as a pilot experiment, whether these cultured mesothelial cells adhered specifically to the lesion or randomly, over the entire peritoneum. The cultured mesothelial cells were suspended in methylene blue (10mg/ml glucose solution) for two minutes, centrifugated and re-suspended in 1ml of DPBS. The mesothelial cells were injected after a surgical lesion and 60 minutes of CO_2 pneumoperitoneum. Blue staining was evaluated after two days using a stereomicroscope.

In a fourth experiment, designed to evaluate whether methylene blue injected intraperitoneally did color the entire peritoneal layer or rather specifically the surgical lesion and the retracted areas between the mesothelial cells following 60 minutes of CO_2 pneumoperitoneum, 1ml of methylene blue was injected intraperitoneally either in a control

mouse without pneumoperitoneum, or in a mouse with a standard lesion and 60 minutes of CO2 pneumoperitoneum. Both mice were evaluated after 2 days using a stereomicroscope.

RESULTS: Mesothelial derived from the mice were easily cultured and were initially bipolar or multipolar in appearance but at confluence they adopted a polygonal configuration as described by Yung et al.[88] Viability of cells as estimated by tryptan blue exclusion was over 95%. The cells had intercellular connections and were positive for cytokeratin staining confirming their mesothelial nature.

Cells obtained from the first washing did not grow, but remained viable as estimated by tryptan blue exclusion after three weeks of culture. Their number, however, was too low for subsequent experiments.

In the first experiment the injection of 400.000 cultured mesothelial cells caused a reduction of the proportion of adhesion formation from 19% to 7% ($p<0.046$) and a reduction of the total adhesion score from 3.1 (+/- 1.74) to 1.7 (+/-1.68) (NS, $p<0.086$).

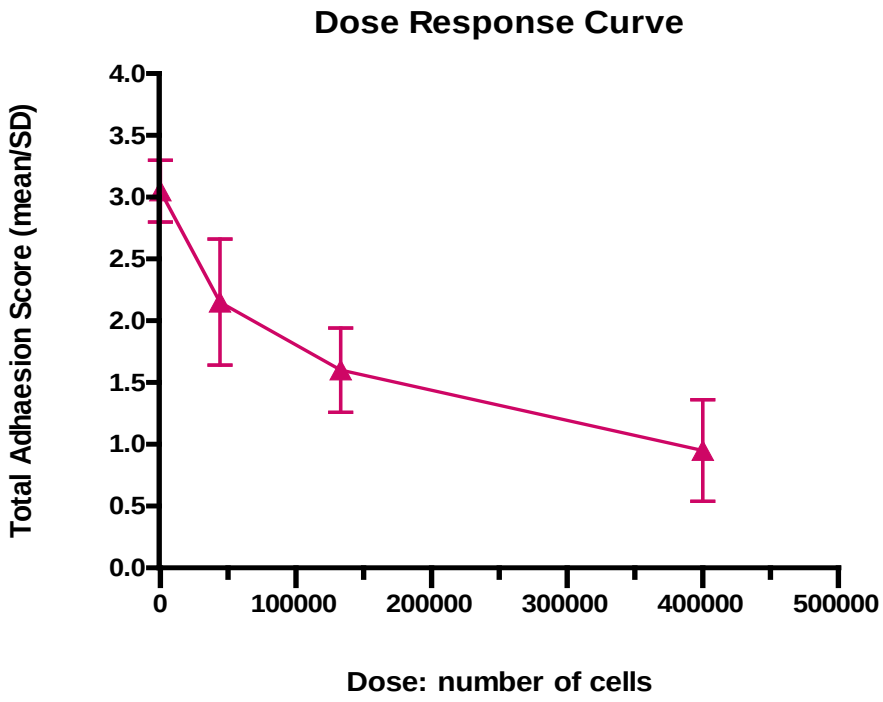
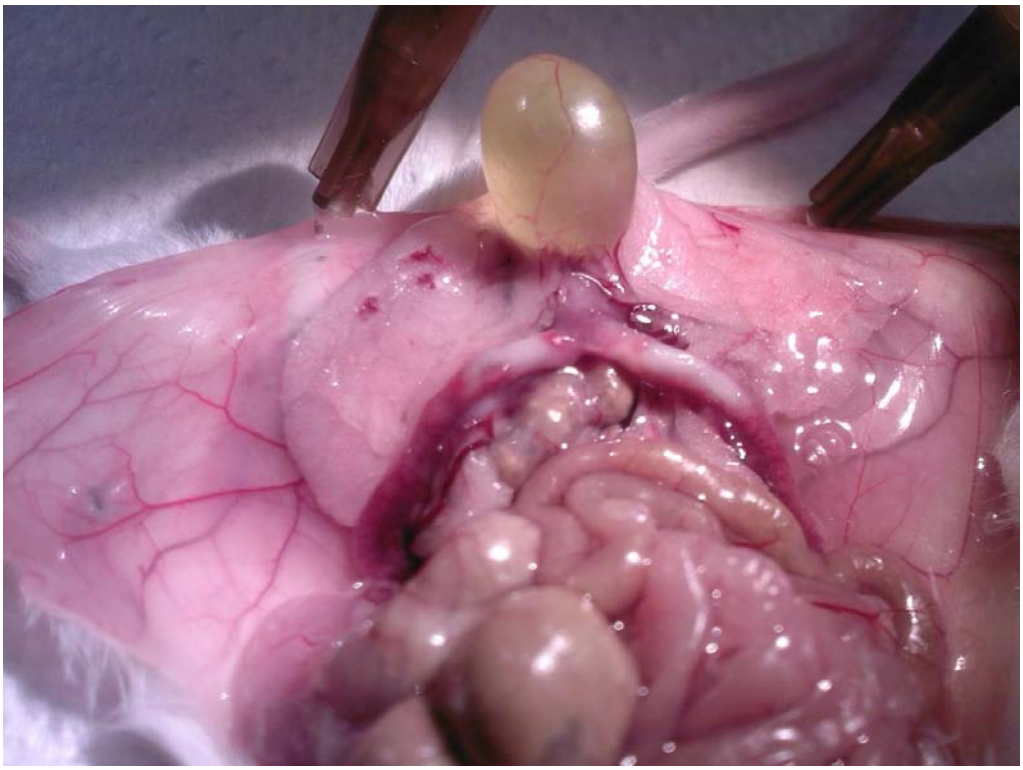


Figure 8: dose-response curve

The second experiment confirmed that mesothelial cells can reduce CO_2 pneumoperitoneum enhanced adhesion formation. With 44.000, 133.000 and 400.000 cells the proportion of adhesions dropped from 13.5% to 11.5% (NS), 8% ($P<0.055$) and 5% ($P<0.007$) respectively. The total adhesion score showed a similar decline being for the 44.000, 133.000 and 400.000 cells not significant, $P=0.0093$ and $P=0.0025$ respectively. (Figure 8) When adhesions are plotted against the logarithm of the concentration of cells, the amount of cells required to decrease adhesion formation by half was estimated at 100.000 cells.

Following injection of methylene blue stained mesothelial cells, after a standard lesion and 60 min of CO_2 pneumoperitoneum, blue spots were visualised on the lesion site (picture 1) while faint blue spots were found over the entire parietal peritoneum but not on the bowel. In the control mouse however, without surgery nor pneumoperitoneum, no blue staining was seen after injection of methylene blue stained mesothelial cells.



Picture 1: Following injection of methylene blue only a similar blue staining was found on the surgical lesion and all over the parietal peritoneum after 60 minutes of pneumoperitoneum

III. Tumor implantation

Verguts J, Corona R, Binda M, Koninckx P. Adding 3% of oxygen to the CO_2 pneumoperitoneum reduces tumor implantation in a laparoscopic mouse model. Clin Exp Metastasis, submitted, 2011

AIM: Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and will decrease tumor growth

MATERIALS & METHODS: Mouse CT26 (colon adenocarcinoma) and RENCA (renal adenocarcinoma) cell lines, syngeneic to Balb/c mice, were generously provided by Dr. Markus Steinbauer from the University of Regensburg, Regensburg, Germany and Dr. Takumi Takeuchi from the University of Tokyo, Japan respectively. Before use, cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine at 37°C in a humidified environment with 5% CO_2. Cells were harvested, suspended in culture medium and rinsed by centrifugation at 3000 RPM (1509g) for 10 minutes. Cell viability was assessed by the tryptan Blue exclusion test (which always exceeded 95%) and counted in a Neubauer counting chamber. The final concentration was diluted using culture medium to the desired concentration for each experiment. Cells were injected intraperitoneally (i.p.) in 1.0 ml culture medium immediately after deflation of pneumoperitoneum and closure of ports.

Animals were sacrificed by cervical dislocation. The abdominal cavity was exposed using a xyphopubic midline incision and examined for tumor nodules under stereomicroscopic vision x10. Nodules of more than 1 mm present in the abdominal wall and mesentery were scored blindly by two independent investigators, the codes of groups and inoculations being broken only at the end of the entire experiment.

All experiments were performed using block randomization by day. Therefore, a block of animals comprising one animal of each group was always operated the same day, avoiding day-to-day variability. Within a block, experiments were performed in random order, varying each day. Laparoscopy was performed as described earlier (cfr supra), but no lesions on the uterus or abdominal wall were made in this experiment.

Dose-response curve experiments with CT26 ("*experiment A"*) and RENCA cells ("*experiment B"*).

These experiments were designed to estimate the optimal amounts of cells to be used in further experiments by injecting increasing amount of tumor cells intraperitoneally. Animals were anaesthetized and a variable amount of cells, suspended in 1 ml of medium, were inoculated i.p. 2 cm caudal to the xyphoid. In experiment A and B, 63 mice (n=9 in each group, seven groups) were injected with 10^3, $3x10^3$, 10^4, $3x10^4$, 10^5, $3x10^5$ or 10^6 CT26 or Renca cells (Groups: G_1, G_2, G_3, G_4, G_5, G_6 and G_7 respectively). From each group, 3 mice were examined for tumor growth after respectively 7, 14 and 21 days.

Experiments to evaluate the effect of pneumoperitoneum and of adding 3% of oxygen to the pneumoperitoneum using CT26 and RENCA cell lines ("*experiment C*" and "*experiment D*") upon tumor implantation.

The mice of the control group (CG) were anaesthetized and ventilated only, whereas in mice of Group 1 (G_1) and Group 2 (G_2) a pneumoperitoneum was maintained for 60 minutes with CO_2 + 3% O_2 and with pure CO_2 respectively.

In experiment C, 20 mice in each of the 3 experimental groups (CG, G_1 and G_2) (n=60) were injected with 10^3, $3x10^4$, 10^5 or 10^6 CT26 cells (5 mice/concentration/experimental group). Mice inoculated with 10^5 and 10^6 cells were sacrificed and examined after 7 days and mice inoculated with $3x10^3$ and $3x10^4$ cells were sacrificed and examined after 14 days.

In experiment D, 15 mice in each of the 3 experimental groups (CG, G_1 and G_2) (n=45) were injected with $3x10^4$ and 10^5 cells (5 mice/$3x10^4$ cell concentration/experimental group; and 10 mice/10^5 cell concentration/experimental group). Mice inoculated with $3x10^4$ cells were sacrificed and examined after 14 days whereas mice inoculated with 10^5 cells were sacrificed and examined after 14 (n=15) and 21 days (n=15) respectively.

Experiments were performed in blocks. Each block comprised the three experimental groups (CG, G_1 and G_2) with the 4 (CT26) or 3 (RENCA) cell concentrations i.e. 1 mouse/experimental group/cell concentration. Cells were inoculated i.p. 2 cm caudal to the xyphoid immediately after deflation and closure of port sites.

RESULTS: **Experiments A and B**: Following inoculation of exponentially increasing concentrations of CT26 or RENCA cells, tumor implantation occurred mainly in the mesentery, pancreas, subhepatic area, bladder, ovarian fat and abdominal wall. For both cell

types, the number of visible tumor nodules increased linear with increasing number of inoculated cells (for CT26 cells after 7, 14 and 21 days: R2= 0.9283, R2= 0.8679 and R2= 0.4316 respectively; for RENCA cells after 14 and 21 days: R2 = 0.9795 and R2 = 0.9910 respectively). After 21 days, all animals with more than 10^4 injected cells had died. In mice inoculated with RENCA cells and examined after 7 days, no tumor nodules were found, whereas after 21 days tumor nodules were evident within mice injected with 10^3 cells. (Figure 9)

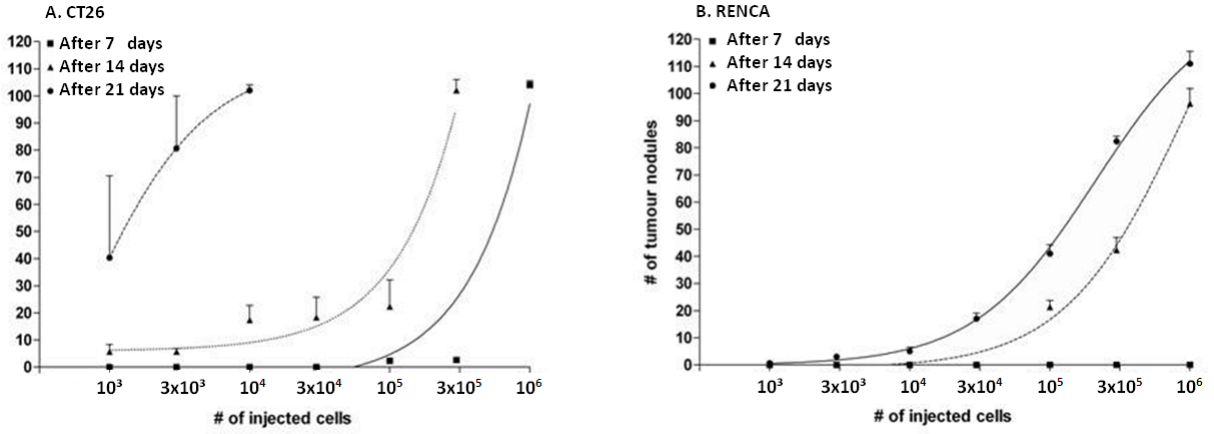


Figure 9: Dose response curves for CT26 and RENCA

The size of tumor nodules increased with time and with the number of cells injected. **Experiments C**. Effect of 60 min of pneumoperitoneum upon CT26 cells implantation. In comparison with the control group, tumor implantation increased after one hour of pure CO_2 pneumoperitoneum for all doses used. This increase was found for total number of tumor nodules (Figure 10) and for number of nodules in mesentery and abdominal wall (Table 2) ($3x10^3$: Mesentery: P= .04, Abdominal wall: P= .04, Total: P= .04; 10^5: Mesentery: P= .03, Abdominal wall: P= .03, Total: P= .03 and 10^6: Total: P= NS). In comparison with pure CO_2 pneumoperitoneum, tumor implantation decreased with the addition of 3% of oxygen to the CO_2 pneumoperitoneum for all doses used. This decrease was found for total number of tumor nodules and for number of nodules in mesentery and abdominal wall (Table 2) ($3x10^3$: Mesentery: P= NS, Abdominal wall: P= NS, Total: P= .05; 10^5: Mesentery: P= .NS, Abdominal wall: P= .NS, Total: P= .04 and 10^6: Mesentery: P= NS, Abdominal wall: P= NS, Total: P= NS). In comparison with the control group, tumor implantation increased after one hour of CO_2 pneumoperitoneum and 3% of oxygen for all doses used. This increase was

found for total number of tumor nodules (Figure 10) and for number of nodules in mesentery and abdominal wall (Table 2) ($3x10^3$: Mesentery: P= .04; Abdominal wall: P= .04; Total: P= .04; 10^5: Mesentery: P= .04, Abdominal wall: P= .04, Total: P= .04 and 106: Mesentery: P= NS, Abdominal wall: P= NS, Total: P= NS).

	# of inoculated cells	Groups	Abdominal wall	Mesentery	Total
CT 26 cell line	3×10^3	Control	1.2 ± 0.5	6.6 ± 2.4	7.8 ± 2.7
		CO_2 + 3% O_2	16.5 ± 2.1 [b]	38 ± 4 [b]	54.5 ± 5.6 [b]
		CO_2	24.25 ± 4.2 [b]	63 ± 5.3 [b]	87.25 ± 7.9 [a, b]
	10^5	Control	1.8 ± 0.5	6.8 ± 0.8	8.6 ± 1.2
		CO_2 + 3% O_2	20.75 ± 2.6 [b]	33 ± 1.8 [b]	53.75 ± 4.3 [b]
		CO_2	33 ± 4 [b]	53.6 ± 6.9 [b]	86.6 ± 6.7 [a, b]
	10^6	Control	7 ± 1	14.25 ± 1.8	21.25 ± 2.7
		CO_2 + 3% O_2	51.5 ± 8.7	83 ± 5.8	134.5 ± 12.6
		CO_2	56.25 ± 4.3	88.75 ± 5.1	145 ± 9.1
RENCA cell line	3×10^4	Control	0.2 ± 0.2	0.4 ± 0.2	0.6 ± 0.4
		CO_2 + 3% O_2	4.2 ± 0.8 [b]	4 ± 0.7 [b]	8.2 ± 1 [b]
		CO_2	37 ± 5.5 [a, b]	42.6 ± 4.8 [a, b]	79.6 ± 6.9 [a, b]
	10^5	Control	5 ± 1.7	5.2 ± 1.7	10.2 ± 2.6
		CO_2 + 3% O_2	11.2 ± 0.8	15.8 ± 2.4 [b]	27 ± 2.7 [b]
		CO_2	45.4 ± 5	58.2 ± 6.9 [a, b]	103.6 ± 11.2 [a, b]
	10^6	Control	2.4 ± 0.8	40.2 ± 2.6	43.2 ± 3.3
		CO_2 + 3% O_2	11.4 ± 1.6 [b]	41.2 ± 2.8	52.6 ± 3.4
		CO_2	58 ± 9.1 [a, b]	113.6 ± 21.6 [a, b]	183.6 ± 15.2 [a, b]

Table 2: nodules per type of tumor and per site, [a] P vs. Group + 3%oxygen <.05; [b] P vs. Control <.05.

In the group injected with $3x10^3$ cells, one mouse in G1 and G2 died 1 day before the examination. The group injected with $3x10^4$ cells had a high mortality rate which didn't allow any difference to appear, a total of 11 animals died after 12 days, 2 animals in CG, 4 animals in G1 and 5 animals in G2. In the group injected with 10^5 cells one animal from G1 died 1 day before the examination.

In the group injected with 10^6 cells, three animals died, two animals before the examination from CG and G1 respectively and one animal two days before the examination from G2.

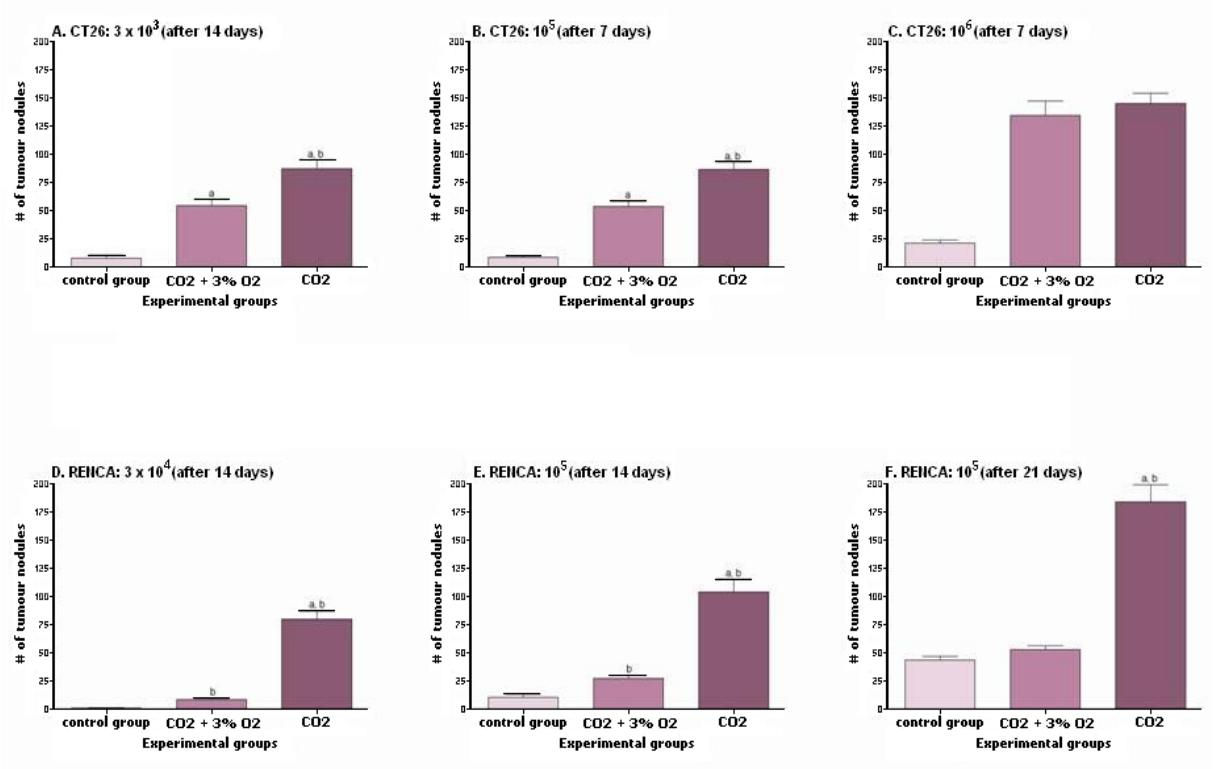


Figure 10: effect of pneumoperitoneum upon tumorimplantation and with addition of 3% oxygen

Experiment D. Effect of 60 min of pneumoperitoneum upon RENCA cells implantation.

In comparison with control group, tumor implantation increased after one hour of pure CO_2 pneumoperitoneum for all doses used. This increase was found for total number of tumor nodules (Figure 10) and for number of nodules in mesentery and abdominal wall (Table 2) ($3x10^4$: Mesentery: P= .03, Abdominal wall: P= .02, Total: P= .03; 10^5 evaluated after 14 days: Mesentery: P= .03, Abdominal wall: P= .03, Total: P= .03 and 10^5 evaluated after 21 days: Mesentery: P= .03, Abdominal wall: P= .03, Total: P= .03). In comparison with pure CO_2 pneumoperitoneum, tumor implantation decreased with the addition of 3% of oxygen to the CO_2 pneumoperitoneum for all doses used. This decrease was found for total number of tumor nodules and for number of nodules in mesentery and abdominal wall ($3x10^4$: Mesentery: P= .03, Abdominal wall: P= .03, Total: P= .03; 10^5 evaluated after 14 days: Mesentery: P= .03, Abdominal wall: P= .03, Total: P= .03 and 10^5 evaluated after 21 days: Mesentery: P= .03, Abdominal wall: P= .03, Total: P= .03). In comparison with control group, tumor implantation increased after one hour of CO_2 and 3% of oxygen for all doses used. This increase was found for total number of tumor nodules and for number of nodules in mesentery and abdominal wall (Table 2) ($3x10^4$: Mesentery: P= .03, Abdominal wall: P= .02,

Total: P= .03; 10^5 evaluated after 14 days: Mesentery: P= .03, Abdominal wall: P= NS, Total: P= .03 and 10^5 evaluated after 21 days: Mesentery: P= NS, Abdominal wall: P= .03, Total: P= NS).

IV. Nitrous oxide

Mailova K, Corona R, Verguts J, Adamian L,Koninckx P. The addition of N_2O to the CO_2 pneumoperitoneum reduces adhesion formation in a laparoscopic mouse model. Manuscript prepared

AIM: To investigate the effect of different concentrations of nitrous oxide pneumoperitoneum on adhesion formation in a laparoscopic mouse model.

MATERIALS & METHODS: As was stated before (*cfr. supra*) N_2O may have a beneficial effect on the mesothelial barrier and thus on adhesion formation. The exact concentration to obtain these beneficial effects was never established.

All mice in this experiment received 4 standard bipolar lesions and one hour of pneumoperitoneum as described above. Pneumoperitoneum with variable concentrations of N_2O was established by two insufflators (Thermoflator Plus, Karl Storz, Tüttlingen, Germany): one for insufflation of CO_2 and one for insufflation of N_2O. To obtain a homogenous mixture, the output of both insufflators was mixed in a mixing chamber which was connected to a water valve with free escape of gas for a continuous flow and insufflation pressure. The insufflation pressure for both insufflators was set at 16 mm Hg. Mixture with 5% of nitrous oxide in carbon dioxide was achieved by 9,5 l/min of CO_2 and 0,5 l/min N_2O, similarly 10% of N_2O by 4,5 l/min CO_2 and 0,5 l/min N_2O, 25% of N_2O by 3,0 l/min CO_2 and 1,0 l/min N_2O, 50% of N_2O by 1,0 l/min CO_2 and 1,0 l/min N_2O.

Experiment I (n=18) was a designed as an exploratory pilot experiment evaluating 100% CO_2 (group I; n=9) to 100% N_2O (group II; n=9) upon adhesion formation. Two animals died in group II during the procedure because of intubation problems.

Experiment II (n=30) was as a dose finding study, to establish the lowest effective concentration of N_2O upon prevention of adhesion formation. Six groups (n = 5 in each group) were used with 100, 50, 25, 10, 5 and 0% N_2O.

Statistical analyses were performed with the SAS software (SAS System, SAS Institute, Cary, NC) using the non-parametric Kruskal-Wallis.

RESULTS: Experiment I demonstrated that 100% N_2O for the pneumoperitoneum instead of CO_2 decreased adhesion formation, both the quantitative score ($p<0,012$), and the qualitative score. Total adhesion score, type, tenacity and extent decreased from 3,0±0,3 to 1,6±0,4 ($p<0,009$), from 10,8 to 5,5 ($p<0,023$), from 10,8 to 5,6 ($p<0,025$) and from 11,3 to 4,9 ($p<0,005$) respectively.

Experiment II confirmed the effect of 100% N_2O on adhesions while surprisingly the effect persisted until as little as 5% of N_2O was used. Indeed the proportion of adhesion formation decreased (Figure 11) already with addition of 5% of N_2O to the CO_2 pneumoperitoneum (P=0,0138). The same decrease was observed for the total adhesion scores being 2,6 ±1,4; 0,7 ±0,9; 1,0±0,3; 1,8±0,8; 0,5±0,7; 0,4±0,9 for groups VI to I respectively. Significances in comparison with pure CO_2 were P=0,0339, P=0,0259, NS, P=0,0150 and P=0,0236. Proportion scores were significantly lower in groups I, II, IV and V compared to group VI where pure CO_2 was used for insufflation, being significant (P=0,0634), (P=0,0138), (P=0,0245), (P=0,0138) respectively.

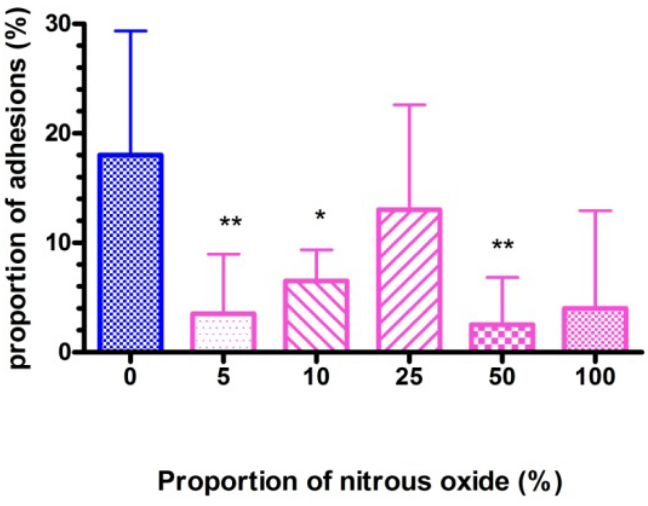


Figure 11: Effect of the addition of different proportions of nitrous oxide to CO_2 pneumoperitoneum on adhesion formation, scored 7 days after surgery. Significances in comparison with pure CO_2 are indicated: * P<0.05, ** P=0.01

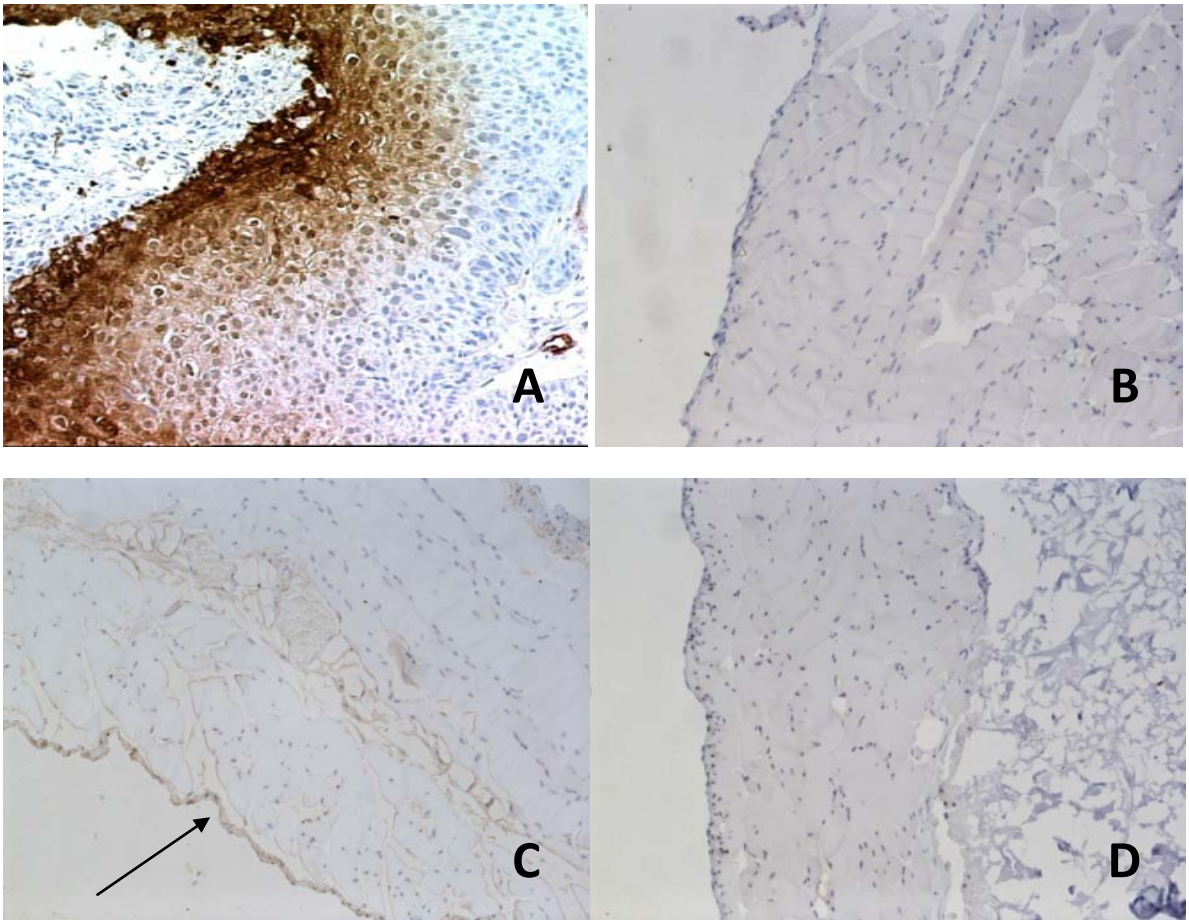
V. Depth of Hypoxia

Verguts J,Binda M, Corona R, Koninckx P. Manuscript in preparation

Aim: to investigate the depth of hypoxia caused by the pneumoperitoneum with pure CO_2 and after addition of 3% of oxygen to the pneumoperitoneum

MATERIALS & METHODS: The depth of the hypoxia caused by the CO_2 pneumoperitoneum was measured by the Hypoxyprobe™-1 immunoperoxidase staining for hypoxia. Hypoxyprobe™ kits consist of two parts. The first part is a substituted 2-nitroimidazole whose chemical name and only ingredient is 1-[(2-hydroxy-3-piperdinyl)propyl]-2-nitroimidazole hydrochloride which was injected in the peritoneum before surgery. The second part contains a primary antibody reagent that recognizes Hypoxyprobe adducts in hypoxic cells for which it is stained after fixation. Biopsies from the peritoneum were formalin-fixed, paraffin-embedded and a peroxidase immunohistochemistry technique was used for staining.
Three situations were examined: control without laparoscopy, laparoscopy with 60 minutes of carbon dioxide pneumoperitoneum and laparoscopy with 60 minutes of carbon dioxide with 3% oxygen. Samples were taken from the peritoneum and stained with Hypoxyprobe™-1 immunoperoxidase. Slides were evaluated for hypoxia in the mesothelial and submesothelial layer. Hypoxic cells should stain brown for hypoxia. (Picture 2)

RESULTS: The mesothelial layer clearly stained for hypoxia after 60 minutes of pneumoperitoneum with CO_2. (Picture 2, arrow) There was no staining in the control without laparoscopy or when 3% of oxygen was added to the CO_2 pneumoperitoneum. (Picture 2) These data confirm that pure CO_2 creates mesothelial hypoxia as concluded from previous experiments on adhesion formation. These experiments furthermore, demonstrate that the depth of hypoxia is limited and confined to the mesothelial layer.



Picture 2: **A**. Hypoxia control example from Hypoxyprobe™-1: hypoxic cells are colored brown (peroxidase staining), **B**. Control without laparoscopy, **C**. Staining after 60 minutes of CO_2 pneumoperitoneum, **D**. Staining after 60 minutes of CO_2 pneumoperitoneum + 3% oxygen

B. Clinical studies

I. Adding 4% oxygen to the pneumoperitoneum

a. CO_2 absorption

Verguts J, Corona R, Vanacker B, Binda M, Koninckx P. Adding 4% of oxygen to the CO_2 pneumoperitoneum decreases CO_2 absorption. Manuscript prepared

AIM: Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and will decrease CO_2 absorption.

MATERIALS & METHODS: In this prospective randomized trial using pure CO_2 or CO_2 + 4% of oxygen we included twenty women undergoing laparoscopy for at least one hour, N=10 for each subgroup. Laparoscopy was standardized by adjusting the insufflation pressure exactly to 15 mm of Hg. A Trendelenburg position of 30° was maintained. If during surgery more Trendelenburg or a higher insufflation pressure would be required, the patient would not be used in the analysis.
Endpoints of the trial were heart rate, blood pressure, ventilation frequency, stroke volume and end tidal CO_2 and they were recorded every minute for the duration of the experiment. Arterial $PaCO_2$ was measured at the start and after 60 minutes of surgery. The area of surgical deperitonealization was clinically estimated every 10 minutes.
Following induction of anesthesia, a pneumoperitoneum was created and parameters (i.e. end tidal CO_2, blood pressure or heart rate) were recorded in 20 women during laparoscopy for at least 60 minutes.

RESULTS: Regarding systolic blood pressure and heart rate there were no significant differences between both groups. The initial raise in systolic blood pressure was approximately 30% and was comparable for both groups. (Figure 12) Also heart rate did not alter between the two groups.

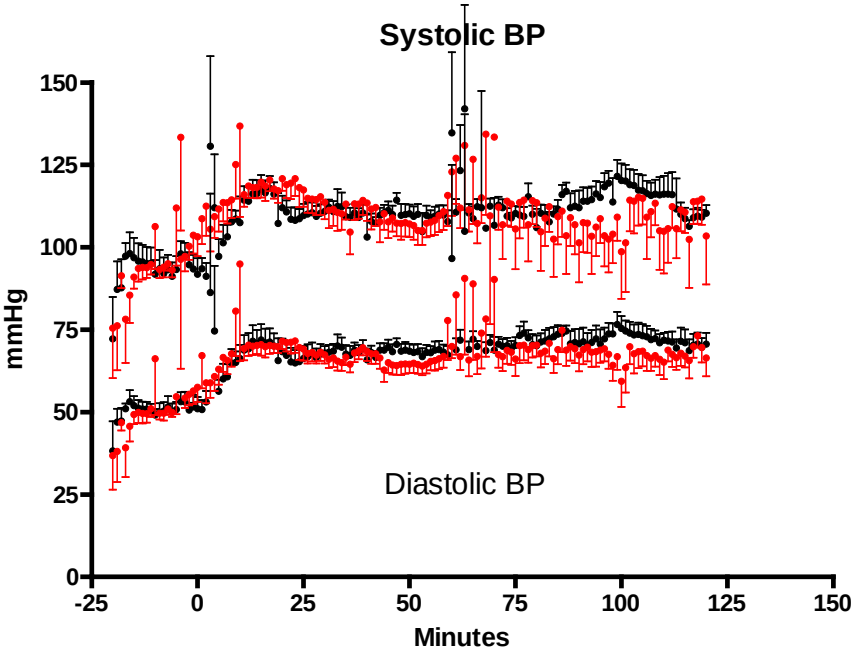


Figure 12: Changes in systolic and diastolic blood pressure during laparoscopy with CO_2 pneumoperitoneum (black) or with PP + 4% oxygen (red) after creation of the peritoneum (time=0)

Arterial oxygenation at the beginning of surgery or after 60 minutes of pneumoperitoneum did not show a difference between the two groups, indicating an optimal anaesthesiologic correction during laparoscopy. (Data not shown)

End tidal CO_2 (ET CO_2) had a comparable increase in the two groups of about 20% during the first 15 minutes of laparoscopy. (Figure 13) In the control group where 100% CO_2 was used, a further increase in ET CO_2 was seen. In the group where 4% of oxygen was added to the CO_2 pneumoperitoneum we noted a significant ($p<0.001$) decrease in ET CO_2 during the rest of the procedure. The two groups showed a remarkable divergence in ET CO_2 starting only 15 minutes after creation of the pneumoperitoneum.

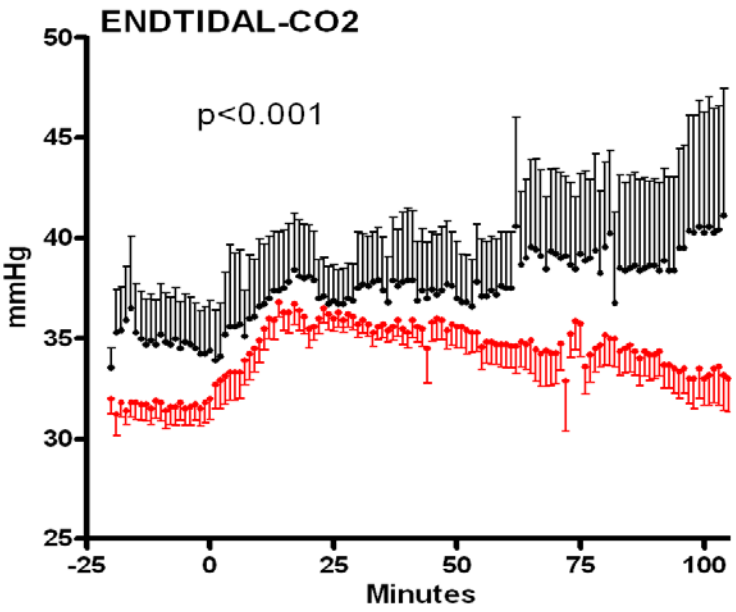


Figure 13: End tidal CO_2 during laparoscopy with CO_2 pneumoperitoneum (black) or with PP + 4% oxygen (red) after creation of the peritoneum (time=0)

Minute volume had a comparable increase of about 25% during the first 15 minutes of laparoscopy. (Figure 14) After 15 minutes of laparoscopy we also noted a significant divergence of the minute volume in favor of the group where 4% of oxygen was added to the CO_2 pneumoperitoneum (p<0.0001). After 60' the groups were no longer comparable as the number of women still having a pneumoperitoneum was low.

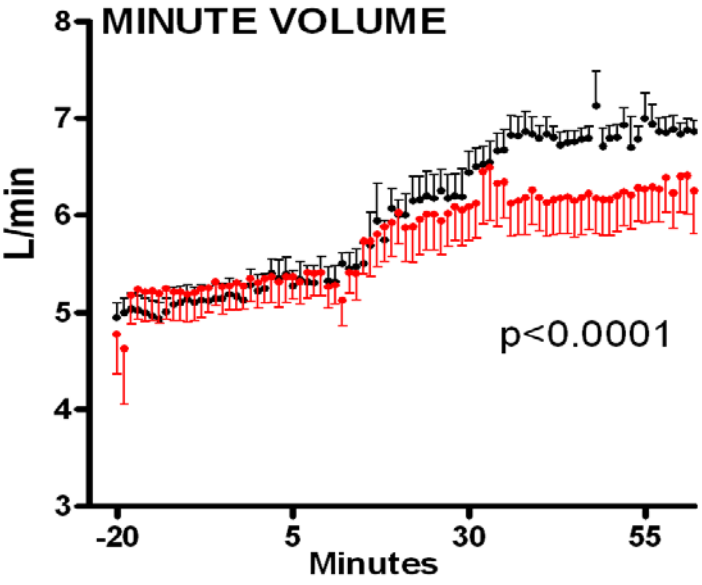


Figure 14: Minute volume measured during laparoscopy with CO_2 pneumoperitoneum (black) or with PP + 4% oxygen (red) after creation of the peritoneum (time=0)

Conclusion: The addition of 4% of oxygen to the CO_2 pneumoperitoneum decreases the progressive increase in CO_2 resorption during laparoscopic surgery.

b. Pain and inflammation

Verguts J, Corona R, Vanacker B, Binda M, Deprest J, Koninckx P. Adding 4% of oxygen to the CO_2 pneumoperitoneum decreases pain and inflammation in the post-operative phase after laparoscopic sacrocolpopexia. Manuscript prepared

AIM: Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and decrease post-operative inflammation and pain.

MATERIALS & METHODS: All patients undergoing a laparoscopic colpopexia from May 2008 till November 2009 at least 18 years of age were eligible for this trial. In order not to have any interference with other inflammatory conditions or pre-existing pain, women would be excluded as stated above or in case of chronic pain (i.e. peripheral neuropathy, pathology of the vertebral column, osteo-articular disease) or acute pain (i.e. trauma).
This was a randomized, double-blind, controlled study. Randomization 1:1 into one of the two groups (CO_2 or CO_2 + 4% oxygen) was based on block randomization using a sealed envelope system of 6 envelopes per block. The envelopes were kept sealed until induction of anesthesia. The envelope was opened and the appropriate gas was used for the laparoscopy. The details of the study were kept blinded to the investigator that collected the post-operative data. Patients were discharged as soon as they were adequately ingesting orally and were mobile.
All operations were carried out by the same team of surgeons. Laparoscopy was standardized by adjusting the insufflation pressure exactly to 15 mm of Hg in all patients. A Trendelenburg position of 30° was maintained. If during surgery more Trendelenburg or a higher insufflation pressure would be required, the patient would not be used in further analysis.
Promontofixation started by dissecting the peritoneum of the promontory, paracolic gutter up to the vaginal vault and the posterior vaginal vault up to the level of the levator ani

muscle. A polypropylene mesh was sutured anterior and posterior to the vault by PDS 0 sutures (Ethicon, Inc.) and fixed at the level of the promontory by stapling. The peritoneum was closed using a monocryl 3/0 (Ethicon, Inc.) running suture.

Anesthetic technique: An intravenous (IV) cannula was inserted into a vein of the forearm for the administration of anesthesiologic drugs and fluids. Standard monitoring consisted of electrocardiogram, non-invasive blood pressure measurements, and pulse oximetry, as well as end tidal CO_2 and sevoflurane (%) measurements.

Anesthesia was induced with an IV opioid (choice was left to the discretion of the investigator) and IV propofol, and maintained using sevoflurane at 1–2 minimum alveolar concentration (MAC) end tidal and opioids, according to the patient's need. After induction of anesthesia, a neuromuscular blocking agent was administered to facilitate orotracheal intubation. During 2-3 minutes patients were ventilated through a face mask with oxygen/air at normocapnia and than intubated; a nasogastric tube was inserted. Neuromuscular block was monitored using a TOF-watch (Organon-Oss, The Netherlands). Prophylactic post-operative nausea and vomiting medication was provided.

Central body temperature was maintained at ≥35ºC (Bair Hugger). Heart rate and noninvasive blood pressure measurements were performed continuously and recorded.

Post-anesthetic heart rate, arterial blood pressure, oxygen saturation and respiratory rate were monitored as part of clinical routine in the recovery room.

Peri-operative analgesia: The local analgesic protocol as designed by the department of anesthesiology (J. De Coster MD and E. Vandermeulen MD, 2006) was used for all patients.

1/ all patients will be loaded in the operating room with

Perfusalgan® (max. 2000mg):	30	mg/kg IV
Taradyl®:	0.5	mg/kg IV
Dipidolor®:	0.05-0.1	mg/kg IV

2/ in the PAZA therapy is continued

Perfusalgan® (max. 1000mg):	15	mg/kg IV / 6 h.
Taradyl®:	0.25	mg/kg IV / 6h
Dipidolor®:		
>6y:	0.2-0.3	mg/kg IM
>80y:	0.15-0.2	mg/kg IM

Limitation in the use of Perfusalgan®, Taradyl® or Dipidolor® as described by the manufacturer.

For each patient, age, body mass index (BMI) and duration of surgery (from incision to closure of the laparoscopic ports) were recorded. To measure pain intensity, the Visual Analogue Score (0 – 100 mm scale) pain score was measured by a blinded investigator at 4, 24, 48 and 72 hours after surgery to asses pain intensity in the shoulder, trocar wound, subcostal and visceral pain. VAS pain scores of 30 mm or less are categorized as mild, scores from 31-69 mm as moderate and scores of 70 mm or more as severe pain.

A blood sample was taken the day before surgery and on day 1, 2, 3 and 4 after surgery as is standard in our centre for patients undergoing a laparoscopic colpopexia. C-reactive protein (CRP), white blood cell count (WBC) always expressed as 10^9/L and CA-125 always expressed as kU/L was evaluated.

Statistics: Statistical analysis was performed with the SAS System (SAS institute, Cary, NC) using the nonparametric Kruskal-Wallis test to compare individual groups. All data are presented as the mean ± SE.

RESULTS: Patients (n=24) were randomized to pneumoperitoneum with carbon dioxide or to pneumoperitoneum with carbon dioxide + 4% oxygen.

Groups were comparable for age, BMI, laparoscopic operating time and pre-operative scores for pain, CRP, WBC's and CA 125. (Table 3)

Variable (mean +/- SD)	**Oxygen-group**	**Control-group**
Age (years) (mean +/- SD)	63.4 +/- 13.4	66.4 +/- 14.0
BMI (mean +/- SD)	23.9 +/- 2.5	25.9 +/- 2.9
Operating time (minutes) (mean +/- SD)	170 +/- 35	200 +/- 50
General pain score (VAS) (mean +/- SD)	0.09 +/- 0.3	0 +/- 0
CRP (mg/L) (mean +/- SD)	2.9 +/-2.7	2.8 +/- 3.5
WBC (10E9 /L) (mean +/- SD)	6.8 +/- 1.6	6.2 +/- 1.3
CA 125 (kU/L) (mean +/- SD)	10 +/- 5	15 +/- 13

Table 3: comparison of women having laparoscopy with CO_2 pneumoperitoneum (control group) or with PP + 4% oxygen (oxygen group)

Pain-scores in the groups where oxygen was added to the CO_2 pneumoperitoneum was significantly lower ($p<0.03$) compared to the control group on the day of surgery (day 0). (Figure 15) The first day after surgery the difference was not statistically significant anymore.

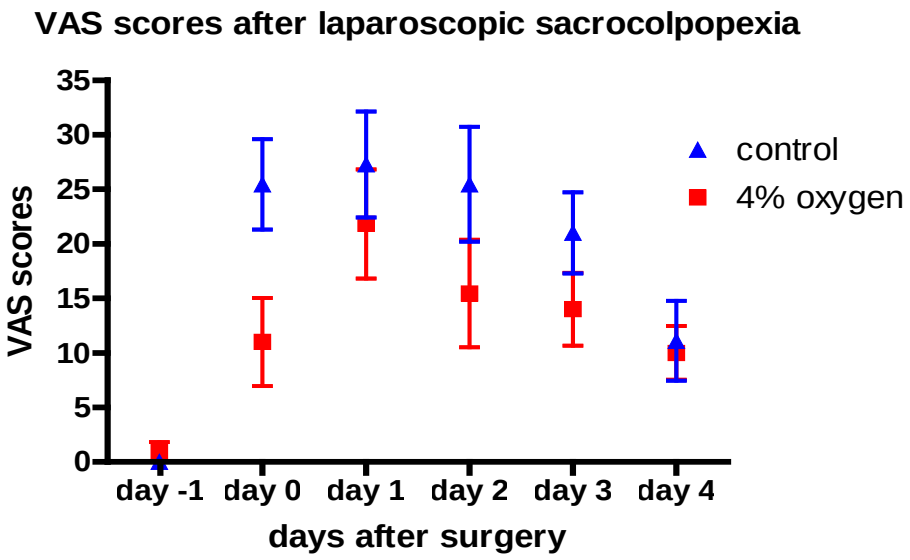


Figure 15: General VAS scores for group 1 (oxygen) and group 2 (carbon dioxide): mean and SEM.

Inflammatory reaction as defined by CRP and WBC showed no significant decrease in the group where oxygen was added to the CO_2 pneumoperitoneum. Levels of CA-125 were not different between the two groups. The means for CRP were however twice as high in the control group. (Figure 16)

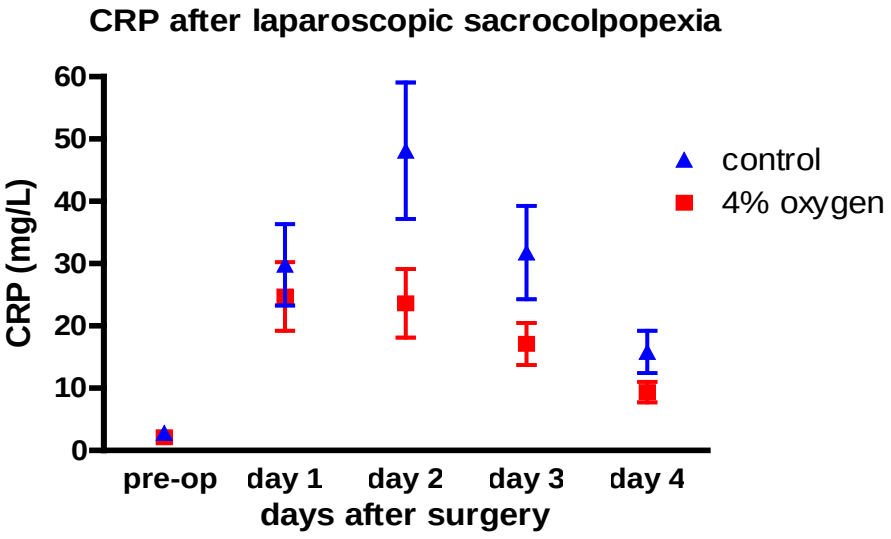


Figure 16: Means and SEM for C-reactive proteine (CRP) before surgery (pre-op) and the first 4 days after.

The use of pain killers after surgery was not significantly different between the two groups although less pain medication was used by the study group receiving oxygen on all post-operative days. The difference was the greatest for the use of paracetamol at the day of the surgery ($p<0.09$) and at the second day after surgery for ketorolac ($p<0.16$).
Conslusion: The addition of 4% of oxygen decreases postoperative pain and CRP at least on day 1-2 after surgery.

c. Port-site metastases

Verguts J, Vergote I, Amant F, Moerman P, Koninckx P. The addition of 4% oxygen to the CO(2) pneumoperitoneum does not decrease dramatically port site metastases. J Minim Invasive Gynecol. 2008 Nov-Dec;15(6):700-3. - addendum

AIM: Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and will decrease tumor implantation in laparoscopic port sites.

MATERIALS & METHODS: For detection of port-site metastases (PSM) after diagnostic laparoscopy with or without addition of 4% of oxygen to the carbon dioxide pneumoperitoneum all patients presenting with an ovarian mass, suspected to be malignant were included (n=35). Of these 22 fulfilled the criterium of malignancy with the necessity of a second look laparotomy. 13 women were excluded since two had an endometrial cancer, two did not have a malignancy whereas in nine no primary or interval debulking was performed.
An exploratory prospective randomized controlled trial was initiated using pure CO_2 or 96% CO_2 + 4% of oxygen, using the Thermoflator plus for insufflation (Karl Storz Tuttlingen). Pressure was standardized at exactly 15 mm Hg. At subsequent debulking, the laparoscopic ports were excised and examined by the pathologist for PSM. PSM were defined as the presence of at least 1 microscopic cluster of tumor cells observed in a single microscopic slide made from the port site. Body mass index, type of tumor, stage according to the FIGO classification and duration of laparoscopy were used for subgroup analysis.

This trial was not powered to detect minor decreases in port site metastases. Indeed considering the 17% incidence of PSM, 192 women would be needed to detect a 50% reduction. Therefore an interim analysis after one year was done in order to evaluate continuation.
Analysis was done with the SAS system, with the use of a Spearman r correlation.

RESULTS: As expected both groups were comparable for demographic data as length, weight, BMI, duration of surgery, type of tumor and pathology. No major complications such as bowel perforations, bowel fistula, major bleedings, or infections were observed.
The interval between diagnostic laparoscopy and initiation of therapy (primairy debulking or neo-adjuvant chemotherapy) was 18 days for the control group (n=11) and 12 days for the study group (n=11). In the control group 19 port sites were excised, and 9 (47%) showed microscopical PSM. In the oxygen group 16 port sites were excised and 8 (50%) had PSM. In both groups 5 women had PSM (45%). PSM were not higher in the 12 millimetre umbilical port than in the 5 millimetre ports. PSM were not higher in ports through which specimens or biopsies had been extracted.
PSM correlated with the tumor grade ($P<0.05$) and negatively with length ($P<0.018$), but not with BMI. Also after correcting for these variables oxygen addition did not have any effect on the incidence of PSM
Conclusion: The addition of 4% of oxygen to the CO_2 pneumoperitoneum does not decrease port site metastases.

d. Ultramicroscopic alterations in laparoscopy

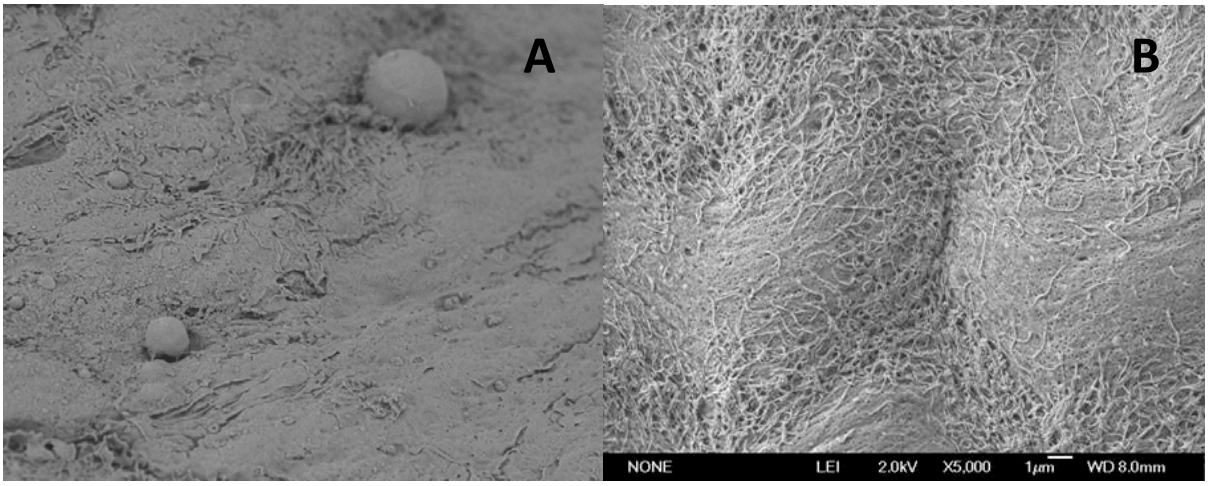
Riiskjaer M, Binda M, Verguts J, Corona R, Forman A, Koninckx P. Peritoneal morphology after laparoscopic surgery in a mouse model. Manuscript prepared

AIM: Addition of 4% of oxygen to the pneumoperitoneum will prevent mesothelial hypoxia and prevent retraction of the mesothelial cells and exposure of the extracellular matrix.

MATERIALS & METHODS: Women undergoing a laparoscopic sacrocolpopexia were eligible and randomized in this trial.

After installation of the pneumoperitoneum a biopsy of approximate 1cm² was taken and fixated in 2% formaldehyde + 2,5% glutaraldehyde in Sörensen buffer. Further processing consisted of rinsing in Sörensen buffer without fixative and placement in 1% osmium for 1 hour. Further dilution in Sörensen buffer and dehydration in ethanol 50-70-90-100 % (each percentage for 3 x 20 minutes) and gradually replacing by acetone. The ethanol/acetone was removed and pure HMDS was added and removed. The sample was mounted on aluminum stubs with silver paint, dried thoroughly and coated with platinum. All samples were viewed by an experienced lab worker.

RESULTS: After 4 patients the trial was stopped early as specimens obtained in this trial were not representative. No mesothelial cells were visible in the pre- or post-pneumoperitoneum biopsies and only fibrous tissue was seen at scanning electron microscopy (SEM). (Picture 3)



Picture 3: **A**. SEM of human peritoneum after 60 minutes of pneumoperitoneum with CO_2 + 4% oxygen **B**. SEM of mouse peritoneum after 60 minutes of pneumoperitoneum with CO_2 + 3% oxygen

The SEM pictures obtained in animals by the same technique were showing some effect of the addition of 3% of oxygen to the pneumoperitoneum. Pure CO_2 pneumoperitoneum had a deleterious effect on the mesothelial morphology and the effect increased with the duration of the pneumoperitoneum. This was observed immediately after the pneumoperitoneum and after 24 hours. When 3% oxygen was added to the 60 minutes pneumoperitoneum with humidification of the gas, mesothelial cells slightly retracted and microvilli were still present. (Picture 3)

e. Simultaneous analysis of inflammation and surgery related complications when 4% of oxygen is added to the CO_2 pneumoperitoneum

MATERIALS & METHODS: We reviewed the effect of 4% of oxygen in all 66 patients from the 3 RCT's as described above, comprising, 20 from the trial of CO_2 absorption, 24 from the trial on pain and inflammation and 22 from the trial on port-site metastases.

RESULTS: As expected patients were comparable for age, BMI and duration of surgery. (Table 4)

Parameter	**Oxygen group**	**Control**	**p-value**
Total, n=66	33	33	NS
Age (y), mean +/- SD	51+/- 17	51 +/- 19	NS
BMI, kg/m² +/- SD	25 +/- 5.7	25 +/- 3.7	NS
Duration of surgery (minutes), mean +/- SD	163 +/- 89	203 +/- 113	NS
Time to first flatus (hours), mean +/- SD	32 +/- 18	44 +/- 25	NS
Time to first stool (hours), mean +/- SD	66 +/- 32	61 +/- 29	NS

Table 4: comparison of women having laparoscopy with CO_2 pneumoperitoneum (control group) or with PP + 4% oxygen (oxygen group)

A two way analysis of variance, evaluating simultaneously the effect of the addition of 4% of oxygen, and the trial (thus compensating for eventual inter trial variability) failed to reach statistical significance. There was a trend however of a decreased time to first flatus after surgery being 12 hours earlier in the oxygen group. In women where CRP and WBC were recorded after surgery (n=56), similarly pain killer intake and CRP were slightly less in the oxygen group. (Table 5)

Parameter	**Oxygen group**	**Control**	**p-value**
Total, n=56	25	31	NS
Ibuprofen (any use)	7	12	p<0.125
	Mean +/- SD	Mean +/- SD	
CRP pre-operative	2.5 +/- 2.1	2.4 +/- 2.3	NS
CRP day 1	21 +/- 15	33.6 +/- 35.2	NS
CRP day 2	35.8 +/- 31	49 +/- 30	NS
WBC pre-operative	6.8 +/- 2.3	7.3 +/- 2.9	NS
WBC day 1	8.3 +/- 2.1	8.9 +/- 3.5	NS
WBC day 2	7.8 +/- 4.1	9.0 +/- 4.9	NS

Table 5: comparison of women having laparoscopy with CO_2 pneumoperitoneum (control group) or with PP + 4% oxygen (oxygen group) for pain killer intake, C-reactive protein (mg/L) and white blood cell count (10^9/L)

Interestingly, in the group with carbon dioxide only three complications occured: two bowel perforations and one wound eventration with none in the oxygen group (p<0.137). Conclusion and discussion: although with 4% of oxygen added to the CO_2 pneumoperitoneum, women had less pain, we failed to demonstrate in the entire group a decrease in CRP, and in time to first flatus. There were no serious adverse events recorded in the group where oxygen was added to the pneumoperitoneum, adding to the safety of this altered gas.

The observed difference in complications should be interpreted carefully, since based upon three events only. If confirmed however, this will be of utmost clinical importance. Although to a large extent speculative today, we suggest that these complications are not strictly related to the use of carbon dioxide, but the repair of the peritoneum and/or the viability of the peritoneum having to cover the wound and allowing for subperitoneal repair processes may be involved. A viable peritoneum able to cover a superficial wound on the bowel or the peritoneal wall will protect against macrophages which are present in the abdominal cavity to adhere to the wound. Macrophages will thus not be able to degrade the matrix covering the lesion, making a restitutio ad integrum more likely. This may seem to be a paradox as macrophages are also needed to attract mesothelial cell, but processes in covering a

denudated area is speculated to be different from covering a necrotic area. This is in concordance with the fact that CO_2 pneumoperitoneum decreases production of IL-1 and TNFalfa (decreased inflammation), but increases adhesion formation[89].

II. Full conditioning of the peritoneal cavity

a. CO_2 absorption

Verguts J, Corona R, Vanacker B, Binda M, Koninckx P, Manuscript in preparation

AIM: Full conditioning of the pneumoperitoneum will prevent mesothelial damage and will decrease CO_2 absorption

MATERIALS & METHODS: In this prospective randomized trial using pure CO_2 or full conditioning we included women undergoing a laparoscopy for an indication of total laparoscopic hysterectomy, laparoscopic colpopexia, laparoscopic adhesiolysis, laparoscopic myomectomy or laparoscopic resection of deep endometriosis. CO_2 absorption was measured during laparoscopy by end-tidal CO_2 as in the previous trial were only 4% of oxygen was added to the pneumoperitoneum.
Reduction in CO_2 absorption: given previous data that adding 4% of oxygen significantly reduces CO_2 absorption in a group of 20 women undergoing laparoscopic surgery for endometriosis, a prospective series of 20 patients in each arm (i.e. 40 in total) should be sufficient to reach statistical significance per stratum.
Women were followed for anesthesiologic changes during surgery i.e. end tidal CO_2 and expiratory N_2O, and for inflammatory response (CRP and WBC) and pain as measured by VAS in the post operative phase. (cfr. infra)

RESULTS: Up to date 20 women were recorded. Measurements showed a significant decrease in end tidal CO_2, even more then with addition of 4% oxygen to the CO_2 pneumoperitoneum. The increase in ET CO_2 was not as high as in the first trial for both groups and occurred some 10 minutes later. (Figure 17)

Nitrous oxide as measured in the expiratory phase of ventilation did not show any sign of absorption. The 10% of nitrous oxide present in the insufflation gas thus did not enter the bloodstream as it was not detectable in the expirated gas.

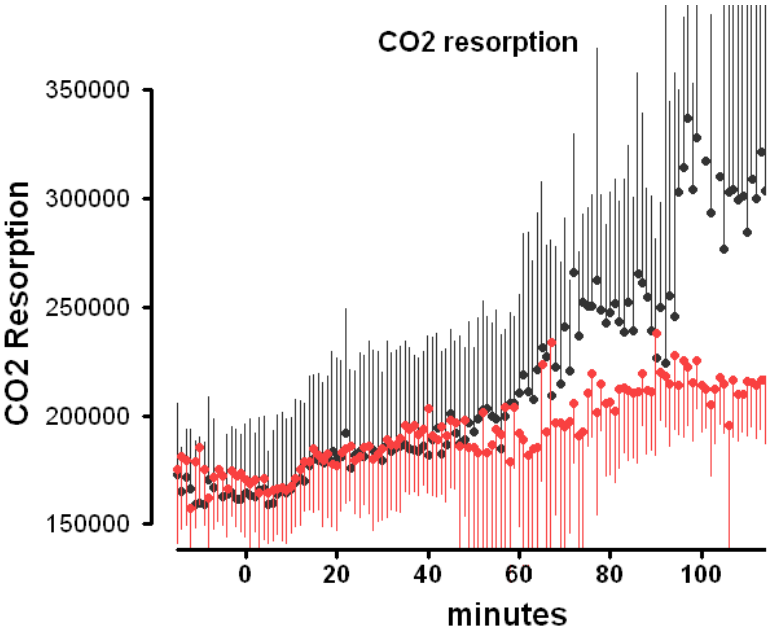


Figure 17: CO_2 resorption during laparoscopy with CO_2 pneumoperitoneum (black) or with full conditioing (red) after creation of the peritoneum (time=0)

The safety of full conditioning was demonstrated for these women. No serious adverse events occurred during or after surgery.

b. Pain and inflammation

Verguts J, Corona R, Vanacker B, Binda M, Koninckx P, Manuscript in preparation

AIM: Full conditioning of the pneumoperitoneum will prevent mesothelial damage and decrease post-operative inflammation and pain.

MATERIALS & METHODS: We wanted to demonstrate reduced postoperative pain and a decreased inflammatory reaction following full conditioning. Secondly we anticipated a shorter time to resumption of transit i.e. time to first flatus and time to first stool.

Postoperative pain was assessed by Visual Analog Scales (VAS). Patients were asked to locate their pain-symptoms (shoulder, subcostal, trocar wound and visceral pain) and score the severity. VAS was collected 2-4 hours after surgery and every post-operative day until discharge. Patients were free to use pain medication after surgery but preferentially ibuprofen was used in order to permit easier comparison of pain killer intake. Postoperative inflammatory reaction was assessed by inflammatory parameters as CRP, WBC, and temperature and were recorded as done routinely. CRP and WBC were measured pre-operative and every post-operative day until day 3 or until discharge.

Patients were randomized 1:1. Half of them received standard treatment (CO_2 pneumoperitoneum), the other half was randomized to full conditioning of the peritoneum. In *trial 2* women undergoing laparoscopy for total or subtotal hysterectomy, myomectomy, colpopexia or adhesiolysis were included. Women in *trial 1* undergoing laparoscopy for deep endometriosis were also included, but separately analyzed as they also received dexamethasone 5mg at the induction of pneumoperitoneum and application by the end of surgery of Hyalobarrier®gel at the surgical wound.

Surgery and postoperative follow-up were performed as standard. Antibiotics were given as specific for the surgery performed and immediate post-operative analgesia as is standard. (see local analgesic protocol, *cfr. supra*)

A prospective series of 20 patients in each arm (i.e. 40 in total) should be sufficient to reach statistical significance per stratum. Statistical analysis was performed as an intention to treat analysis in a 2 way ANOVA. This will allow analysis per stratum and per type of gas used. If in one procedure the effect of full conditioning is not obtained, this will be visible in the analysis.

RESULTS: VAS was significant lower in the group receiving full conditioning. (Figure 18) This was as expected regarding the data from the trials where only 4% of oxygen was used. Shoulder tip pain 2-4 hours after surgery was significantly lower in the group receiving full conditioning: 1/22 vs. 11/27 ($p<0.0001$).

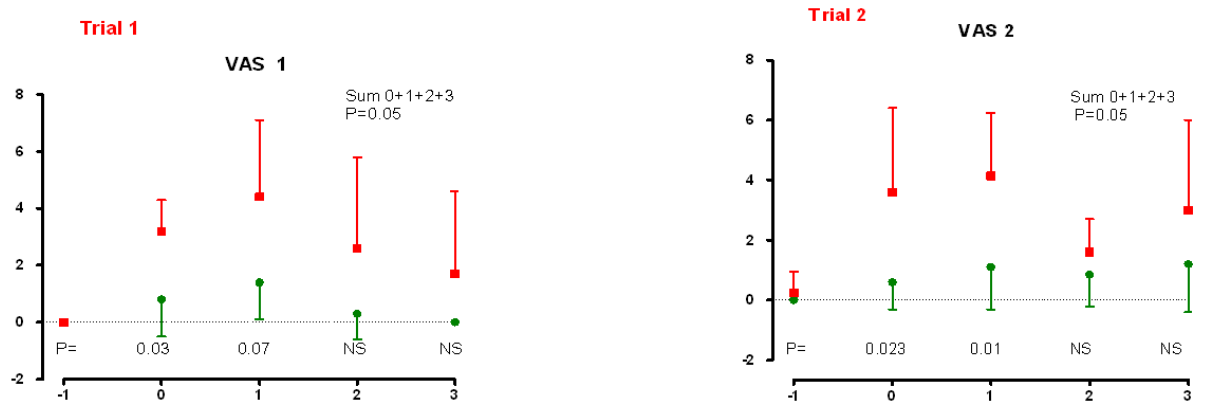


Figure 18: General VAS scores (mean and SD) for women having laparoscopy with CO_2 PP (square, red) or with full conditioning (dot, green) for *Trial 1 (endometriosis)* and *Trial 2 (rest)*

Difference in CRP was not significant, also not after correction for duration of surgery. Leucocytosis was unaffected by full conditioning of the peritoneal cavity.

There was a trend of decreased pain killer intake (ibuprofen) in the group with full conditioning. (Figure 19)

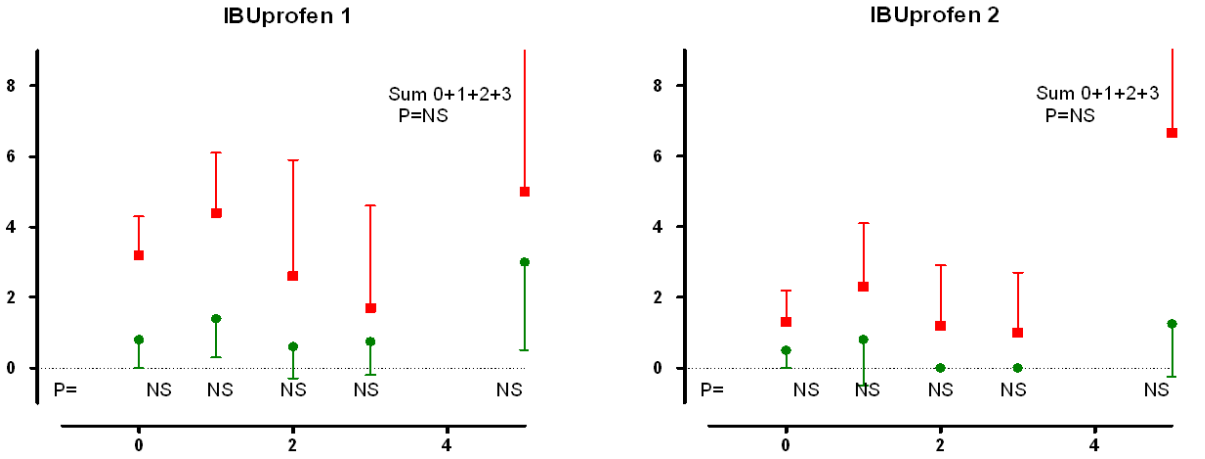


Figure 19: Pain killer intake (mean and SD) for women having laparoscopy with CO2 PP (square, red) or with full conditioning (dot, green) for *Trial 1* and *Trial 2*

c. Adhesion prevention

Verguts J, Binda M, Corona R, Koninckx P. Carbondioxide pneumoperitoneum prevents postoperative adhesion formation in a rat cecal abrasion model, PhD. J Laparoendosc Adv Surg Tech A. 2011; author reply, in press- addendum

Koninckx P, Verguts J, Binda M, Vanacker B, Craessaerts M, Corona R. Manuscript in preparation

AIM: Full conditioning of the pneumoperitoneum will prevent mesothelial damage and decrease adhesion formation.

MATERIALS & METHODS: Women undergoing laparoscopic excision of deep endometriosis were randomized in this study since this is severe surgery, associated with severe postoperative adhesions and since for most of these women fertility is important and hence a second look laparoscopy can be beneficial. This trial is referred to as *trial 1*.
40 women should be randomized 1:1. 20 women receiving standard treatment with a CO_2 pneumoperitoneum. 20 women being randomized to full conditioning of the peritoneum plus rinsing with heparinised saline (5000 U per Liter) plus injection of 5mg dexamethasone at creation of the pneumoperitoneum plus application of an anti adhesion barrier (Hyalobarrier Gel Endo®) at the site of surgical wound by the end of surgery.
Second look laparoscopy to assess adhesion formation was performed some two weeks after surgery under general anaesthesia using a 5 mm laparoscope. Early repeat laparoscopy for atraumatic lysis of pelvic adhesions is used routinely in many centers for infertility patients. During this early repeat laparoscopy any existing adhesions were lysed and adhesions were scored by the surgeon.
11 women were randomized 1:1. 6 women received standard treatment with a CO_2 pneumoperitoneum, 5 women were randomized to full conditioning of the peritoneum plus rinsing with heparinised saline.

Scoring of adhesions: A new scoring system for adhesion formation was developed based upon the lessons learned from previous classification systems as used in the human and in

animal models. Adhesions can be scored quantitatively, as the area of the surgical lesions covered by adhesions[90]. This however could only be done in animal models with a well standardized lesion. Adhesions generally are scored qualitatively by scoring separately extent, tenacity and type at a given localization[91]. Past experience in clinical trials and during surgery, however, made us aware of the strong inter-correlation between extent and tenacity and type, filmy adhesions generally having low tenacity and covering a small area only, whereas the rather thin, long, dense and vascularized adhesions are too rare to be picked up in any classification system. In addition, from the statistical evaluation of adhesion data we learned that scoring too specifically at too many locations generally is counterproductive[56]. Finally scoring systems should be sufficiently practical to permit immediate scoring after surgery by the surgeon. Otherwise subsequent blind review and scoring of videorecordings will be necessary. Although this may at first glance be considered the most objective method, reviewing videorecordings balances between reviewing and scoring short video clips, and reviewing and scoring the complete recording of an intervention. The former has the drawback that not all adherences can be grasped in short videoclips, since the extent of a frozen pelvis can only be judged during and after surgery. Reviewing complete registrations of the entire intervention, requiring advanced videocompression and accelerated replay only recently has become technically realistic, while the time required to review still might be prohibitively long to become practically realistic.

We therefore scored adhesions as type/tenacity together with 7 areas involved. Type and tenacity were scored as 0 to 3 when adhesions were absent, filmy with easy lysis and little or no coagulation, mixed filmy and dense, and dense adhesions requiring sharp dissection and coagulation respectively. The area involved was scored quantitatively as the diameter of a theoretical circle comprising all adhesions. This area in fact describes the sum of all denuded areas caused by adhesiolysis. Filmy adhesions thus almost invariably will cover a small area, ie often long but very thin. The areas scored separately were kept to a minimum and were based upon clinical importance and surgical difficulty. Were scored separately 1. vesico-uterine fold, 2. adhesions between abdominal wall and bowels or omentum (almost invariably a consequence of previous surgery) and 3. adhesions between the back side of the uterus and the pouch of Douglas or bowels (as a consequence of previous surgery, PID of deep endometriosis). Areas 3 and 4 comprised adhesions around

left and right ovaries and oviducts together and areas 5 and 6 scored adhesions between bowels and lateral side-walls in the pelvis. Finally 7 grouped all other adhesions outside the pelvis without further specification. This was added for completeness.

Statistical analysis and validation of the scoring system: To the best of our knowledge all the available adhesion scoring systems are based upon common sense, surgical difficulty and anticipated impairment of fertility but have not been validated. It indeed is unclear whether the scoring of extent, type, tenacity can be simply added or whether some should get more weight in order to predict or measure risk of occurrence after surgery, or to predict recurrence after lysis, or to predict fertility, pain or reoperation. For these trials we calculated the adhesion load at each site by multiplying scoring and area of denudation (mm^2).

With collection of more data it is the aim to model the relative importance of area involved and type of adhesions in order to obtain a more predictive total score of adhesions at any of the clinically important areas.

RESULTS: Although numbers are still small, statistically significant differences between the two groups were observed. In the control group severe adhesions were systematically found, whereas in the full conditioning group, adhesions were either minimal or not existent. (Figure 20)

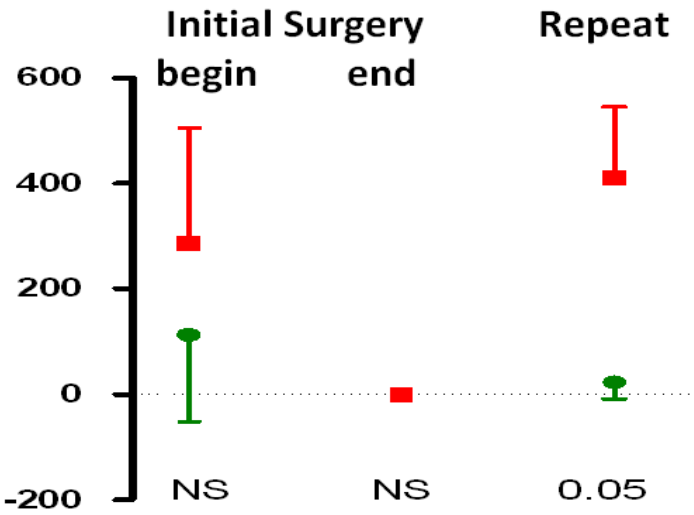


Figure 20: Adhesions scored (type x area) at the initial laparoscopy (beginning and end of surgery) and at repeat laparoscopy two weeks later.

d. Absorption of fluid from the peritoneal cavity

Koninckx P, Verguts J, Timmerman D. Assessment of measurement validity. Fertil Steril. 2006 Jan; 85(1):268; author reply 268. - addendum

Verguts J, Timmerman D, Bourne T, Lewi P, Koninckx P. Accuracy of peritoneal fluid measurements by transvaginal ultrasonography. Ultrasound Obstet Gynecol. 2010 May; 35(5):589-92. - addendum

Verguts J, Corona R, Timmerman D, Koninckx P. Absorption of Ringer lactate and Adept after full conditioning of the peritoneal cavity during laparoscopy. Manuscript in preparation

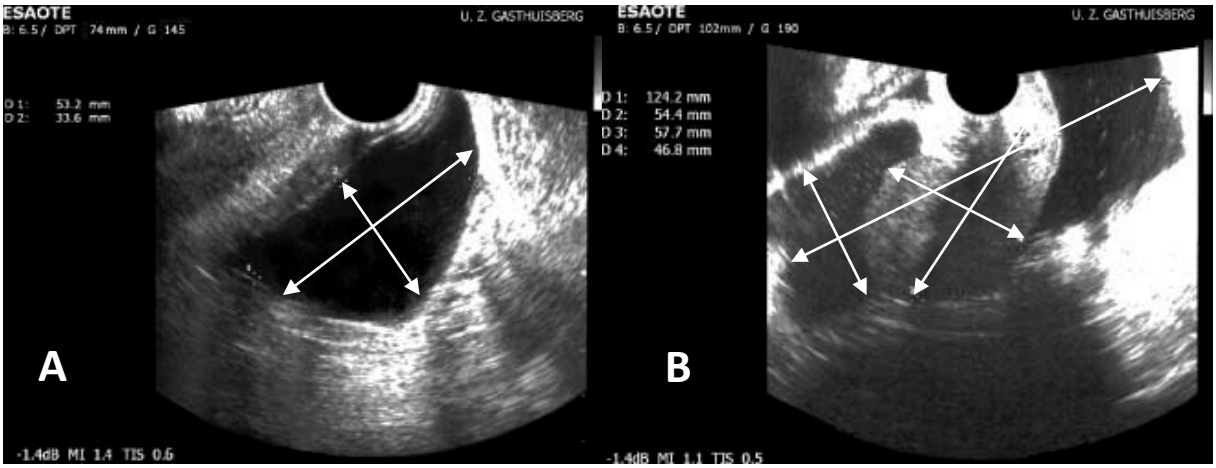
AIM: Full conditioning of the pneumoperitoneum will prevent mesothelial damage, decreasing speed of absorption of fluid (Ringer lactate or Adept®).

Measurement of volume

MATERIALS & METHODS: A non invasive method to measure accurately the volume of intra-abdominal fluid would be clinically useful to assess retention time of anti-adhesion flotation agents believed to be a key factor in effectiveness.

A previous trial by Muzii et al.[92] only included volumes up to 300 ml and suggested a linear correlation between volume measured by ultrasound and real volume. We think this correlation is not linear[93] and designed a study to 15 patients undergoing minor laparoscopic surgery (tubal sterilization, diagnostic laparoscopy) were enrolled after informed consent. Besides age, also weight, length and BMI were recorded, since these could obviously influence the collection of larger amounts of fluid in the pelvis. After laparoscopy all peritoneal fluid was aspirated from the pelvis and carbon dioxide was carefully removed. The patient was then placed in a 30° reverse Trendelenburg position and a catheter was placed 5 centimeters through the umbilical port in the direction of the pouch of Douglas through which Ringer lactate was infused using a Wolf 2220 Hystero Pump® in four discrete steps of 100, 250, 500 and 1000 ml (increments of 100, 150, 250 and 500mL) i.e. a logarithmic increase. An equilibration time of 2 minutes was used since in initial pilot experiments little to no changes occurred after this period. The 3 dimensions of the pocket

were measured by ultrasound (Esaote Technos with a 6.5-MHz transvaginal probe). Each measurement was repeated 3 times with one minute interval and the mean of each measurement was used for the final calculation. At larger volumes, the fluid spread above the pouch of Douglas anterior and posterior of the uterus. When this occurred the volume of the uterus was subtracted from the estimated volume. The volume of the uterus was calculated from its three dimensions considering the uterus as an ellipsoid (length x height x depth x pi/6) (8, 9). (see Pictures 8 and 9). At the end of the measurements the instilled fluid was drained with the catheter.



Picture 4. **A**. Transvaginal ultrasound image of the pelvic pocket, without involvement of the uterus, following infusion of 250 mL of Ringer's lactate. Two dimensions—D1 and D2—are indicated. These were used together with the third dimension to calculate the volume. **B**. Transvaginal ultrasound image of the pelvic pocket, extending above the uterus, following infusion of 500 mL of Ringer's lactate. The volume of the pocket was calculated from its three dimensions (D1 and D2 indicated), from which the volume of the uterus was subtracted (D3 and D4 indicated).

Means of the volume measurements performed in triplicate were used to calculate the relationship between the known instilled volumes and the calculated volumes. Several models were evaluated using JMP Version 7, SAS Institute Inc., Cary (NC), 2005.

RESULTS: Women (n=15) undergoing minor laparoscopic surgery participated in this trial and at the end of surgery fluid was instilled in discrete steps and measured by ultrasound.

The measurements of the largest pockets performed after 2, 3 and 4 min of equilibration were not significantly different, confirming that 2 min of equilibration was sufficient for reliable estimations. Using untransformed instilled volumes and the corresponding

ultrasound-calculated volumes the best prediction of the measured peritoneal fluid volume proved to be a second degree or quadratic regression curve, providing the best trade-off between goodness of fit and complexity.

The measured ultrasound volume (Y (mL)) was found to be related to the instilled volume (V (mL)) by means of the quadratic regression equation: Y = −31.9 + 1.3429 V − 0.0005023V2. Using this equation the volume V can be estimated from a measured ultrasound volume Y: V = 1336.7 − (1 723 204.9 − (1990.8421 × Y)) 1/2, for values of Y between 0 and 865.6 mL. (Figure 21) The fitted quadratic curve was significant (P < 0.0001 for the second-degree coefficient) and accurate (r2 = 0.89, P = 0.0001).

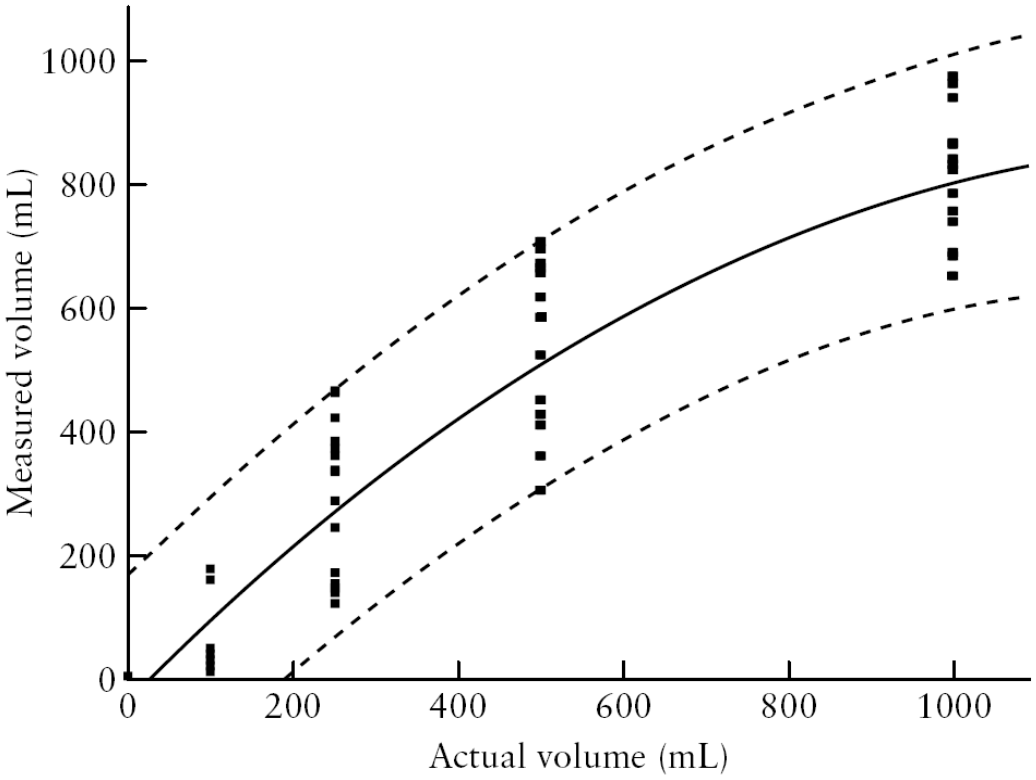


Figure 21. Quadratic regression curve Y = 31.9 + 1.3429 V − 0.0005023 V2 (—) of the volumes measured by ultrasound (Y (mL)) versus the actual instilled volumes (V (mL)) with 95% prediction intervals (i.e. the mean ±1.96 times the SD of the individual measurements) (----).

When the sets of measurements obtained in each patient were considered separately, the coefficient of variation was 7.3% (within-patient variation), which is significantly lower than the overall coefficient of variation (F (12, 51) = 7.0, p<0.001).

The volume of intra-abdominal fluid can be quantified reasonably accurately, at least up to 1 L, by measuring the largest pocket of fluid by transvaginal ultrasonography in a patient in the 30◦ anti-Trendelenburg position for at least 2 min. The procedure was standardized with the women in this position, with direct instillation, in order to collect most fluid present in the pouch of Douglas, for which we found an equilibration time of only 2 min.

Since the within-patient variability was found to be only 7.3%, this method can be used to detect changes in peritoneal fluid volume over time. Indeed, a study involving serial measurements in individual patients, e.g. when estimating resorption of instilled peritoneal fluid, would need only 10 patients to detect decreases of 15% in volume at the 0.05 significance level with a power of 80%.

Measurement of absorption

MATERIALS & METHODS: Primary endpoints of this were to evaluate whether the clearance rate of peritoneal fluid is exponential or linear over time. (absorption rate expected for Adept ~30-60 ml/h) and to evaluate the role of the mesothelial barrier in this clearance rate. Knowing that diffusion to and from peritoneal fluid decreases with increasing molecular weight (MW), our hypothesis is that clearance rate will decrease following peritoneal conditioning. Indeed a decreased mesothelial trauma (either through hypoxia or through denudation) and retraction will expose less the basal membrane.

The study was conceived as a *Latin Cross*. (Table 6)

	Ringer-lactate n = 10	Adept® n = 10
Control with CO_2 n = 10	n = **5**	n = **5**
Full conditioning n = 10	n = **5**	n = **5**

Table 6: Study design fluid absorption: *Latin Cross*

Horizontally: Prospective randomised trial using pure CO_2 or full conditioning. 20 women were randomized 1:1. N=10 for each subgroup. Vertically: Prospective randomised trial using 1000 ml of fluid: Ringer-Lactate or Adept® left in the abdomen following laparoscopic

hysterectomy. 20 women were randomized 1:1. N=10 for each subgroup. The volume was estimated by ultrasound at times 0, 24h, 48h and 72h as described later.

All women undergoing a laparoscopic subtotal hysterectomy were eligible for this trial. No limitation in body mass index or uterine size was made.

Laparoscopy was standardized by adjusting the insufflation pressure exactly to 15 mm of Hg in all patients. Rinsing of the abdomen was performed with the randomized product (Ringer-Lactate or Adept®). One liter of the fluid was left in place after surgery.

The first measurements were performed immediately after surgery in order to account for any differences in instilled fluid. We estimated the residual volume of fluid in the abdominal cavity in a 30° anti-Trendelenburg position after 1 minute in this position.

In a small pilot-study of 3 patients we estimated the time to have fluid accumulated in the Pouch of Douglas (data not shown). On the first post-operative day after we instilled Adept® for the prevention of adhesions we obtained the patients consent to have the ultrasound performed. Three ultrasonografic measurements after 1, 5 and 15 minutes (9 in total per patient) in a 30° anti-Trendelenburg position or after some walking down the hall showed no differences in measured volume. After this we decided to estimate the residual volume of fluid in the abdominal cavity in a 30° anti-Trendelenburg position after 1 minute.

The volume was estimated by ultrasound immediately after surgery (time 0) and after 24, 48 and 72 hours.

RESULTS: Fluid absorption was fastest in the carbon dioxide group when Ringer lactate was used: no detectable fluid after 48 hours and the slope of the curve was the steepest. This was as expected. The slowest resorbtion occurred however in the carbon dioxide group where Adept® was used where we still had a pocket of fluid after 72 hours. Resorbtion in the full conditioning group when Adept® was used was slower then when Ringer lactate was used. (Figure 22)

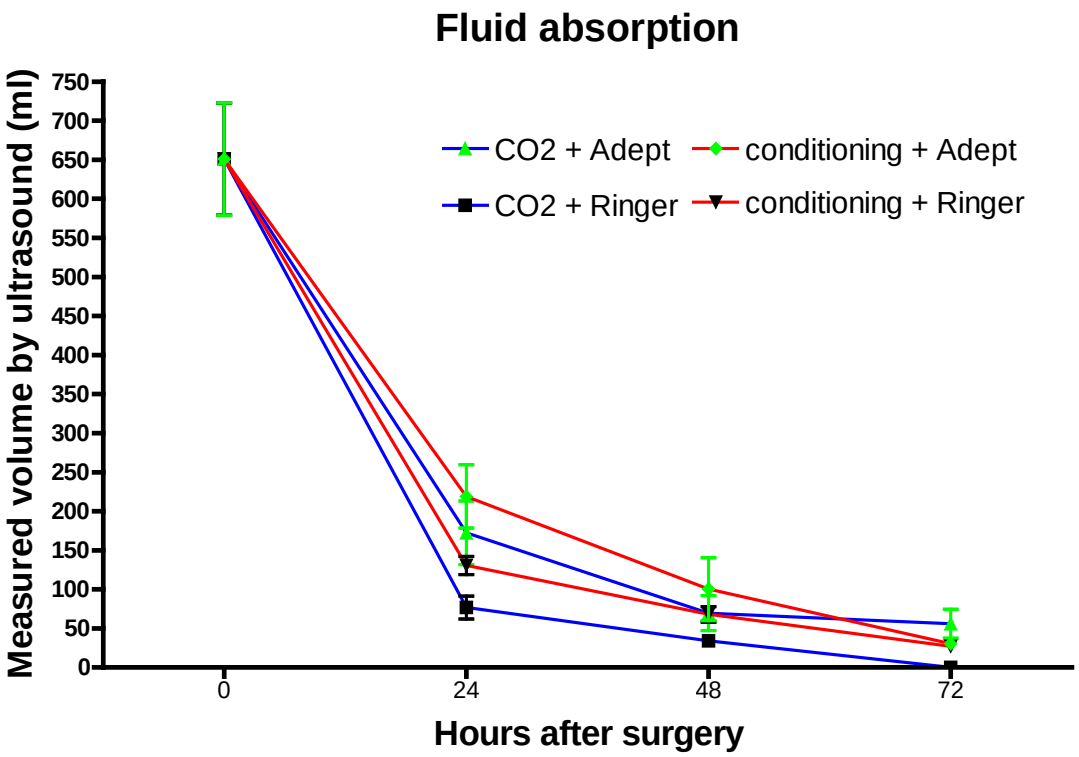


Figure 22: Fluid absorption as calculated by consecutive ultrasound measurements, mean and SEM

III. Lavage of the peritoneal cavity

De Cicco C, Corona R, Schonman R, Verguts J, Ussia A, Koninckx P. Extensive abdominal lavage during laparoscopic discoid excision of 1 deep endometriosis decreases bowel complications rate. Submitted

AIM: Extensive lavage after laparoscopic surgery will decrease acute inflammation

MATERIALS & METHODS: With the concept of acute inflammation of the entire peritoneal cavity, we decided to investigate the effect of extensive lavage after full thickness bowel resection upon postoperative CRP, leucocytosis and the incidence of late bowel perforations. To assess the effect of lavage on residual peritoneal fluid and cells and subsequent inflammation, we designed a prospective trial where we compared either standard rinsing of the abdomen to extensive lavage with 8 liters of saline. Lavage was started in the upper abdomen with the patient in a 30° anti-Trendelenburg position and continued until the liquid

around the liver was completely clear. To achieve this, usually some 4 liters of saline were required. Subsequently the patient was placed horizontally and the lavage continued in the lower abdomen.

Lavage of the peritoneal cavity after surgery was performed in 20 women with randomization to standard lavage or extensive lavage with 8 liters of saline.

Women in the lavage group and in the control group were comparable for age, weight and duration of surgery.

RESULTS: Postoperative CRP was significantly lower in the lavage group from day 1 onwards to day 7 (P=0.01). Moreover, in the lavage group, CRP declined faster, being less than 13 mg/l on day 5, whereas in the control group CRP still was markedly elevated on day 6 with a mean of 22 mg/l. (Figure 23) WBC counts did not show any difference.

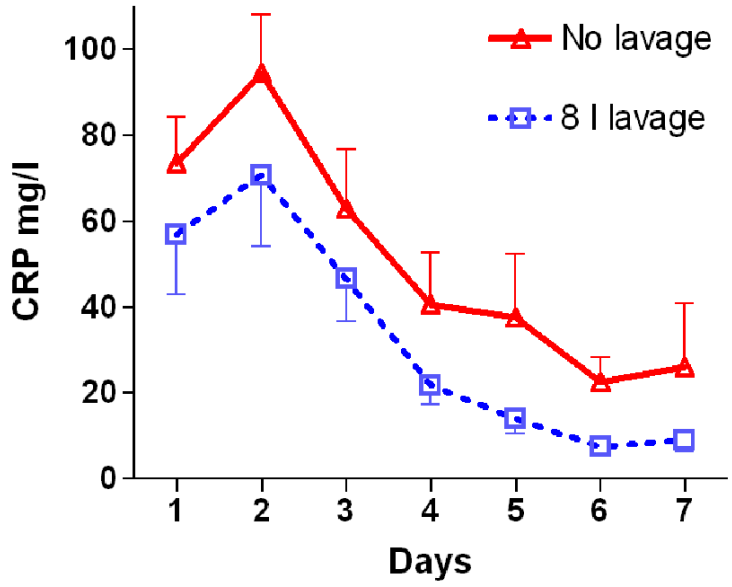


Figure 23: Mean and standard error of CRP concentrations following full thickness resection of deep endometriosis of the rectum in case of extensive lavage with extensive lavage (8 L) or standard (no) lavage. Overall significance: P=0.01 (repeated measurement ANOVA)

After implementation of extensive lavage of the peritoneal cavity, complications following full thickness resection of bowel endometriosis such as late bowel perforations disappeared. We prospectively compared all cases thereafter to historic controls. In 65 patients who received 8 liters of extensive abdominal lavage, no bowel complications occurred (0/65). In

the historical series of 84 patients who received the standard abdominal rinsing, 8 bowel complications occurred (8/84). (P<0.02 Chi-Square test).

With extensive lavage, CRP is significantly decreased and late bowel perforation did no longer occur. These finding can be placed in the protection of the mesothelial layer, as debris and other inflammatory cells, i.e. T lymphocytes[94], are washed out of the cavity with subsequent decreased adherence to the surgical wounds. We hypothesize that if fewer of these inflammatory cells adhere to the surgical wound, mesothelial cells may cover the wound completely within a few days, resulting in an increased healing.

IV. pH of irrigation fluid and wet tongue-tip pain

As pain after laparoscopy is multifactorial, and as pH of the different gasses may have an influence on pain experienced during laparoscopic surgery we wanted to investigate how pH of irrigation fluids (Saline or Hartman) changed, when put under pressure (15 mm Hg) of carbon dioxide, nitrous oxide or our mixture of 86% CO_2 + 4%O_2 + 10% N_2O. Pain sensation when carbon dioxide (i.e. soda water) comes in contact with the mucosa of the mouth is believed to be the result of the lowering of the pH.

MATERIALS & METHODS: We heated the irrigation fluid (Saline or Hartman) up to 32°C and measured pH at 1 Atm with an electronic pH meter (pHep®, HI98127, Caprock Developments Inc. Morris Plains, USA) with a deviation of +/- 0.02. Fluid was then put in a sealed cup and carbon dioxide, nitrous oxide or our mixture of 86% CO_2 + 4%O_2 + 10% N_2O was insufflated at 15 mm Hg into the fluid with a continuous outflow through a small needle valve above the level of the fluid. After five minutes the cup was opened and pH was measured.

To correlate this with sensation of pain, the three gasses were blown on the tip of a wet tongue of 15 volunteers (health care workers) and pain scores were measured using a visual analogue scale (0-100).

RESULTS: The pH of the two fluids did not alter when nitrous oxide was blown through the fluid under pressure and remained 7.0 +/- 0.1. When blowing carbon dioxide through the fluid in saline or Hartman, pH decreased significant to a mean of 4.2 +/- 0.04 and 5.2 +/- 0.04

respectively. (Figure 24) Insufflation of the mixture of 86% CO_2 + 4%O_2 + 10% N_2O through saline or Hartman decreased pH to a mean 4.3 +/- 0.04 and 5.3 +/- 0.04 respectively. Differences between saline and Hartman are due to an increased buffering capacity of Hartman.

Pain scores for the different gasses were significantly different, with practically no pain when nitrous oxide was used. (Figure 25) Pain scores given for carbon dioxide were higher compared to the gas mixture (p<0,0498).

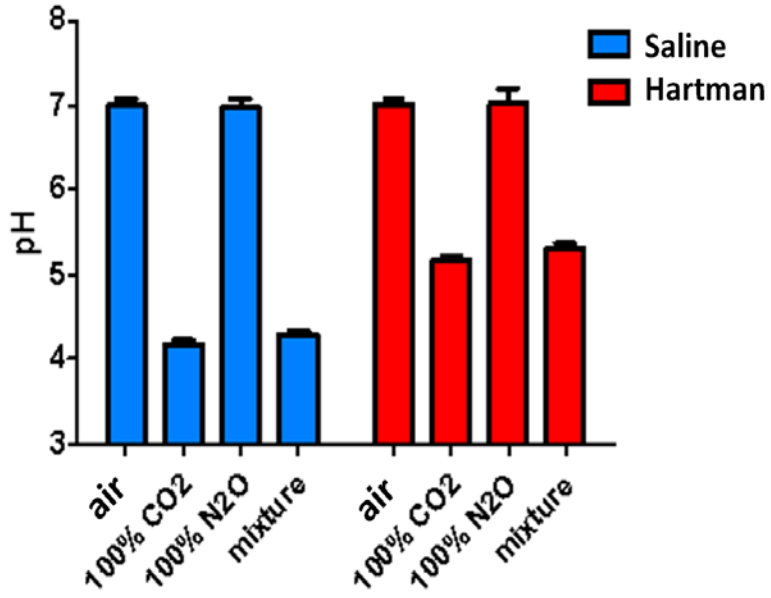


Figure 24: mean pH and standard deviation of irrigation fluid in open air (air), or under 14mmHg pressure of carbon dioxide, nitrous oxide or the 86% CO2 + 4%O2 + 10% N2O (mixture)

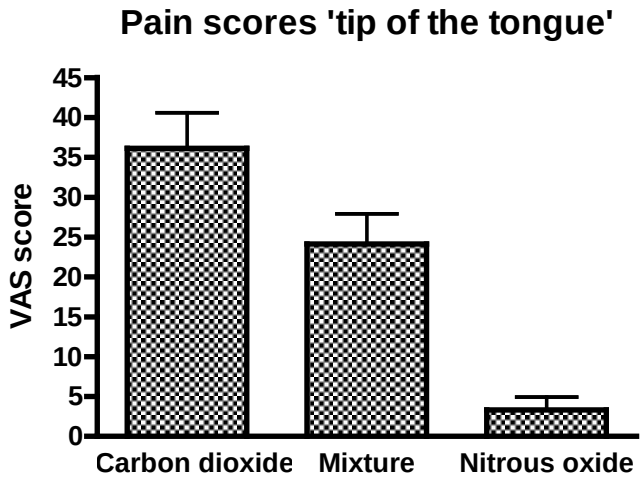


Figure 25: Pain scores (mean +/- SD) as measured by VAS with insufflation of 100% carbon dioxide, 100% nitrous oxide or the 86% CO2 + 4%O2 + 10% N2O (mixture) on the tip of the tongue.

Chapter 4

Discussion and conclusions

A. Understanding the peritoneal cavity

I. The mesothelial layer: a fragile border

A pneumoperitoneum using carbon dioxide creates a hypoxic environment for the mesothelial cells. In our mouse model the hypoxic effect on the mesothelial cells was clearly demonstrated. This hypoxia is superficial, but the effects can be profound regarding adhesion formation.

Prevention of hypoxia of the mesothelial cells is essential but this can be achieved in different ways. Addition of oxygen to the insufflation gas used during endoscopic surgery as demonstrated to be beneficial in animals and human is logic. As was demonstrated by Matsuzaki et al, hyperventilation of the mouse with oxygen may influence adhesion formation by oxygenation directed through the blood.[95] As during laparoscopy the bloodflow may be reduced due to intraperitoneal pressure, hypoxia may not be prevented and addition of oxygen to the gas seems essential.

The mesothelial layer is a single cell layer which is subject not only to hypoxia, but also to damage inflicted by i.e. lavage, manipulation, dessication and too low or too high temperatures. These detrimental effects were well addressed in our laparoscopic mouse model, but are more difficult to confirm in the human. Direct proof of decreased damage upon the human peritoneum by altering the insufflation gas up to date does not exist.

Ultramicroscopic alterations in the peritoneum which is of course the ultimate proof that the gas used is beneficial to its integrity is not feasible in humans as this would require in vivo fixation with a toxic product. Previous trials by Volz et al used fixation in vivo to obtain representative specimens of the peritoneum in animals[42].

A larger specimen could also be suggested, but this larger deperitonealisation would have altered our results on CO_2 absorption. New studies to directly look at the integrity of the mesothelium will be designed in the near future. Alternative ways to show the influence on

the peritoneum were used in this doctoral thesis such as tumor implantation, CO_2 absorption, inflammation, pain en fluid absorption. These are indirect way to show the effect on the peritoneum, but these end points all are proven reliable alternatives in animal experiments.

The fact that addition of 4% oxygen to the pneumoperitoneum or lavage completely prevented late bowel perforations may indicate that inflammatory cells (i.e. macrophages) play an important role in the healing process of the peritoneum. One may argue that this is due to the surgical technique, but by adding oxygen to the pneumoperitoneum, we hypothesize that the mesothelial barrier stays intact and thus is less subject to inflammatory cells and also produces less exudate. By this reduced inflammation the weakend bowelwall may heal in a more rapid way without having macrophages interfering with this process. A viable peritoneum able to cover a superficial wound on the bowel or the peritoneal wall will protect against macrophages which are present in the abdominal cavity to adhere to the wound. The same hypotesis on healing can be true for extensive lavage where macrophages are depleted through this extensive lavage. Macrophages will thus not be able to degrade the matrix covering the lesion, making a restitutio ad integrum more likely.

In our laparoscopic mouse we saw that prevention of inflammation did indeed prevent adhesion formation[96]. This study is not included in my doctoral thesis, but it proves that inflammation by all means plays a central in the healing process of the peritoneum. Administration of dexamethasone or addition of 4%oxygen to the pneumoperitoneum did decrease adhesion formation in a linear way.

Peritoneal infections are not covered by any study so far with this new gas. There may be a hypothetical risk that aerobic bacteria present in the abdominal cavity may benefit from these new conditions. It was previously studied however for different gasses. There was a significant increase in bacteremia in a CO_2 pneumoperitoneum group compared with the laparotomy-only and helium groups at 1 and 2 hours after surgery in a bacterial infection model from Erenoglu[97]. The risk of bacterial translocation in an E. coli rat peritonitis model was increased with insufflation using CO_2 or helium, and this effect was more significant at lower pressures (3 mmHg) than at higher pressures (14 mmHg)[98]. The results suggest that CO_2 insufflation may promote the growth of intra-abdominal anaerobic bacteria. Such bacterial growth may lead to intra-abdominal abcess formation or cause localized peritonitis to develop into generalized peritonitis[99]. Macrophages and by consequence adhesion

formation are strongly induced by enteric bacteria and their antigens such as lipopolysaccharides[100]. Prevention of this cascade by administration of a recombinant bactericidal agent with endotoxin neutralizing activities reduced adhesions in a rat cecal abrasion model[100].

The use of another insufflation gas other then CO_2 thus seems favorable in order to prevent transperitoneal bacterial translocation and it was suggested that laparoscopy without pneumoperitoneum may be preferred in patients with peritonitis. This new gas with or without full conditioning is therefore as safe as a CO_2 pneumoperitoneum or even safer, but studies are needed.

II. Mesothelial cells in prevention of adhesions

Harvesting and growing mesothelial cells for three weeks is so time consuming that large scale experiments become difficult.

These experiments confirm and extend the observations that cultured mesothelial cells obtained after trypsinization of the peritoneal cavity can reduce adhesion formation as described in rabbits and rats with mesothelial cells obtained from the omentum[28-30]. The effect moreover is not limited to pure surgical models such as the abrasion model[29;30], but also in the CO_2 pneumoperitoneum enhanced adhesion model. The effect is moreover dose dependent. Since we know that following a 60 minute CO_2 pneumoperitoneum mesothelial cells retract thus exposing directly the basal membrane[14], we may speculate that injected mesothelial cells may fill the gaps between the retracted mesothelial cells thus attenuating the adhesion enhancing effect of the CO_2 pneumoperitoneum by preventing the deleterious effect of the peritoneal cavity. Considering that 100.000 cells are needed for a half maximal effect, the hypothesis that the cells affect the entire peritoneal cavity seems attractive, as 100.000 cells exceed what is necessary to cover the injury which only measures 1cm by 1.6mm. These 100.000 cells indeed cover 10 cm2 since a confluent culture flask of 25cm² contains 250.000 cells. That 100.000 cells are similar to the number of cells recovered from the entire peritoneal cavity of 1 mouse further lends support to the hypothesis that these cells affect the entire peritoneal cavity. Whether this observation on CO_2 pneumoperitoneum enhanced adhesions can be extended to hyperoxia, desiccation, or manipulation enhanced adhesions is likely but remains to be established. Indeed considering

that the driving mechanisms of all these factors are micro denuded areas all over the peritoneal cavity the effect of cultured mesothelial cells is expected to be similar in all. A (surgical) trauma is essential to inititate adhesion formation but these adhesions are enhanced by factors from the peritoneal cavity, the latter being quantitatively the most important. The importance of factors in the peritoneal cavity was unequivocally proven by the observation manipulation in the upper abdomen increased adhesions at the site of the surgical lesion[101]. This moreover was recently proven to be mediated by acute inflammation of the entire peritoneal cavity and also explains that de novo adhesions (outside the surgical lesion) were never observed.

The mechanisms of adhesion reduction by cultured mesothelial cells can only be speculated upon. Whereas in pure surgical models, incorporation in the lesion as demonstrated in rats[102] seems logic, in the peritoneal cavity enhanced models, incorporation in between retracted mesothelial cells of the entire peritoneal cavity cannot be excluded. Moreover, it is unclear whether the origin of the mesothelial cells and the culture conditions could be important or whether these cells actively secrete substances in the peritoneal fluid. We even cannot ascertain that the effect is specific for mesothelial cells since Alpay et al. demonstrated that also fibroblasts display different immunologic characteristics with an effect on adhesion formation[103].

It is unclear why the cells from the first washing did not grow although remaining viable. The same culture medium was used as for the detached mesothelial cells. The only difference between cells from the first washing and cells obtained after trypsination is the fact that the former were kept for a much longer period in PBS, which indeed may damage mesothelial cells by making them lose their ability to divide (oral communication S. Mutsaers).

The effect of mesothelial cells in reducing adhesion formation might seem less important in the pilot experiment (400.000 cells caused a 50% reduction in adhesions) than in the dose-response experiment where some 100.000 cells caused a 50% reduction in adhesions. This difference may be a consequence of the fact that cultured mesothelial cells probably stayed longer in PBS in the pilot study since our experience in handling cells was less at that moment. Also variability in adhesion formation has to be taken into account. Indeed, although total adhesion scores were comparable in the two control groups of both experiments (3.1) the proportion of adhesions was lower in the second experiment (13.5%) than in the first experiment (19%). Although overall very reproducible, this variability in

proportion of adhesions ranging from 45% to 15% between experiments had been observed previously[86;104]. This indeed prompted us to block randomise by day while standardising all experimental conditions as temperature, pneumoperitoneum conditions and duration, anaesthesia and experience of the surgeon as much as possible.

The clinical implications of these experiments are far reaching whereas the use of mesothelial cells for adhesion prevention becomes attractive. Extensive lavage during and after surgery might remove besides debris and fibrin also mesothelial cells and macrophages, and the latter might be detrimental. These animal experiments combined with clinical observation at least suggest that these effects should be evaluated in detail in specific experiments. Whereas collecting and culturing mesothelial cells for adhesion prevention in the same patient is not realistic today, this method could become useful if fibroblast would be useful, or if some cell lines with similar effects could be developed, i.e. stem cells. In case mesothelial cells could be preserved from the peritoneal lavage during surgery as is the case with peroperative cell salvage for blood, this could be a reliable method to prevent adhesions and the detrimental effects it may cause.

III. Nitrous oxide

The beneficial effect of N_2O upon post-operative pain, intra-operative ventilation is well known, but the underlying mechanism of action remains unclear[66], as well as the lowest effective dose to obtain these advantages.

The low effective concentration of only 5% N_2O in our laparoscopic mouse model was somehow surprising, but this allowed us to implement a safe (<30%) concentration of 10% N_2O in clinical trials.

The mechanism of action of N_2O, however has to be different from the effect of oxygen, since the effect of even 100% of N_2O is comparable to the effect of 5%, whereas oxygen concentrations above 10% clearly increased adhesions[73]. We therefore anticipated that N_2O and low concentrations of oxygen will have additive effects. This additive effect cannot be seen in our data with full conditioning, as other factors such as humidification and control of temperature are interfering.

The central effect on pain of N_2O is known to be the result of the stimulation of opioid receptors, but peripheral nociception in the peritoneum is still under investigation

IV. Training and experimental models

Training is not only important to improve quality of surgery, but is also directly related to prevention of adhesion formation as shown in our laparoscopic mouse model. A previous study performed in rabbits showed that post operative adhesion formation, duration of surgery and complication rate decreases with the surgeon training expressed by the consecutive number of procedures performed.

When comparing experienced and non-experienced surgeons, we saw that experienced surgeons achieved a plateau in an early stage (after 10 procedures) whereas non-experienced surgeons didn't achieve the level of experienced surgeons even after 80 consecutive procedures. To obtain a general level of experience it thus seems not possible to achieve this within one training model, but it may consist of a larger exposure to surgery to improve skills.

Improved skills by experience probably indicates a less traumatic, more precise and gentle surgical technique gained by this experience and appears to be important since post operative adhesion formation was already lower in the ten first procedures within the group of experienced surgeons compared to non-experienced surgeons. This confirms the data of the effect of manipulation-enhanced adhesions[38] .

Other studies on the effect of CO_2 or Helium pneumoperitoneum upon post operative adhesion formation have shown an important effect of the time of exposure to CO_2 pneumoperitoneum upon adhesion formation. So less surgical skills correlates with a longer operation time and a longer exposure to the CO_2 pneumoperitoneum with increased mesothelial hypoxia.

Different strains of mice showed a different proportion in adhesion formation, suggesting that a genetic predisposition influences the process of adhesion formation. Recently we noted that the Balb/c mice in identical control groups over our different experiments had a different mean number of adhesions. Research confirmed that the company by which the mice were delivered did change over time. Strain differences have been reported for other processes involving fibrosis and healing responses such as hepatic, lung, and colorectal fibrosis [105-107], myocardial and ear wound healing[108;109], and bone regeneration[110].

B. Translation to clinical practice

I. Addition of oxygen

The translation of the mouse experiments showing beneficial effects on adhesion prevention by addition of 4% oxygen to the CO_2 pneumoperitoneum started five years ago. Since then the addition of 4% of oxygen was further improved by full conditioning with addition of 10% nitrous oxide, humidification and temperature control.

To study the direct effect of this altered gas on the integrity of the peritoneum is not clinically feasible, but indirect ways of proving our concept is. We looked at secondary effects of this gas on the mesothelial barrier as CO_2 absorption, pain, inflammation and port-site metastases and tried to connect this to the preserved integrity of the bassier.

The results on the addition 4% of oxygen are very clear regarding CO_2 absorption. Our study demonstrated a small but beneficial effect on pain and inflammation with adding 4% of oxygen to the CO_2 pneumoperitoneum during laparoscopy. This effect is thought to be mediated through different mechanisms.

We have demonstrated that by adding 4% of oxygen to the CO_2 pneumoperitoneum the inflammatory response will decrease. We can hypothesis that as the peritoneal lining will lose its integrity during laparoscopy, the extracellular matrix will come in contact with the peritoneal cavity and produce different cytokines that are released into the abdomen. These cytokines will induce an inflammatory cascade, resulting in elevated CRP and white blood count and post-operative pain. The effect of the oxygen on the inflammatory cascade can also be direct through mediation of reactive oxygen species.

In the global understanding of adhesion formation in the peritoneal cavity, this study clearly adds the value of maintaining a certain homeostasis in the oxygen level of the mesothelial cells. As the use of oxygen has already demonstrated a beneficial effect on adhesions and on a parallel inflammatory reaction in mice, one can speculate that the effect on adhesions in humans will be parallel to the decreased inflammatory reaction which was demonstrated by this study.

The group which received the oxygen-carbon dioxide mixture did have lower VAS scores and used less pain medication to maintain mean pain scores less than 3 on the VAS scale.

This is the first study to demonstrate the pain scores by VAS for a laparoscopic colpopexia. Pain scores are demonstrated to be generally low, but even then a difference was observed. This study should be extended to other types of laparoscopic surgery, were more post-operative pain and inflammation is to be expected. The difficulty to overcome will be the standardization of the study populaition. In our study, the population underwent a standardized procedure, but for more extensive types of surgery (oncologic surgery, endometriosis surgery,…) this will be impossible. A large number of patients is therefore required.

II. Full conditioning of the peritoneal cavity

All findings from previous trials where only 4% of oxygen was added to the CO_2 pneumoperitoneum were confirmed.

End tidal CO_2 as a measurement for CO_2 absorption further decreased to an almost steady state after some 20 minutes. From this we may confirm that the mesothelial layer maintains its integrity. This finding also adds up to the safety of the full conditioning where hypercarbia is almost unthinkable under these conditions.

Pain was improved by full conditioning with decreased VAS especially during the first post-operative days. This also resulted in a decreased use of pain killers (i.e. ibuprofen). Pain was examined for the different gasses on the tongue, where the mixture used for full condition provoked the least amount of pain. Differences in pH when the gasses were blown through Hartman or physiologic serum did not differ that much to explain the difference in pain as experienced on the tip of the tongue.

Looking at fluid absorption rates in the different groups, we saw the slowest absorption in the women who received Adept®, and the fastest absorption when Ringer Lactate was used. There was however an inversed correlation with the use of full conditioning, where Adept had an increased absorption when full conditioning was used and a decreased absorption of Ringer lactate when full conditioning was used.

We can hypothesize that the full conditioning keeps mesothelial cells more intact and thus providing more active transport of Adept® whereas Ringer is retained with an intact mesothelial layer, explaining the somehow inversed relation. With the use of carbon dioxide

and the thus damaged mesothelial layer the Ringer lactate is easily absorbed through third space where on the other hand there is no more active transport for Adept® possible and thus retained for a longer period.

III. Oncologic implications

Our laparoscopic mouse model was designed to detect differences in adhesion formation under different circumstances. Over the years we added progressively, a control of blood pH which is affected by ventilation rate and tidal volume, a strict control of body temperature which can vary widely with anaesthesia and the environmental temperature, a strict control of duration of manipulations such as intubation and initiation of laparoscopy, the duration of pneumoperitoneum and the type of gas used for the pneumoperitoneum, its humidification, temperature and the flow rate or leakage through the abdomen. Each of these variables were found to affect adhesion formation[39;41;111]. This should be kept in mind when performing studies on tumour implantation in mice and it seems important that in any study these technical details should be listed in detail.

Two different cell lines were used in our study to avoid erroneous conclusions through observations which would be cell line dependent. The cell lines used, CT26 and RENCA, are moreover well characterized and have been used in different cancer models[112-115]. The number of tumour cells used in these experiments varied from 10^3 to 10^6. Survival in all experiments was excellent up to two weeks. The survival of the mice is inversely related to the number of cells used. With low number of tumour cells (10^3) mice survived up to three weeks. With high number of cells (10^6) mice would only survive up to 5 days. However, these studies did not indicate whether this resulted in a controlled and standardized tumour growth or whether an uncontrolled tumour spreading was observed. We therefore first established the optimal dose for our experiments, as the laparoscopic setting with addition of oxygen is new in oncologic literature. We observed a tumour implantation pattern that was reproducible in the 3 different animals from each concentration group.

The suspension vehicle for the cells injected could be an important issue but some authors do not provide any information on this solution[116;117]. Those studies indicating the suspension solution, have used either Hanks balanced saline solution (HBSS), phosphate

buffered saline solution (PBS) or culture medium[113;118;119]. One study showed a difference in the number of tumour implants depending on the solution used for suspension as it seems that injecting the cells in PBS yields less tumour implants than when culture medium is used[120]. We also observed this detrimental effect of PBS when collecting mesothelial cells for culture from mice compared to collection in culture medium. Mesothelial cells collected in culture medium survived up to three weeks in contrast to cells collected in PBS which would not grow. (article submitted) In addition, studies using these tumour cell lines have different injection routes i.e. intra peritoneal, tail vein, portal vein, subcutaneous or intra splenic and different methods to assess the outcome, i.e. by directing counting tumour nodules, by weight or by tumour mass. The data from the studies where tumour nodules were directly counted was comparable to ours[117;118].

Adhesions increase with duration and insufflation pressure of pneumoperitoneum and this increase is prevented by adding small amounts of oxygen[41;54;121]. Pneumoperitoneum, especially at higher insufflation pressure will compress the capillary flow in the superficial peritoneal layer which together with diffusion of pure CO_2 from the abdominal cavity will create a gradient of hypoxia in the superficial layers. Adding 3% of oxygen to the CO_2 pneumoperitoneum may result in a partial oxygen pressure (pO_2) of 23 mm Hg, which is similar to normal intracellular pO_2. Insufflation with pure CO_2 or helium during laparoscopy has been shown to induce a hypoxic environment, with oxygen tensions as low as 5 mmHg in the abdominal wall. In addition, a recent study showed a 82% reduction of blood flow in the abdominal wall (rectus muscle) after the creation of a 10 mmHg air pneumoperitoneum for 45 minutes in rats as measured using the laser Doppler technique and a concurrent significant increase in tumour development after the injection of adenocarcinoma cells into the abdominal wall rectus muscle compared to the control group (no insufflation)[122]. A second study, showed similar results with a 71% reduction of blood flow when CO_2 pneumoperitoneum was induced together with an increase in tumour implantation when compared to the control group (not insufflated group)[123]. Suggesting that ischemia as a factor contributing to an increased risk for tumour implantation and progression after laparoscopic surgery with pneumoperitoneum.

The hypoxic environment created by the CO_2 pneumoperitoneum may cause upregulation of matrix metalloproteinase (MMP) activity[124]. These enzymes have the capability of degrading the extracellular matrix (ECM), which enables invasion of cancer cells, tumour progression

and ultimately metastasis to occur[125]. Also, the in vitro laparoscopic hypoxic environment reduces both cell-cell and cell-ECM adhesion due to a down regulation in cell surface adhesion molecules, these effects being readily reversible once normal conditions are returned[126]. Scanning electron microscopic examinations confirmed that the induction of a pneumoperitoneum induces diffuse damage of the entire mesothelial cell layer with exposure of the basal lamina and extensive repair mechanisms[42]. The exposure of extracellular matrix proteins, including laminin, fibronectin, and vitronectin, to the tumour cell surface is a possible mechanism for increased tumour cell adherence.

Another possible mechanism may be the promotion of intraperitoneal tumour cell growth by increasing interleukin-1 (IL-1) production of peritoneal macrophages, which are involved extensively in the repair mechanisms[127]. Hypoxia could modulate directly or indirectly the production of cytokines and growth factors by peritoneal mesothelial cells, macrophages and fibroblasts. Macrophages can exert both negative and positive effects on tumour growth, which is greatly affected by the microenvironment. Insufflation with CO_2 has been shown to compromise intraperitoneal macrophage activity and decrease immunologic responses[128]. Yet another study demonstrated that laparoscopy with CO_2 significantly reduces plasma tumour necrosis factor-α (TNF) production and increases IL-10 levels, confirming that CO_2 pneumoperitoneum exerts an influence on the local peritoneal environment[129].

In conclusion, this study confirms the role of a CO_2 pneumoperitoneum in tumour implantation and extends supporting data pointing at mesothelial hypoxia as an important mechanism in tumour implantation and adhesion formation. Further investigations concerning changes at molecular level under hypoxic laparoscopic environment are required.

When trying to translate these significant effects on tumor implantation to clinical practice, circumstances become difficult as most of the important factors controlled in the lab such as type of tumor, number of tumor cells, time to evaluation and immunologic response of the patient are no longer determined by us.

Port site metastases (PSM) are one of the consequences following laparoscopic oncologic surgery. The overall incidence of PSM in this trial, ie 47%, is much higher than reported previously. This might be related to the study design which was prospective in contrast with previous retrospective studies, with probably a risk of underreporting. As predictive factors,

we found a correlation with tumor grade, something not observed before. Although this seems not to be surprising, it should, however, be interpreted with caution, given the risk of spurious correlations in a small series. Factors as type and stage of tumor, duration of laparoscopy and primary debulking therapy or neo-adjuvant chemotherapy were not predictive for PSM. Stratification for randomisation obviously is imposible since these factors will only be known after laparoscopy.

The addition of 4% of oxygen to the CO_2 pneumoperitoneum unfortunately did not affect dramatically the incidence of PSM. Given the strong beneficial effects of adding 4% of oxygen observed in mouse models, we wanted to test the hypothesis of a reduced incidence of PSM through a direct effect upon the tumor cells exposed to the pneumoperitoneum, or by an indirect effect through effects upon peritoneal fluid such as an increase of angeogenic factors as was demonstrated for adhesion formation. We fully realize that the trial was not powered to detect minor differences, but given the absence of any effect and the high prohibitive number of patients necessary to detect even differences of 50% we did not consider continuation of the trial indicated. The pathophysiology of PSM is poorly understood. With this trial we may at least conclude that the CO_2 used for the pneumoperitoneum is not causally related, given the absence of effect of adding 4% of oxygen. This trial also did not confirm the hypotheis of direct soiling from ports through which tumor had been extracted. Aerosolisation and gas leaks, known as 'the chimney effect', also could not be supported, since we did not observe a higher incidence of PSM in thin women (correlation with BMI not significant) in whom the risk of gas leaks is probably higher. Also the negative correlation with length could be related to gas leaks although spurious correlations cannot be ruled out given the borderline significance and the small series. It needs to be emphasized that in this trial insufflation pressure was strictly standardized, whereas in retrospective studies the insufflation pressure might have been increased in obese women.

Whether macroscopical port site metastases affect survival remains debated given the controversial data reporting no effect and decreased survival[130]. Whether microscopic port site metastasis affect survival is unknown, and this series obviously is too small to answer the question. A fortiori, the effect of adding oxygen to the pneumoperitoneum upon patient survival in advanced stage ovarian cancer cannot be excluded today. In Balbc mice we observed a significant decrease in number and size of metastasis following injection of 2

types of syngeneic tumors, an effect that we interpreted as a consequence of the decreased trauma upon the mesothelial cell lining. Since besides an effect of the peritoneum, a direct effect upon tumor cells cannot be excluded the addition of oxygen still might affect tumor cell implantation and growth in the abdominal cavity and patient survival. In this study we did not look at this aspect as this is very subjective and would require far more inclusions.
In conclusion, this is a negative trial which nevertheless seems useful reporting, given the strong effect of adding 4% of oxygen to the CO_2 pneumoperitoneum upon tumor implantation observed in an experimental mouse model and upon adhesion formation.

C. Conclusions and future perspectives

The peritoneum is a delicate organ which is influenced by its environment in a direct way. Homeostasis of the environment is essential to decrease adhesion formation, post-operative pain and to minimize anesthesiologic problems.
Some new means of adhesion prevention such as injection of cultured mesothelial cells were proven in our laparoscopic mouse model and the findings on adhesion prevention from our model were translated to tumor implantation and most important to clinical practice. Today it is clear that conditioning of the peritoneal cavity improves recovery after surgery, with decreases post-operative pain and improves restoration of bowel transit, and CO_2 absorption. For other aspects such as adhesions and ultimately fertility and chronic pelvic pain the beneficial effects are anticipated, as they are also expected for open surgery and tumor metastasis.
Further clinical development of full conditioning of the peritoneal cavity with a new device incorporating humidification, temperature regulation and our new gas mixture will make future trials more feasible. Cooperation with industry (i.e. e-Saturnus®, Storz®, and Fisher and Paykel®) was and still is essential in the development of this new approach to laparoscopic surgery, but also the collaboration with anesthesiology (Prof. Dr. B. Vanacker), surgery (Prof. Dr. F. Penninckx and Prof. Dr. M. Miserez) and Leuven Research and Development is key to successful trials. When all parties work together in this "*Surgery Valley of Europe*", we can dream of inter university –industry collaboration putting Belgium on the surgical map.

Large randomized controlled trials are still needed to confirm these first promising, but already significant findings, but these first results make it clear that peritoneal cavity conditioning is here to stay and will become a new standard of care in surgery.

Preserving is better than restoring and with this new way of looking at and entering the peritoneal cavity, laparoscopic surgery may step into a new era.

D. Summary - English

Laparoscopic surgery advanced over the last years as it holds the promise of reduced invasiveness and thus of reduced adhesion formation. Results on reduced adhesion formation were however not as good as expected and new approaches to prevent the deleterious complications such as bowel obstruction, infertility and chronic pelvic pain were investigated.

Over the last years we developed a laparoscopic mouse model on which preventive measurements concerning gas (temperature, humidification, oxygen) and other means (i.e. dexamethasone, barriers) were tested and the most promising were selected. These favorable actions needed to be brought into clinical practice to reduce adhesion formation and to bring laparoscopic surgery to a new standard.

Tumor implantation reduced with the addition of 3% of oxygen to the CO_2 pneumoperitoneum in our laparoscopic mouse model. There was however no clinical correlation with reduced port site metastases by adding 4% of oxygen to the CO_2 pneumoperitoneum in women undergoing laparoscopy for ovarian malignancies.

Intraperitoneal injection of cultured mesothelial cells did decrease adhesion formation in a dose-response dependable way. To bring this approach to clinical practice will need the development of other means to safely remove, culture and inject mesothelial cells in humans in the peri-operative phase.

The direct beneficial effect on the peritoneum of the addition of oxygen to the CO_2 pneumoperitoneum was demonstrated in mice with scanning electron microscopy, but these experiments were not reproducible in humans. Indirect ways to measure the influence of the addition of oxygen to the CO_2 pneumoperitoneum were investigated.

Absorption of CO_2 was investigated by the study of end tidal CO_2 (ET CO_2) where we noticed a significant decrease when 4% oxygen was added to the CO_2 pneumoperitoneum. Perhaps related to this, was the significant decrease in post-operative pain and inflammation in women who received the oxygenated gas.

From ongoing mice experiments we learned that *full conditioning* of the peritoneal cavity with humidification of the gas at 32°C and with addition 4% of oxygen and 10% of nitrous oxide further reduced adhesion formation.

Clinical experiments with full conditioning of the peritoneal cavity studying ET CO_2, pain, inflammation and fluid resorption as we carried out with the addition of 4% of oxygen all showed a beneficial effect, with even some more pronounced results on pain and inflammation.

To preserve the integrity of the mesothelial layer, injection of cultured mesothelial cells or altering the insufflation gas did provide evidence, but also extensive lavage of the peritoneal cavity did improve inflammatory parameters, suggesting a depletion of inflammatory cells able to degrade the surgical lesion.

The peritoneum is a delicate organ which is influenced by its environment in a direct way. Homeostasis of the environment is essential to decrease adhesion formation, post-operative pain and to minimize anesthesiologic problems.

Preserving is better than restoring and with this new way of looking at, and entering the peritoneal cavity, laparoscopic surgery may step into a new era.

E. Summary - Dutch

Laparoscopische heelkunde kent een belangrijke opmars in de geneeskunde met het doel om ingrepen minder traumatisch te maken en zo het risico op adhesies te verminderen. De verwachtingen werden evenwel niet ingelost en de nieuwe manieren om complicaties van adhesies zoals darmobstructie, infertiliteit en chronische pelvische pijn, te voorkomen, dienen te worden onderzocht.

Over de laatste jaren ontwikkelde we een laparoscopisch muismodel, waarin verschillende maatregelen met betrekking tot het gas (temperatuur, bevochtiging, toevoegen van zuurstof) en andere (bvb. dexamethasone, barriers) werden onderzocht. De meest gunstige maatregelen werden geïdentificeerd, maar translatie naar de klinische praktijk is nodig om zo laparoscopische heelkunde naar een nieuwe standaard te brengen.

Tumorimplantatie bij muizen wordt op dezelfde manier beïnvloed als adhesievorming met een verminderen van de implantatie door toevoegen van 3% zuurstof aan het CO_2 pneumoperitoneum. Er is hiervoor echter nog geen klinische correlatie te zien op de vermindering van poortmetastasen bij vrouwen die een laparoscopie ondergaan wegens een onderliggende ovariële maligniteit.

Intraperitoneale injectie van gekweekte mesotheelcellen verminderde het aantal adhesies exponentieel met de dosis. Deze benadering van adhesiepreventie naar de kliniek brengen vergt echter de ontwikkeling van nieuwe technieken om de mesotheelcellen veilig te verwijderen, te kweken en opnieuw in te brengen in de perioperatieve fase.

Het directe gunstige effect op het peritoneum door toevoegen van zuurstof aan het CO_2 pneumoperitoneum werd aangetoond bij muizen door scanning elektron microscopie, maar dit was niet reproduceerbaar bij de mens. Indirecte manieren om het gunstige effect van het toevoegen van zuurstof aan het CO_2 pneumoperitoneum te onderzoeken werden gebruikt.

Absorptie van CO_2 werd gemeten met behulp van end tidal CO_2 (ET CO_2), waar we een significante daling zagen door het toevoegen van 4% zuurstof aan het CO_2 pneumoperitoneum. Misschien hieraan gerelateerd is de eveneens significante daling van de postoperatieve pijn en inflammatie bij vrouwen die het geoxygeneerde gas kregen.

Uit de doorgaande muizenexperimenten leerden we dat *full conditioning* met bevochtiging van het gas op 32°C en met toevoegen van 4% zuurstof en 10% lachgas de adhesies verder reduceerde.

Klinische experimenten met full conditioning van de peritoneale holte met evaluatie van ET CO2, pijn, inflammatie en vochtresorptie waren alle gunstig in het voordeel van de full conditioning, met een zelfs nog gunstiger effect op pijn en inflammatie dan met enkel toevoegen van 4% zuurstof.

Om de integriteit van de mesotheliale barrière te vrijwaren, kan injectie van mesotheelcellen of het veranderen van het laparoscopiegas gunstig zijn, maar eveneens kan uitgebreid spoelen van het abdomen de inflammatoire parameters gunstig beïnvloeden, wat suggereert dat inflammatoire cellen weggespoeld worden die een chirurgische wonden kunnen degraderen.

Het peritoneum is een delicaat orgaan dat beïnvloed wordt door zijn omgeving op een zeer directe manier. Homeostase van de omgeving is essentieel om adhesies, postoperatieve pijn en anesthesiologische problemen te verminderen.

Beschermen is beter dan herstellen en met deze nieuwe manier om naar de buikholte te kijken en om er in te gaan, kan de laparoscopische chirurgie in een nieuw tijdperk stappen.

Chapter 5

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Chapter 6

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Curriculum vitae

Personal data

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Education:

1980 - 1992:	Grammar school at the European school in Mol, Belgium
1992 - 1999:	Medicine at the K.U. Leuven., M.D., Graduation 31-7-1999
Courses 1997:	Tropical diseases, Adolescent psychiatry
1998:	Review paper: 'Verborgen anaplastisch stromaal sarcoom' with Prof. dr. I. Vergote
1999:	Review study: Five years retrospective study on vulvacarcinomas (Prof. dr. I. Vergote en dr. Katja Gaarenstroom)

Specialization Obstetrics and Gynecology:

1.8.1999 - 31.7.2001	Virga Jesse Hospital, Hasselt (B), dr. J.P. Nolens
2001	Interval examination after 2nd year (K.U. Leuven): passed cum magna laude
1.8.2001 – 31.7.2002	U.Z. Gasthuisberg, Leuven, Prof. dr. A. Van Assche
1.8.2002 - 31.7.2003	Catharina Hospital, Eindhoven, dr. P.A. van Dop
1.8.2003 - 31.7.2004	U.Z. Gasthuisberg, Leuven, Prof. dr. A. Van Assche and Prof. dr. I. Vergote
1.8.2004-onward	Gynecologist at UZ Gasthuisberg, Leuven
Sub specialization	uro-gynaecology (2 years) and gynecologic oncology (2 years) till 8/2008
7-2007	Award: Pfizer Educational Grant
7-2008	Doctoral seminar Peritoneal repair and stem cells

Societies and journals:

2000 till present	Active member of the section 'Premalign cervical lesions' of the Flemish Group of Gynecologic Oncology (FGOG)
2005 till present	GSK - CEVEB board-member (Cervarix ® vaccination evaluation board)
1-2006 till present	Member of the Gynecological infectious platform, FRIDOG - FGOG
1-2006 till present	Member of the Gynecological endoscopic platform (GEP), FGOG
5-2006	Initiation of Cervical cancer screening program (VIA / VILI) and treatment in Cameroon, Njinikom, Catholic Hospital

2006 till present	Council member of Gerson Lehrman Group
2008	contribution to “100 Clinical Cases in Obstetrics and Gynecology” by Hodder Arnold
2010 till present	Chairman of the Interest Group in Infectious diseases in ObGyn of the FGOG

Research:

2005 till present	Patricia-trial (co-investigator): HPV vaccine (GSK)
2005 till present	Laboratory work: P. Koninckx: experimental work on adhesion formation, tumor implantation, mesothelial cells and laparoscopy in mice
2006	PDA / CRF training course + certificate
2006	ERB-trial Wyeth (co-investigator): endometriosis study
2006	Macro version 3.0 training (Organon)
2006 - 2008	NOMAK-trial Organon (co-investigator): oral contraceptive study
2007 - 2009	HORMOS-trial (co-investigator): SERM
2009 - 2010	Rifaximin (co-investigator): Bacterial Vaginosis
2009 - 2010	HPV vaccination trial (Roche) (co-investigator)
2010	Hot Flushes (Merck) (co-investigator)
2010	In-Choice study (Schering-Plough) (principal investigator)
2010	Libido-trial (Bayer-Schering) (co-investigator)
2011 ongoing	LCS12 vs COC, user satisfaction study (Bayer-Schering) (principal investigator)

Teaching / Congress organization:

2001 - ongoing	Faculty and instructor of the yearly colposcopycourse of the FGOG
2004 - ongoing	Faculty and instructor of the biannual vulvacourse of the FGOG
2004 - ongoing	instructor at the Centre for experimental Surgery, CHT (laparoscopic animal models)
2004 - ongoing	training and teaching of assistants and co-assistants
5-2006	Uro-gynaecology and cervical cancer screening, Njinikom, Cameroon
28-30 sept. 2006	Faculty of the 7th PAX meeting, Universiteitshallen, Leuven
2006 - ongoing	6th year medicine: endoscopy in gynecology, urgencies in gynecology
2007	Female Cancer Program – Asia Link (EuropeAid): HPV vaccination and laparoscopic techniques
28- 31 aug. 2008	Faculty of the 6th European Conference of ESIDOG
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Chapter 7

Addenda

Gynecol Surg
DOI 10.1007/s10397-011-0658-8

ORIGINAL ARTICLE

Intraperitoneal injection of cultured mesothelial cells decrease CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model

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Received: 18 August 2010 / Accepted: 6 January 2011
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Abstract Mesothelial cells are involved in peritoneal repair, and they modulate adhesion formation. Intraperitoneal injection of cultured mesothelial cells can decrease adhesion formation. The importance of the amount of cells, of the culture methods or of the origin of the cells is still unknown. Female Balb/c mice were used in a CO_2 pneumoperitoneum-enhanced adhesion formation model. Mesothelial cells were obtained from Balb/c mice and cultured over a 3-week period. Following a pilot experiment (n=10) with injection of 400,000 mesothelial cells a dose–response study was performed with 400,000; 133,000; and 44,000 cells (n=40). Adhesion formation decreased after the injection of cultured mesothelial cells as evidenced in the first (proportion $p<0.046$, total adhesions: NS) and second experiment ($p<0.001$ for proportion and total adhesions). The reduction was moreover dose dependent with a half maximal effect with some 100,000 cells. Intraperitoneal injection of cultured mesothelial cells can decrease CO_2 pneumoperitoneum-enhanced adhesion formation in mice and the effect is dose dependent, the half maximum effect being obtained with some 100,000 cells, a number which is surprisingly similar to the number of cells harvested from one mouse.

Keywords Laparoscopy · Adhesions · Mesothelial cells · Mouse model

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Background

The pathophysiology of postoperative adhesions is not yet fully understood. Generally, peritoneal repair has been considered as a happening process at the traumatized areas consisting of an inflammatory reaction, exudation with fibrin deposition and appearance of macrophages, and mesothelial cells on the lesion within 24 h [1, 2]. Macrophages are believed to regulate healing through local secretion of cytokines and growth factors. They disappear within 8 days and are not incorporated into the repair [3]. When the fibrin matrix persists too long, constituting a scaffold for cellular ingrowth of fibroblasts, vessels and nerves, adhesion formation occurs.

Over the last decade, the entire peritoneal cavity has become recognized as a co-factor modulating adhesion formation at traumatized sites [4]. Identified as strongly adhesiogenic are pure CO_2 pneumoperitoneums, the addition of more than 4% oxygen to the CO_2 pneumoperitoneum, desiccation, and surgical trauma [5–8]. The mechanisms involved are believed to be mesothelial cell damage by hypoxia, by hyperoxia and reactive oxygen species, or directly by desiccation or trauma. These effects are moreover strongly attenuated at lower temperatures [9].

Prevention of adhesion formation in humans is based upon a model that considers interaction between traumatized areas. Since adhesions decreased when denuded areas were kept separated for more than 24–36 h, non-irritative

Published online: 28 January 2011

and resorbable barriers were developed or traumatized areas were kept separated by flotation agents [10].

The recognition of the peritoneal cavity as a co-factor in adhesion formation has resulted in minimizing mesothelial trauma during surgery through cellular normoxia (a partial oxygen pressure of 28 mm of Hg achieved by adding 4% of oxygen to the CO_2 pneumoperitoneum), humidification, slight cooling, and minimal tissue handling [4]. Mesothelial cells obviously play an important role in peritoneal repair. Within hours, a peritoneal defect (i.e., caused by trauma during surgery) is covered with macrophages and mesothelial cells, previously described as tissue repair cells [11]. The healing process starts from multiple spots over the defect from where mesothelial cells proliferate. Hence, small and large defects heal in the same short time (7–10 days) [12]. It is an attractive concept that mesothelial cells at the edge of a lesion and mesothelial cells of the opposing mesothelial surface have an increased dividing capacity, a phenomenon being maximal within 2 days suggest locally secreted factors [13].

The origin of these mesothelial or repair cells remains unclear. Macrophages could transform to mesothelial cells when in contact with a defect, subserosal mesothelial cells could migrate into the wound, free-floating mesothelial cells could implant while these cells could originate from bone marrow precursors [14]. Indeed, subserosal mesothelial cells are pluripotent, since they can differentiate to mesenchymal and epithelial cells. After peritoneal injury, they differentiate and start expressing cytokeratin while losing expression of vimentin [15, 16]. The exudate after injury contains a high number of fibroblast and epithelioid-like cells. These cells have low proliferative activity for at least 48 h [17]. That free-floating mesothelial cells are important is attractive since they are present at all times, their number increases 12 times in 2 to 5 days after injury [2], these cells were demonstrated to implant at the peritoneal lesion, and since extensive lavage with removal of these free-floating cells slows down peritoneal healing [18]. That mesothelial cells might originate from bone marrow precursors in blood, probably is less important since total body radiation has no effect upon peritoneal wound healing [19].

Intraperitoneally, injected exogenous mesothelial cells can implant into the injured area and reduce adhesion formation in the human by injection of cultured mesothelial cells obtained from the omentum. This confirms the observations in a staphylococcal peritonitis rabbit model [20, 21]. In a rat abrasion model, the injection of cultured mesothelial cells or mesenchymal stem cells derived from skeletal muscles of newborn rats decreased adhesion formation [22, 23]. Recently, an artificial peritoneum with collagen gel, fibroblasts, and overlying mesothelial cells significantly reduced adhesions [24]. Mesothelial cells grown from adhesions moreover are different from fibroblasts derived from normal peritoneum [25].

The concept that cultured mesothelial cells can decrease adhesion formation in an abrasion model thus seems well established. The amount of cells required, however, the culture method and the origin of the cells are still unclear [22]. Since an abrasion model is a pure surgical lesion requiring laparotomy, we therefore wanted to evaluate whether cultured mesothelial cells also affected CO_2 pneumoperitoneum-enhanced adhesion formation.

Materials and methods

Collection and culture of mesothelial cells

Mesothelial cells were obtained from 9 to 16-week-old inbred female Balb/c mice. Following anesthesia with intradermal pentobarbital (Nembutal, Sanofi Sante Animale, Brussels, Belgium; 0.06 mg/g) the abdomen was shaved, the animal was secured to the table in supine position, and the skin disinfected with a 50% alcohol solution. A 14-gauge catheter (Insyte, Vialon, Becton Dickinson) was inserted into the abdomen at the umbilicus and fixed leak proof with a Prolene 5/0 purse suture.

The cells were collected via a two-step procedure per mouse. First, 10 ml of phosphate-buffered saline pH 7.3 (Dulbecco's phosphate-buffered saline, DPBS) was injected, of which at least 8 ml could be re-aspirated. This first set of "free-floating cells" was centrifuged at 200 g for 10 min and the pellet was then suspended in culture medium RPMI 1640 (Lonza, BioWhittaker) supplemented with 15% fetal calf serum (CSL UK Ltd., Andover, UK), 4 mM L-glutamine (Gibco, Paisley, UK), 0.4 mg/ml of hydrocortisone (Sigma-Aldrich, Poole, UK), and antibiotics: penicillin, 120 mg/l, amphothericine (2.5 mg/l), and gentamicin (4 mg/l) as used by Foley-Comer et al. [3]. Secondly, 10 ml of DPBS with 0.25% trypsin and 1 mM EDTA 4Na at 37°C was injected through the same catheter and the mouse was softly shaken. After 10 min, the instilled fluid was re-aspirated. Next, the abdominal cavity was washed with 5 ml culture medium; to finish, the abdominal cavity was opened and gently rinsed eight times with 2 ml culture medium. All mesothelial cells obtained via this second procedure were collected together, centrifuged for 10 min at 200 g and re-suspended in culture medium. Each time two mice had undergone the procedure, cells of those mice were pooled, keeping cells of step 1 and 2 separately. Cells were then put in an incubator at 37°C with 5% CO_2. If cells were confluent, which was normally the case after 1 week, cells were split at a ratio of 1:2, after having them detached with trypsin–EDTA. After 3 weeks, cells were detached and counted for further use (pilot, 400,000 cells; dose–response, 400,000 cells; 133,000 cells; and 44,000 cells).

Immunohistochemistry

To ascertain the growing of mesothelial cells, cells from 3-week-old cultures were seeded on slides. After 2 days of culture, the slides were fixed and endogenous peroxidase activity was blocked by 0.5% H_2O_2 in methanol. Cells were evaluated for intercellular connections and for cytokeratin staining.

Mouse model

Laparoscopic surgery

Our laparoscopic mouse model has been described and validated extensively [5]. Briefly, a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity of 9–16-week-old inbred female Balb/c mice.

CO_2 pneumoperitoneum-enhanced adhesions were induced by performing two standardized 10×1.6-mm lesions in the antimesenteric border of both right and left uterine horns and on the pelvic sidewalls with bipolar coagulation (BICAPTM, bipolar hemostasis probe, BP-5200A, 5-Fr, 200 cm; IMMED Benelux, Linkebeek, Belgium) at 20 W (Autocon 200, Karl Storz, standard coagulation mode) after which the pneumoperitoneum with pure CO_2 was maintained for exactly 60 min without desiccation (using humidified gas) at 37°C in all mice. All mice in the first experiment were operated on by RC, in the second experiment some were operated by JV who was blind for the dose of mesothelial cells injected per mouse.

Adhesions were qualitatively and quantitatively scored blindly (the investigator was not informed of the group being evaluated) 1 week later by JV during laparotomy using a stereomicroscope (Wild Heerbrugg M7A, Gais, Switzerland). The quantitative scoring system assessed the percentage of the lesions covered by adhesions as follows: adhesion proportion (%)=(sum of the length of the individual attachments/length of the lesion)×100. The qualitative scoring system assessed: extent (0, no adhesions; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% of the injured surface involved, respectively); type (0, no adhesions; 1, filmy; 2, dense; 3, capillaries present); tenacity (0, no adhesions; 1, easily fall apart; 2, require traction; 3, require sharp dissection). Total adhesion score was the sum of extent, type, and tenacity. All results represent the average of the adhesions formed at the four individual sites i.e., right and left visceral and parietal peritoneum.

Experimental design

All mice underwent four standard bipolar lesions and 1 h of CO_2 pneumoperitoneum as described above. After all laparoscopic ports had been closed, 1 ml of DPBS with or without mesothelial cells was injected intraperitoneally.

The first experiment was designed to prove that cultured mesothelial cells could prevent CO_2 pneumoperitoneum-enhanced adhesions. Mesothelial cells were collected in four mice, and cultured in eight flasks of 25 cm². Two weeks later, 2×10^6 cells were harvested. In order to investigate the effect in five mice, 400,000 cells per mouse were used. The control group, which only received DPBS, consisted of five mice. The second experiment was designed to obtain a dose–response curve of adding different amounts of cultured mouse mesothelial cells in adhesion reduction. Considering the effect of 400,000 cells from the first experiment and taking into account the number of cells available, we decided to inject per group of five mice 400,000 cells; 133,000; and 44,000 cells, i.e., a logarithmic decrease. The control group which only received DPBS consisted of five mice. The study was approved by the Institutional Review Animal Care Committee.

Statistics

Statistical analysis was performed with the SAS System (SAS Institute, Cary, NC) using the non-parametric Kruskal–Wallis test to compare individual groups. All data are presented as the mean±SE.

Findings

Culturing mesothelial cells

Culturing the harvested mesothelial cells did not provide any specific difficulty. Cultured cells were initially bipolar or multipolar in appearance, but at confluence they adopted a polygonal configuration as described by Yung et al. [26]. Viability of cells as estimated by trypan blue exclusion was over 95%. The cells had intercellular connections and were positive for cytokeratin staining confirming their mesothelial nature.

The cells from the first washing did not grow but remained viable as estimated by trypan blue exclusion after 3 weeks of culture. Their number, however, was too low for subsequent experiments.

Adhesion reduction

No mice died before the end of the experiment. In the first experiment, the injection of 400,000 cultured mesothelial cells caused a reduction of the proportion of adhesion formation from 19% to 7% ($p<0.046$), and a reduction of the total adhesion score from 3.1 (±1.74) to 1.7 (±1.68) (not significant (NS), $p<0.086$).

The second experiment confirmed that mesothelial cells can reduce CO_2 pneumoperitoneum-enhanced adhesion formation. With 44,000; 133,000; and 400,000 cells the proportion of adhesions dropped from 13.5% to 11.5% (NS), 8% ($p<0.055$), and 5% ($p<0.007$), respectively. The total adhesion score (Fig. 1) showed a similar decline being for the 44,000; 133,000; and 400,000 cells not significant, $p=0.0093$ and $p=0.0025$, respectively. When adhesions are plotted against the logarithm of the concentration of cells, the amount of cells required to decrease adhesion formation by half was estimated at 100,000 cells.

Discussion

Harvesting and growing mesothelial cells for 3 weeks is so time consuming that large scale experiments become difficult. These experiments confirm and extend the observations that cultured mesothelial cells obtained after trypsinization of the peritoneal cavity can reduce adhesion formation as described in rabbits and rats with mesothelial cells obtained from the omentum [20–22]. The effect, moreover, is not limited to pure surgical models such as the abrasion model [20, 21], but also in the CO_2 pneumoperitoneum-enhanced adhesion model. The effect is moreover dose dependent. Since we know that following a 60-min CO_2 pneumoperitoneum mesothelial cells retract thus exposing directly the basal membrane [27], we may speculate that injected mesothelial cells may fill the gaps between the retracted mesothelial cells thus attenuating the adhesion-enhancing effect of the CO_2 pneumoperitoneum by preventing the deleterious effect of the peritoneal cavity. Considering that 100,000 cells are needed for a half maximal effect, the hypothesis that the cells affect the entire peritoneal cavity seems attractive, as 100,000 cells exceed what is necessary to cover the injury which only measures 1 cm by 1.6 mm. These 100,000 cells indeed cover 10 cm^2 since a confluent culture flask of 25 cm^2 contains 250,000 cells. That 100,000 cells are similar to the number of cells recovered from the entire peritoneal cavity of one mouse further lends support to the hypothesis that these cells affect the entire peritoneal cavity. Whether this observation on CO_2 pneumoperitoneum-enhanced adhesions can be extended to hyperoxia, desiccation, or manipulation, enhanced adhesions are likely but remain to be established. Indeed, considering that the driving mechanisms of all these factors are micro-denuded areas all over the peritoneal cavity, the effect of cultured mesothelial cells is expected to be similar in all. A (surgical) trauma is essential to initiate adhesion formation, but these adhesions are enhanced by factors from the peritoneal cavity, the latter being quantitatively the most important. The importance of factors in the peritoneal cavity was unequivocally proven by the observation manipulation in the upper abdomen increased adhesions at the site of the surgical lesion [8]. This, moreover, was recently proven to be mediated by acute inflammation of the entire peritoneal cavity and also explains that de novo adhesions (outside the surgical lesion) were never observed.

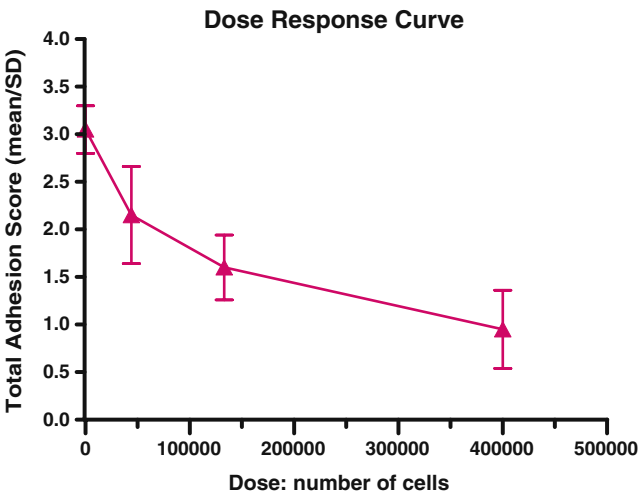

Fig. 1 Dose–response curve. *X*-axis, number of cells used per mouse

The mechanisms of adhesion reduction by cultured mesothelial cells can only be speculated upon. Whereas in pure surgical models, incorporation in the lesion as demonstrated in rats [3] seems logical, in the peritoneal cavity enhanced models incorporation in between retracted mesothelial cells of the entire peritoneal cavity cannot be excluded. Moreover, it is unclear whether the origin of the mesothelial cells and the culture conditions could be important or whether these cells actively secrete substances in the peritoneal fluid. We cannot even ascertain that the effect is specific for mesothelial cells since Alpay et al. demonstrated that also fibroblasts display different immunologic characteristics with an effect on adhesion formation [28].

It is unclear why the cells from the first washing did not grow although remaining viable. The same culture medium was used as for the detached mesothelial cells. The only difference between cells from the first washing and cells obtained after trypsinization is the fact that the former were kept for a much longer period in PBS, which indeed may damage mesothelial cells by making them lose their ability to divide (oral communication, S. Mutsaers).

The effect of mesothelial cells in reducing adhesion formation might seem less important in the pilot experiment (400,000 cells caused a 50% reduction in adhesions) than in the dose–response experiment where some 100,000 cells caused a 50% reduction in adhesions. This difference may be a consequence of the fact that cultured mesothelial cells probably stayed longer in PBS in the pilot study since our experience in handling cells was less at that moment. Also, variability in adhesion formation has to be taken into

account. Indeed, although total adhesion scores were comparable in the two control groups of both experiments (3.1) the proportion of adhesions was lower in the second experiment (13.5%) than in the first experiment (19%). Although overall very reproducible, this variability in proportion of adhesions ranging from 45% to 15% between experiments had been observed previously [29, 30] This indeed prompted us to block randomize by day while standardizing all experimental conditions as temperature, pneumoperitoneum conditions and duration, anesthesia, and experience of the surgeon as much as possible.

The clinical implications of these experiments are far reaching, whereas the use of mesothelial cells for adhesion prevention becomes attractive. Extensive lavage during and after surgery might remove besides debris and fibrin also mesothelial cells and macrophages, and the latter might be detrimental. These animal experiments combined with clinical observation at least suggest that these effects should be evaluated in detail in specific experiments. Whereas collecting and culturing mesothelial cells for adhesion prevention in the same patient is not realistic today, this method could become useful if fibroblast would be useful or if some cell lines with similar effects could be developed. In case mesothelial cells could be preserved from the peritoneal lavage during surgery as is the case with peroperative cell salvage for blood, this could be a reliable method to prevent adhesions and the detrimental effects it may cause.

Conclusions

Cultured mesothelial cells can prevent adhesion formation in mice and the effect increases with the number of cells. In Balb/c mice, the number of cells needed to reduce adhesions by 50% seems to be around 100,000. Considering that 100,000 cells cover in culture 10 cm^2, this suggests that the effect is mediated by incorporation in the entire peritoneal cavity and not only at the lesion site

Acknowledgements We would like to thank Steven Mutsaers for his valued help in preparing the culture medium, Mercedes Binda in reviewing the manuscript, and the Research Foundation–Flanders (FWO) for their grant support (no. 1700409N).

Declaration of interest Drs. J. Verguts, A. Coosemans, R. Corona, M. Praet, K. Mailova, and P. Koninckx have no conflicts of interest or financial ties to disclose.

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THE JOURNAL OF
MINIMALLY INVASIVE
GYNECOLOGY

The Addition of 4% Oxygen to the CO_2 Pneumoperitoneum Does Not Decrease Dramatically Port Site Metastases

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ABSTRACT **Study Objective:** Port site metastases (PSM) after laparoscopic surgery for advanced-stage ovarian carcinoma are a cause of concern, but the pathophysiology is unknown. Because CO_2 pneumoperitoneum was recently demonstrated to be a cofactor in adhesion formation and tumor implantation in a laparoscopic mouse model, and because both could be prevented by the addition of 4% oxygen to the CO_2 pneumoperitoneum, we wanted to test the hypothesis that PSM could be related to tumor cell hypoxia during CO_2 pneumoperitoneum.

Design: A randomized controlled pilot trial to compare the incidence of PSM in women undergoing laparoscopy with a pure CO_2 pneumoperitoneum in comparison with women with CO_2 pneumoperitoneum with the addition of 4% oxygen (Canadian Task Force classification C).

Setting: University Hospital Gasthuisberg, Leuven, Belgium.

Patients: Since January 1, 2007, 22 consecutive women undergoing laparoscopy for suspected ovarian cancer with subsequent debulking laparotomy were included.

Interventions: Diagnostic laparoscopy with 100% CO_2 versus laparoscopy with addition of 4% oxygen.

Measurements and Main Results: In the control group, 9 (47%) PSM found in 19 port sites were excised. In the CO_2+oxygen group, a similar incidence was found, that is, 8 (50%) PSM in 16 port sites. The incidence of PSM was higher in small women ($p < .018$) and in high-grade malignancies. The pathophysiology of PSM is unknown, but besides direct wound contamination, aerosolization of tumor cells and gas leaks have been suggested together with a causal relationship with the CO_2 pneumoperitoneum. Tumor cell hypoxia probably is not an important mechanism because PSM were not prevented by adding small amounts of oxygen to the CO_2 pneumoperitoneum.

Conclusion: The hypothesis of tumor cell hypoxia by the CO_2 pneumoperitoneum as a mechanism for PSM could not be confirmed. Journal of Minimally Invasive Gynecology (2008) 15, 700–703 © 2008 AAGL. All rights reserved.

Keywords: Port site metastases; Laparoscopy; Oxygen

Port site metastases (PSM) are a frequent complication of laparoscopy for malignant conditions [1], especially in advanced ovarian cancer, with an incidence of 17% PSM [2]. The risk of clinical PSM was independent of the type of tumor, tumor grade, and presence of ascites. Different mechanisms have been suggested, such as direct wound contamination during extraction of the malignant specimen through the laparoscopic port, aerosolization of exfoliated tumor cells, gas leaks known as the ''chimney effect,'' and a lowered immune response because of the carbon dioxide [3–22]. Nevertheless, concern remains that laparoscopic surgery, and more specifically CO_2 pneumoperitoneum, might be a predisposing factor for PSM.

Over the years, CO_2 pneumoperitoneum has been identified in a laparoscopic mouse model as a cofactor in adhesion formation through mesothelial hypoxia, an effect that could be prevented by adding 4% oxygen to the CO_2 pneumoperitoneum, thus restoring a physiological partial O_2 tension of approximately 30 mm Hg. This hypoxic effect is pressure and duration dependent and is associated with retraction and bulging of mesothelial cells, thus directly exposing the extracellular matrix. A similar effect was observed for the number of tumor cells implanted after intraperitoneal injection of syngeneic dispersed renal adenocarcinoma or colon-carcinoma cells. This effect could also be prevented by

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Submitted June 3, 2008. Accepted for publication July 19, 2008.
Available at www.sciencedirect.com and www.jmig.org

1553-4650/$ - see front matter © 2008 AAGL. All rights reserved.
doi:10.1016/j.jmig.2008.07.021

adding small amounts of oxygen to the CO_2 pneumoperitoneum [23–25] and Bastidas (unpublished data).

Hypoxia is known to activate the hypoxia induced factor (HIF) cascade leading to enhanced expression of angiogenic factors as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). Also, several other processes such as PGC_1-α [26] are activated. The overall effect can be enhanced cell growth instead of inhibition. Also during CO_2 pneumoperitoneum, this HIF-angiogenesis cascade is activated, as was demonstrated by the absence of peritoneal adhesion in HIF knockout mice, and after treatment with monoclonal antibodies against VEGF and against HIF [27]. We therefore wanted to investigate whether in the human, the addition of 4% oxygen to the CO_2 pneumoperitoneum could reduce the incidence of PSM, by preventing the tumor cell hypoxia caused by CO_2 pneumoperitoneum.

Materials and Methods

Patients

All patients presenting with an ovarian mass suspected to be malignant were included (n = 35). Of these 22 fulfilled the criteria of malignancy with the necessity of a second laparotomy; 13 women were excluded because 2 had an endometrial cancer, 2 did not have a malignancy, and in 9 no primary or interval debulking was performed.

Trial

An exploratory prospective randomized controlled trial was initiated with either pure CO_2 or 96% CO_2 + 4% oxygen, with the Thermoflator Plus for insufflation (Karl Storz, Tuttlingen, Germany). Randomization was done with unmarked and sealed envelopes in block randomization to be opened at the time of the laparoscopy. Pressure was standardized at exactly 15 mm Hg. At subsequent debulking, the laparoscopic ports were excised and examined by the pathologist for PSM. PSM was defined as the presence of at least 1 microscopic cluster of tumor cells observed in a single microscopic slide made from the port site. Body mass index, type of tumor, stage according to the classification of the International Federation of Gynecology and Obstetrics, and duration of laparoscopy were used for subgroup analysis.

Statistics

This trial was not powered to detect minor decreases in PSM. Indeed considering the 17% incidence of PSM, 192 women would be needed to detect a 50% reduction. Therefore an interim analysis after 1 year was performed to evaluate continuation. Analysis was done with the SAS system (SAS Institute Inc., Cary, NC), with the use of a Spearman r correlation.

Results

As expected, both groups were comparable for demographic data as length, weight, body mass index, duration of surgery, type of tumor, and pathologic condition (Table 1). No major complications such as bowel perforations, bowel fistula, major bleedings, or infections were observed.

Table 1
Comparison of the two groups

Parameter	Oxygen-group (n = 11)	Control (n = 11)
Mean height (cm) (±SD, 95% CI)	164 (±6.5,151–166)	167 (±7.5, 152–182)
Mean weight (kg) (±SD, 95% CI)	72 (±19.0, 35–74)	67 (±13.7, 40–94)
BMI (kg/m^2) (±SD, 95% CI)	26.7 (±6.5, 14–29)	24.0 (±4.3, 16–32)
Median duration of laparoscopy (range)	65 min (50–90)	72 min (45–135)
Median L-D interval (range)	12 (6–22)	18 (3–34)
Disease		
Serous papillary carcinoma	8	10
Carcinosarcoma	0	1
Adenocarcinoma	1	0
Other	2	0
Organ with carcinoma		
Ovary	10	9
Tube	0	2
Colon	1	0
FIGO Stage		
1-2	0	2
3-4	11	9
Primary debulking	10	11

BMI = body mass index; FIGO = International Federation of Gynecology and Obstetrics; L-D interval = median time in days between the laparoscopy and the subsequent debulking.

The interval between diagnostic laparoscopy and initiation of therapy (primairy debulking or neoadjuvant chemotherapy) was 18 days for the control group (n = 11) and 12 days for the study group (n = 11). In the control group 19 port sites were excised, and 9 (47%) showed microscopic PSM. In the oxygen group 16 port sites were excised, and 8 (50%) had PSM. In both groups 5 women had PSM (45%). PSM were not higher in the 12-mm umbilical port than in the 5 mm ports. PSM were not higher in ports through which specimens or biopsy specimens had been extracted.

PSM correlated with the tumor grade ($p < .05$) and negatively with height ($p < .018$), but not with body mass index. Also after correcting for these variables, oxygen addition did not have any effect on the incidence of PSM.

Discussion

The overall incidence of PSM in this trial, that is, 47%, is much higher than reported previously. This might be related to the study design, which was prospective in contrast with previous retrospective studies, probably with a risk of underreporting. As predictive factors, we found a correlation with tumor grade, something not observed before. Although this seems not to be surprising, it should, however, be interpreted with caution, given the risk of spurious correlations in a small series. Factors such as type and stage of tumor, duration of

laparoscopy, and primary debulking therapy or neoadjuvant chemotherapy were not predictive for PSM. Stratification for randomization obviously is imposible because these factors will only be known after laparoscopy.

The addition of 4% oxygen to the CO_2 pneumoperitoneum unfortunately did not dramatically affect the incidence of PSM. Given the strong beneficial effects of adding 4% oxygen observed in mouse models, we wanted to test the hypothesis of a reduced incidence of PSM through a direct effect on the tumor cells exposed to the pneumoperitoneum, or by an indirect effect through effects on peritoneal fluid such as an increase of angeogenic factors, as was demonstrated for adhesion formation. We fully realize that the trial was not powered to detect minor differences, but given the absence of any effect and the high prohibitive number of patients necessary to detect even differences of 50%, we did not consider continuation of the trial indicated. The pathophysiology of PSM is poorly understood. With this trial we may at least conclude that the CO_2 used for the pneumoperitoneum is not causally related, given the absence of effect of adding 4% oxygen. This trial also did not confirm the hypotheis of direct soiling from ports through which tumors had been extracted. Aerosolization and gas leaks, known as ''the chimney effect,'' also could not be supported, because we did not observe a higher incidence of PSM in thin women (correlation with body mass index not significant) in whom the risk of gas leaks is probably higher. Also, the negative correlation with height could be related to gas leaks, although spurious correlations cannot be ruled out given the borderline significance and the small series. It needs to be emphasized that in this trial insufflation pressure was strictly standardized, whereas in retrospective studies the insufflation pressure might have been increased in obese women.

Debate remains regarding whether macroscopic PSM affect survival given the controversial data reporting no effect and decreased survival rates [28]. Whether microscopic PSM affect survival is unknown, and this series obviously is too small to answer the question. A fortiori, the effect of adding oxygen to the pneumoperitoneum on patient survival in advanced-stage ovarian cancer cannot be excluded today. In BALB/c mice, we observed a significant decrease in number and size of metastasis after injection of 2 types of syngeneic tumors, an effect that we interpreted as a consequence of the decreased trauma on the mesothelial cell lining. Because, with the exception of the effect of the peritoneum, a direct effect on tumor cells cannot be excluded, the addition of oxygen still might affect tumor cell implantation and growth in the abdominal cavity and patient survival. In this study we did not look at this aspect because this is very subjective and would require far more inclusions. In conclusion, this is a negative trial that nevertheless seems useful, given the strong effect of adding 4% oxygen to the CO_2 pneumoperitoneum on tumor implantation observed in an experimental mouse model and on adhesion formation.

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World J Surg (2011) 35:242
DOI 10.1007/s00268-010-0772-1

World Journal of Surgery

Peritoneal Infusion with Cold Saline Decreased Postoperative Intra-abdominal Adhesion Formation: Letter to the Editor

M. M. Binda · R. Corona · J. Verguts · P. R. Koninckx

Published online: 31 August 2010
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We read with interest the article by Fang et al. [1] demonstrating that peritoneal infusion with cold saline for 30 min after surgery decreased adhesion formation in a mouse model. These results indeed confirm our data demonstrating that, in a laparoscopic mouse model, adhesion formation decreases with hypothermia [2]. The data are, moreover, consistent with our observation in mice that intraperitoneal temperatures higher than 37°C increase adhesion formation [3], as had already been observed after irrigation with saline warmer than 37°C [4].

We would be most interested to have some additional information. From the article by Fang et al., it is unclear what the impact of cold saline infusion was upon body temperature of the mice; i.e., to what extent the infusion with cold saline affects the superficial layers, the abdominal cavity, or the entire body. We also were intrigued by the results of cytokines which are nice, but difficult to judge without standard deviations. Moreover we were surprised that some effects lasted for more than 7 days, although it is generally believed that peritoneal repair is completed by postoperative day 7. Also group III is intriguing. Because we know that desiccation occurs when the abdomen is left open, this can be assumed to be the driving mechanism of the increase of adhesions and of the changes in cytokines observed. Therefore, we wonder whether the authors observed any changes in the mesothelium indicating desiccation. Their data do, however, confirm that the peritoneal cavity is a cofactor in adhesion formation at the level of the surgical trauma [5]. Surprisingly, also in open surgery, Fang et al. did not see de novo adhesions, thus confirming our observations in a laparoscopic mouse model. The time courses of the cytokine concentrations in group IV (desiccation only; no surgical trauma) and group III (desiccation + surgical trauma) are intriguing in that opening the abdomen (and desiccation) induced such an increase in adhesions, yet affected cytokines only slightly.

In conclusion, we appreciate the promising results that expand to open surgery our observations that in laparoscopy temperature affects adhesion formation.

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JOURNAL OF LAPAROENDOSCOPIC & ADVANCED SURGICAL TECHNIQUES
Volume ■■, Number ■, 2011
© Mary Ann Liebert, Inc.
DOI: 10.1089/lap.2011.0010

Letter to the Editor

Regarding: Carbon Dioxide Pneumoperitoneum Prevents Postoperative Adhesion Formation in a Rat Cecal Abrasion Model

Jasper Verguts, MD, Roberta Corona, MD, Mercedes Binda, MSc, and Philippe Koninckx, MD, PhD

Dear Editor:

It is with great interest that we read the article on the prevention of postoperative adhesions by induction of a carbon dioxide pneumoperitoneum prior to a laparotomy.[1] The mechanism by which adhesions were prevented in this model was difficult to explain. Moreover, there seems to be a contradiction with the observation that CO_2 pneumoperitoneum enhances adhesion formation and that this enhancement is pressure and duration dependent.

Traditionally, postoperative adhesion formation has been considered a local process between two injured surfaces. Following injury, exudation and fibrin deposition occur; simultaneously, repair mechanisms are started. If this fibrin is not removed within 2–3 days, the fibrin forms a scaffold for fibroblasts, activated by the repair mechanisms, to form adhesions.

We demonstrated over the last decade that this local phenomenon is essential for adhesion formation, but quantitatively not so important. Factors from the entire peritoneal cavity can stimulate this local process of adhesion formation up to some 20-fold. It was demonstrated that factors that damage the mesothelial cells lining the peritoneal cavity have this stimulating effect upon adhesion formation.[2] Identified so far as stimulating factors are mesothelial hypoxia (e.g., by using CO_2 for pneumoperitoneum), mesothelial hyperoxia (e.g., by exposing the mesothelial cells to air with a pO_2 of 150 mm Hg), desiccation, and direct trauma.[3–5] These factors, alone or combined, result in an acute inflammation of the entire peritoneal cavity, as demonstrated by biopsies, and the severity of this acute inflammation determines the degree of adhesion formation enhancement (Corona et al. Submitted). At a lower peritoneal/mesothelial temperature than 37°C, this inflammatory reaction is decreased, that is, the mesothelial layer is more resistant to damage.

As manipulation of the bowels in the upper abdomen affects in a dose-dependent way, the acute inflammatory reaction in the entire cavity and simultaneously the adhesion formation between injured areas in the lower abdomen, it is clear that the effect is one involving the entire peritoneal cavity and not only a local effect on the injured areas. As all other factors obviously affect the entire peritoneal cavity and thus also the traumatized area, it could have been theoretically argued that the effect is localized only at the injured area and that the acute inflammation is an epiphenomenon. The manipulation experiments in the upper abdomen, enhancing adhesions at distance in the lower abdomen, and the acute inflammatory reaction as a common mechanism for all factors unequivocally demonstrate that the detrimental end beneficial effects upon adhesion formation are mediated through an effect upon the entire peritoneal cavity lining.

A possible mechanism by which it might be explained that CO_2 pneumoperitoneum decreases adhesion formation induced by laparotomy is the ischemic preconditioning. This has been studied over the last two decades in coronary occlusion models, wherein ischemia and reperfusion may activate a cascade of events leading to the death of myocardial cells. There is agreement that reactive oxygen species production by the mitochondria is an essential part in the protective mechanism of ischemic preconditioning.[6] Even brief ischemic preconditioning puts the cardiac cells in a protective phenotype for several hours, but this mechanism is not well understood today. This mechanism of protection is only exerted in the reperfusion phase, not in the ischemic phase. Indeed, we noted that reactive oxygen species (ROS) scavengers protected against adhesion formation in a laparoscopic mouse model exposed to a carbon dioxide pneumoperitoneum.[7]

In conclusion, we suggest that the ischemic condition of a carbon dioxide pneumoperitoneum stimulates ROS production, which will have a protective effect in the reperfusion phase, which obviously occurs during laparotomy after laparoscopy.

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Assessment of measurement validity

To the Editor:

We read with great interest the innovative article, "Ringer's lactate solution remains in the peritoneal cavity after laparoscopy longer than expected" (1). The methodology used to calculate the peritoneal volume from ultrasound measurements is, however, poorly documented. It is unclear why the sum of three pockets was chosen and why the volumes were approximated as a cube, which is an obvious overestimation. We understand that the authors have chosen simple curve fitting without assumption of an underlying model. Simple simulation shows, moreover, that the formula is either not appropriate or unnecessarily complicated. Using the authors' formula to calculate volumes up to 300 mL, the relationship between the measured and calculated volumes approximates a straight line and could thus more easily be approximated as $y = a + bx$. Simulation for volumes of >300 mL reveals an increasingly exponential relationship. That the V2 part of the formula is negative is probably an artefact of the curve-fitting calculations used.

As an hypothesis to be proven, we would suggest that the relationship between measured and calculated volume will be exponential because for lower volumes relatively more fluid will be accumulated between the bowels, leading to an underestimation of the fluid accumulated within the pockets. The advantage of using simple model assumptions to calculate the volume would be that SEs can be estimated, which is useful for clinical conclusions. It would not be surprising if SEs would be much more important at low volumes, and indeed variability of remaining volumes after 24 hours is very important. Therefore, instead of the conclusion that after 24 hours an important amount of Ringer's lactate is still present, the reality might be that after 24 hours the volume is hardly different from that in the control group. This is, moreover, suggested by the disappearance curve of Ringer's lactate, which is more likely to be exponential than linear, as shown.

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doi:10.1016/j.fertnstert.2005.10.003

Reply of the Authors:

We thank Dr. Koninckx and colleagues for their interest in and appreciation of our work (1). We agree that the methodology used to calculate the intraperitoneal volumes according to ultrasound measurements was not described in full detail. However, we do not think the main issue of our article was the method of calculation of fluid volumes but rather the comparison at different times of the volumes remaining in the peritoneal cavity between patients in the experimental (intraperitoneal infusion of 300 mL of Ringer's lactate) and control arms. To highlight any difference and minimize any operator bias, a double-blind, randomized setting was chosen.

In the only similar study published in the literature (2), the comparison of the measured volumes in different groups was reported without any description of the methodology used. We can only guess that the formula adopted was the one for the prolate ellipsoid (volume = 0.523 × width × length × height). The use of a simple formula such as this could have been undoubtedly more convenient; however, the prolate ellipsoid formula has not been validated for the calculation of volumes in the pelvis. This is the reason we conducted a preliminary study (which, we think, is reported in sufficient detail on page 150 of our article), in which we instilled incremental amounts of fluid in the pelvis, up to 300 mL, obtaining real-time measures of any fluid pocket observed at ultrasound. These measurements were given to a statistician, who elaborated the best-fitting mathematical model (not the most practical!) to our set of data. This formula is not intended for volumes >300 mL.

As to the last comment of Dr. Koninckx and colleagues, we are convinced of the opposite: the measurements are more accurate for lower volumes, in which the fluid is confined in a small pocket in the Douglas, with relatively fix boundaries, and in which bowel loops can displace only minimal amounts of fluid. For larger volumes, the surface of the fluid collection that comes into contact with the bowel loops is much greater, and the measurements probably become much more inaccurate and with greater intraobserver and interobserver variability. We therefore cannot assent to the conclusion that after 24 hours the volumes might hardly be different in the two groups.

Again, we thank Dr. Koninckx and colleagues for their thoughtful comments.

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Ultrasound Obstet Gynecol 2010; **35**: 589–592
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/uog.7632

Accuracy of peritoneal fluid measurements by transvaginal ultrasonography

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KEYWORDS: fluid; measurement; ultrasound; volume

ABSTRACT

Objective *To assess the accuracy of the assessment of peritoneal fluid volumes of up to 1 L by transvaginal ultrasonography and to re-evaluate the formula used to calculate total volume from the dimensions of the largest pocket.*

Methods *Patients (*n = *13) enrolled for a minor laparoscopic procedure were prospectively recruited. At the end of the procedure, with the patient in the 30° anti-Trendelenburg position, Ringer's lactate was instilled into the abdomen in discrete steps up to 1 L. Following equilibration the diameters of the single pocket of fluid were measured by transvaginal ultrasonography in order to calculate the volume, and regression models were used to determine the relationship between this and the instilled volume. The body mass index (BMI) of the patient was evaluated as a parameter for predicting the instilled volume more accurately.*

Results *The intra-abdominal fluid volume could be calculated from the measured volume using a quadratic regression equation with an overall coefficient of variation of 19%. In individual patients, changes in volume could be assessed with a coefficient of variation of 7.3%. BMI was not found to be a significant parameter in relating the measured to the instilled volume.*

Conclusion *Transvaginal ultrasound in a standardized setting can accurately estimate the volume of peritoneal fluid, with the accuracy consistent for small and large volumes. Changes in peritoneal fluid volume over time in the same individual can be measured more accurately than the total volume present. Copyright © 2010 ISUOG. Published by John Wiley & Sons, Ltd.*

INTRODUCTION

A non-invasive method of accurately estimating the volume of intra-abdominal fluid would be clinically useful for assessing the volume of intra-abdominal hemorrhage or ascites. It would also permit evaluation of the retention time of anti-adhesion flotation fluids instilled after abdominal surgery, which is believed to be a key factor in their effectiveness. Moreover it would permit comparison of the clearance rates of potential anti-adhesiogenic fluids. For smaller volumes, up to 250 mL, clearance rate data are available[1], but information is scarce for larger volumes. For abdominal tumors, such as ovarian, gastric and mesothelial cancers, an accurate assessment of the ascites volume may improve preoperative assessment and can be helpful in timing adjuvant therapy. Follow-up of cancer treatment may also be evaluated more accurately.

Transabdominal ultrasonography is useful for detecting free peritoneal fluid, whereas the transvaginal approach is highly sensitive in the detection of free fluid in the pelvis[2–4]. In the supine position intra-abdominal fluid tends to accumulate in Morison's pouch, i.e. between the liver in front and the kidney. One study reported that, using transabdominal ultrasound, 1000 mL of instilled peritoneal fluid could be detected in Morison's pouch with a sensitivity of 97%[5]. The position of the patient is important, as another study showed that the median amount of fluid instilled into the abdomen necessary for fluid to be visualized in Morison's pouch on ultrasound was 400 mL for women in the Trendelenburg position and 700 mL for women in the supine position[6].

Recently Muzii *et al.*[7] proposed measurement of the volume of the largest pocket of peritoneal fluid by transvaginal ultrasound for calculating the total volume of intraperitoneal fluid. Pockets were considered cuboid and the peritoneal fluid volume was calculated according

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Accepted: 29 September 2009

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to the formula:

$$y\ (mL) = 34.217 + 2.45V - (1.32 \times 10^{-2}V^2) + (4 \times 10^{-5}V^3) - (4 \times 10^{-8}V^4),$$

where V is the volume calculated from three dimensions of the largest pocket (length, depth and width (cm)). The accuracy of the calculated volumes was not reported and only volumes up to 300 mL were assessed.

We therefore decided to assess the accuracy of the measurement of peritoneal fluid volume by transvaginal ultrasonography in order to extend the range of applicability up to volumes of 1 L and to re-evaluate the formula used, which might be unnecessarily complicated.

PATIENTS AND METHODS

Following approval by the central ethical committee of the University Hospital Leuven, 15 patients undergoing minor laparoscopic surgery (tubal sterilization, diagnostic laparoscopy) were enrolled after informed consent. Two were excluded because of extensive adhesions. As well as age, the weight, height and body mass index (BMI) of the patients were also recorded since these could influence the collection of fluid in the pelvis. After laparoscopy with a pneumoperitoneum pressure of 15 mmHg, all peritoneal fluid was aspirated from the pelvis and carbon dioxide was carefully removed. The patient was then placed in a 30° reverse Trendelenburg position and a catheter was placed 5 cm through the umbilical port in the direction of the pouch of Douglas. Ringer's lactate was infused through the catheter using a Wolf 2220 Hystero Pump® (Richard Wolf GmbH, Knittlingen, Germany) in four discrete steps of 100, 250, 500 and 1000 mL (increments of 150, 250 and 500 mL), i.e. a logarithmic increase. An equilibration time of 2 min was used since in initial pilot experiments little to no change occurred after this period. No pockets containing fluid other than the pouch of Douglas were visualized.

The three dimensions (length, height and depth) of the pocket were measured by ultrasonography using an Esaote Technos system with a 6.5-MHz transvaginal probe (Esaote, Genoa, Italy) (Figure 1). Each measurement was repeated three times with a 1-min interval and the mean of each set of measurements was used for the final calculation of volume. For larger volumes, the fluid spread above the pouch of Douglas anterior and posterior to the uterus. When this occurred the volume of the uterus was subtracted from the estimated volume. The volume of the uterus was calculated from its three dimensions, considering it as an ellipsoid ($V = \text{length} \times \text{height} \times \text{depth} \times \pi/6$)[8,9] (Figure 2). After the ultrasound measurements had been obtained the instilled fluid was drained through the catheter.

Statistical analysis

The means of the volume measurements performed in triplicate were used to determine the relationship between the known instilled volumes and the measured volumes. Regression analysis was performed with the instilled volume as the independent variable and the volume of the fluid pocket calculated using ultrasound measurements as the dependent variable. Several models were evaluated using JMP Version 7.0 (SAS Institute Inc., Cary, NC, USA), with the best selected based on the trade-off between goodness of fit (defined by the r^2 of the regression equation) and complexity.

Subsequently the accuracy of prediction, i.e. the prediction intervals around the regression line, was calculated first for all measurements and then for the sets of measurements obtained in each patient using MlwiN 2.0 (Center for Multilevel Modelling, University of Bristol, Bristol, UK). The former indicates the accuracy

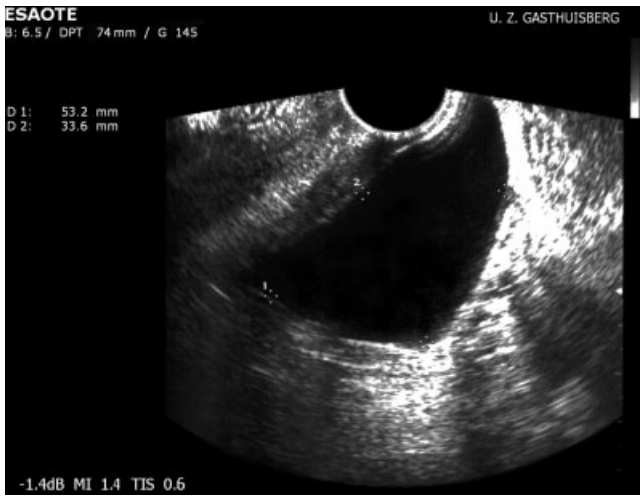

Figure 1 Transvaginal ultrasound image of the pelvic pocket, without involvement of the uterus, following infusion of 250 mL of Ringer's lactate. Two dimensions – D1 and D2 – are indicated. These were used together with the third dimension to calculate the volume.

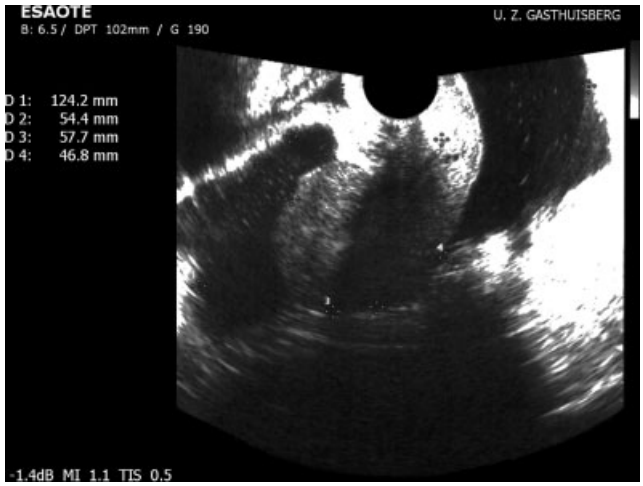

Figure 2 Transvaginal ultrasound image of the pelvic pocket, extending above the uterus, following infusion of 500 mL of Ringer's lactate. The volume of the pocket was calculated from its three dimensions (D1 and D2 indicated), from which the volume of the uterus was subtracted (D3 and D4 indicated).

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of prediction of the absolute volume from the volume calculated using ultrasound measurements; the latter is the accuracy of observed differences between sequential measurements in the same patient. The significance of the difference between overall variance and within-patient variance was determined using an *F*-test. Finally weight, height and BMI were evaluated as contributing factors for the differences between individual patients.

RESULTS

The patients had a mean age (± SD) of 33.4 ± 7.8 years, with a mean height of 1.65 ± 0.06 m and mean weight of 66.5 ± 10.1 kg. The mean BMI was 24.4 ± 3.9 kg/m^2.

The measurements of the largest pockets performed after 2, 3 and 4 min of equilibration were not significantly different, confirming that 2 min of equilibration was sufficient for reliable estimations.

Using untransformed instilled volumes and the corresponding ultrasound-calculated volumes the best prediction of the measured peritoneal fluid volume proved to be a second degree or quadratic regression curve, providing the best trade-off between goodness of fit and complexity. The measured ultrasound volume (Y (mL)) was found to be related to the instilled volume (V (mL)) by means of the quadratic regression equation:

$$Y = -31.9 + 1.3429\ V - 0.0005023V^2.$$

Using this equation the volume V can be estimated from a measured ultrasound volume Y:

$$V = 1336.7 - (1\,723\,204.9 - (1990.8421 \times Y))^{1/2},$$

for values of Y between 0 and 865.6 mL. Using this formula, the mean differences as proposed by Bland and Altman[10] between estimated ultrasound and actual instilled volumes (bias), together with their 95% confidence intervals (i.e. the mean of differences ± 1.96 times the standard error of the mean of differences), were obtained for instilled volumes of 100, 250, 500 and 1000 mL (Table 1). In Figure 3 the 95% band of prediction intervals (i.e. mean ± 1.96 times the SD of individual measurements) around the regression curve is indicated. The fitted quadratic curve was significant ($P < 0.0001$ for the second-degree coefficient) and accurate ($r^2 = 0.89$, $P = 0.0001$).

Table 1 Mean differences between estimated volumes (derived using the ultrasound measurements and regression equation) and actual instilled volumes together with their 95% CI (i.e. the mean of differences ± 1.96 times the SD of the mean of differences)

Instilled volume (mL)	*Mean of differences (95% CI)*
100	−40 (−66 to −14)
250	21 (−44 to 86)
500	23 (−80 to 126)
1000	−79 (−215 to 57)

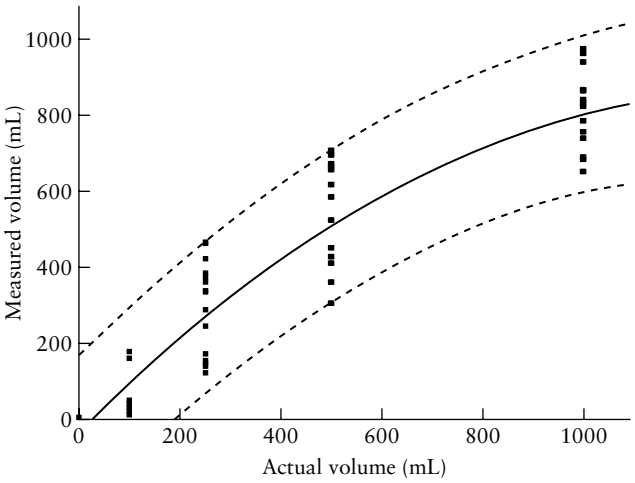

Figure 3 Quadratic regression curve Y = −31.9 + 1.3429 V − 0.0005023 V^2 (——) of the volumes measured by ultrasound (Y (mL)) versus the actual instilled volumes (V (mL)) with 95% prediction intervals (i.e. the mean ± 1.96 times the SD of the individual measurements) (- - - -).

The accuracy of the prediction of a peritoneal fluid volume, calculated from all measured volumes, had an overall coefficient of variation ((SD/mean) × 100) of 19.3% (between-patient plus within-patient variation). When the sets of measurements obtained in each patient were considered separately, the coefficient of variation was only 7.3% (within-patient variation), which is significantly lower than the overall coefficient of variation ($F(12,51) = 7.0$, $P < 0.001$). Given this important difference between within-patient accuracy and overall accuracy we evaluated the patient characteristics of height, weight and BMI as potential contributing factors. However, the results of this analysis did not reach statistical significance.

DISCUSSION

The volume of intra-abdominal fluid can be quantified reasonably accurately, at least up to 1 L, by measuring the largest pocket of fluid by transvaginal ultrasonography in a patient in the 30° anti-Trendelenburg position for at least 2 min. The procedure was standardized with the women in this position, with direct instillation, in order to collect most fluid present in the pouch of Douglas, for which we found an equilibration time of only 2 min. It is highly unlikely that minor variations in anti-Trendelenburg position will affect the measurements, 30° anti-Trendelenburg being chosen as the best compromise in the operating theater. These data, however, do not permit evaluation of how quickly fluid accumulates in the pelvis in patients who have been in the supine position, nor how standing up or walking affects fluid collection. This will be evaluated in subsequent experiments in non-anesthetized patients in order to ascertain the minimum equilibration period needed for reliable fluid measurements, and in order to evaluate how the degree of Trendelenburg position affects fluid collection in the

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pelvis. It is common knowledge that during laparoscopy the 30° anti-Trendelenburg position does not enable fluid in the upper abdomen to collect in the pouch of Douglas. Moreover it cannot be excluded that fluid collection in non-anesthetized patients occurs initially in multiple pockets, and that the equilibration time necessary for all the fluid to collect in one pocket might be much longer.

Peritoneal fluid is present between the bowels and a fraction of it will collect by gravity, the bowels having a lower density because of gas contained in them. Any measurement of peritoneal fluid pockets therefore can be expected to be an underestimation of the real volume of peritoneal fluid. Since using the ultrasound measurements of the largest pocket to calculate peritoneal fluid considering the pocket as an ellipsoid ($V = L \times H \times W \times \pi/6$) resulted in significant underestimation of the real amount of fluid, we decided to consider the pocket as a cuboid ($V = L \times H \times W$). However, as the difference between the two approximations (an ellipsoid and a cuboid) is a constant, i.e. $\pi/6$, this will not have affected the form of the regression models nor the estimated peritoneal volumes derived from them.

The estimation of peritoneal volume from the ultrasound measurement of the largest pocket collected in the pouch of Douglas was accurate, with an estimated overall coefficient of variation of 19.3%. Since the within-patient coefficient of variation, i.e. of sequential measurements of different volumes in the same patient, was only 7.3%, it was suspected that only some individual patient characteristics would contribute to the overall variability. Although it is logical to expect that fluid collection might be different in short and tall women or might be affected by obesity, these factors did not reach statistical significance in our analysis, possibly as a consequence of the small number of patients. It is unclear whether the additional measurement of all smaller pockets, as performed by Muzii *et al.*[7], could improve accuracy. This could not be estimated in our model, since in our set-up fluid accumulated in one major pocket only.

Since the within-patient variability was found to be only 7.3%, this method can be used to detect changes in peritoneal fluid volume over time. Indeed, a study involving serial measurements in individual patients, e.g. when estimating resorption of instilled peritoneal fluid, would need only 10 patients to detect decreases of 15% in volume at the 0.05 significance level with a power of 80%. This obviously is important for evaluation of the retention/clearance time of flotation agents instilled after abdominal surgery to prevent or reduce adhesion formation. Indeed the fast clearance of crystalloids has been suggested as an explanation of their low efficacy, and has been used as a major argument to postulate that efficacy increases with retention time[11]. Whether retention time is affected by the extent of peritoneal lining damage or by the duration of surgery is unknown. We anticipate that this method will permit investigation of the retention time of various fluids and the effect of surgical trauma, for which it is important to understand the effect upon adhesion formation. Similarly this method will permit monitoring of the volume of ascites during chemotherapy in cancer patients. We anticipate that the use of ultrasound machines with a volumetric function could facilitate these calculations. Before routine use, reliability will have to be proven and cost and availability could be other considerations[12,13].

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