

2015

## A rat model to study the effects of gaseous exposure of the peritoneum during open abdominal surgery: tissue oxygenation and mesothelial damage

Jean Kathleen Marshall

*University of Wollongong*

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“A rat model to study the effects of gaseous exposure of the peritoneum during open  
abdominal surgery: tissue oxygenation and mesothelial damage”

A thesis submitted in fulfilment of the requirements for the award of the degree

Doctor of Philosophy (PhD)

from

UNIVERSITY OF WOLLONGONG

by

Jean Kathleen Marshall BE (Biomedical) Hons I, BSc, PGCert (Health Sciences)

Graduate School of Medicine

2015



## Thesis Certification

I, Jean K Marshall, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Graduate School of Medicine, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Jean K Marshall

10 November 2015



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## **List of Abbreviations**

BMI	= body mass index
C	= anaesthesia only control
CO <sub>2</sub>	= carbon dioxide
COX-2	= cyclooxygenase 2 isoform
HMDS	= hexamethyldisilazane
LO:	= laparotomy with controlled air flow
LI:	= laparotomy with insufflation of humidified-warm CO <sub>2</sub>
PaO <sub>2</sub>	= arterial oxygen partial pressure
PO <sub>2</sub>	= oxygen partial pressure
PtO <sub>2</sub>	= tissue oxygen partial pressure
SpO <sub>2</sub>	= arterial haemoglobin oxygen saturation
SSI	= surgical site infection
USA	= United States of America
USD	= United States dollar

## **Abstract**

**Background:** Complications such as surgical site infection and post-operative adhesion formation following abdominal surgery are common and there has been a call for new approaches to reduce these complications. Insufflation of humidified-warm CO<sub>2</sub> to protect the abdominal cavity during open abdominal surgery has been proposed as a promising new therapeutic invention; however mechanisms of action have not yet been fully investigated. After first setting out to develop a controlled animal model, this research has tested the hypotheses that humidified-warm CO<sub>2</sub> can increase tissue oxygen partial pressure and that humidified-warm CO<sub>2</sub> can reduce loss of peritoneal mesothelium that may be caused by exposure of the peritoneum to the dry operating room.

**Methods:** A systematic review was conducted to determine the current state of knowledge regarding the effect of gaseous exposure of the peritoneum during abdominal surgery on loss of peritoneal mesothelium. A rat model was then developed to allow the examination of gaseous exposure of the peritoneum during open abdominal surgery. Using that model, randomised cross-over trials were conducted to investigate the effect of humidified-warm CO<sub>2</sub> on sub-peritoneal tissue oxygen partial pressure, and to elucidate the relative effect of dry versus humidified-warm CO<sub>2</sub>. Finally, a controlled trial was conducted to determine whether exposure of the peritoneum to the operating room environment during open abdominal surgery causes sufficient desiccation to result in loss of peritoneal mesothelium, and

furthermore, whether any mesothelial loss can be reduced by the insufflation of humidified-warm CO<sub>2</sub>.

**Findings:** The systematic review of the literature revealed that the creation of pneumoperitoneum for laparoscopic surgery can cause loss of areas of the mesothelial layer of the peritoneum. The degree of disruption appears to be reduced at lower pneumoperitoneum pressures and by the reduction of desiccation via warming and humidification of the insufflation gas. However, the systematic review revealed a gap in current knowledge regarding understanding of the effect of gaseous exposure during open abdominal surgery, including whether desiccation of the peritoneum during open abdominal surgery is sufficient to damage the peritoneal mesothelium. Therefore, a controlled rat model was developed to investigate the effect of gaseous exposure during open abdominal surgery. Key properties of the model were: assurance of adequate exposure of the peritoneum; modelling of operating room air flow; creation of a CO<sub>2</sub> environment within the constraints of the model; appropriate mechanical ventilation and anaesthetic/analgesic management protocol; measurement of tissue oxygen partial pressure; optimisation of protocols to ensure protection of the mesothelium during tissue fixation and processing. Results of investigations utilising the model showed firstly that insufflation of humidified-warm CO<sub>2</sub> caused an immediate and clinically significant increase in tissue oxygen partial pressure. Two subsequent sets of randomised cross-over trials showed that exposure to CO<sub>2</sub> and exposure to humidity/warmth individually increased PtO<sub>2</sub> and that these effects were additive. Finally, the trial investigating mesothelial loss showed that

simply exposing the peritoneum to standardised operating room airflow during open abdominal surgery led to significant loss of peritoneal mesothelium that was prevented by insufflation of humidified-warm CO<sub>2</sub>. Key methods that were utilised during this research were: design of a rat model and manipulation of conditions during experimental use; endotracheal intubation; blood gas measurement; filtering and displaying signals for monitoring of heart-rate, body temperature and pulse oximetry during general anaesthesia; measurement of tissue oxygen partial pressure; scanning electron microscopy; light microscopy; fluorescent microscopy; biochemical assay.

**Conclusions:** In a carefully designed rat model of open abdominal surgery, humidified-warm CO<sub>2</sub> insufflation increased tissue oxygen partial pressure and prevented loss of peritoneal mesothelium. These are important mechanisms in the prevention of post-operative complications. Humidified-warm CO<sub>2</sub> insufflation during abdominal surgery may have important clinical implications.

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## **Contributions**

With guidance from supervisors, the experiments contained in this thesis were designed, conducted, analysed and interpreted by Jean Marshall with the following additional contributions. Mrs Vanessa Sluyter anaesthetised rats for all the experiments included in this thesis; completed tissue sectioning and light microscopy image capturing. The TUNEL assay was contracted to staff at the microscopy unit at the University of New South Wales, Sydney, with methods and re-agents sourced and supplied by Jean Marshall. COX-2 immunohistochemistry was conducted, while teaching Jean Marshall, by Dr Sandra Carpinteri at the Peter MacCallum Cancer Institute, Melbourne. Dr Carpinteri was also the second observer for the COX-2 data analysis. Ms Pernilla Lindner assisted with data collection for several animals in the tissue oxygenation experiments as part of her undergraduate research training. Professor Noel Tait and Dr Angelique Riepsamen were the second assessors for the morphological image assessment and systematic review, respectively.

## **List of publications**

### **Full peer reviewed publications:**

Marshall, J. K., P. Lindner, N. Tait, T. Maddocks, A. Riepsamen and J. van der Linden (2015). "Intra-operative tissue oxygen tension is increased by local insufflation of humidified-warm CO<sub>2</sub> during open abdominal surgery in a rat model." PLoS ONE 10(4): e0122838.

### **Oral presentations:**

15th Asian Pacific Federation of Coloproctology Congress, Melbourne, Australia:

Marshall, J. K., P. Lindner, A. Riepsamen, T. Maddocks, N. Tait and J. van der Linden (2015). Insufflation of humidified-warm CO<sub>2</sub> during open abdominal surgery increases sub-peritoneal tissue oxygen partial pressure and reduces loss of peritoneal mesothelium in a rat model.

Tripartite Colorectal Meeting, Birmingham, UK: Marshall, J. K., P. Lindner, A. Hoolahan, N. Tait, J. van der Linden and C. Georgiou (2014). "Insufflation of warm, humidified CO<sub>2</sub> during open abdominal surgery reduces loss of peritoneal mesothelium and increases sub-peritoneal tissue oxygen tension in a rat model." Colorectal Disease 16(Suppl 2): 4-37.

### **Poster presentations:**

European Society of Coloproctology 10th Scientific & Annual Meeting, Dublin, Ireland:

Marshall, J. K., P. Lindner, N. Tait, A. Riepsamen and J. van der Linden (2015). Intra-

operative tissue oxygen tension is increased by local insufflation of humidified-warm CO<sub>2</sub> during open abdominal surgery in a rat model.

Royal Australasian College of Surgeons Annual General Meeting, Singapore: Marshall, J. K., P. Lindner, A. Hoolahan, N. Tait, J. van der Linden and C. Georgiou (2014).

Insufflation of warm-humidified CO<sub>2</sub> during open abdominal surgery reduces loss of peritoneal mesothelium and increases sub-peritoneal tissue oxygen tension in a rat model.

The Surgical Research Society of Australasia 48th Annual Meeting, Adelaide, Royal Australasian College of Surgeons: Marshall, J. K., N. Tait and C. Georgiou (2011).

Morphological integrity of the peritoneal mesothelium – How does surgery really affect it? A systematic review.

# 1 Chapter One

## 1.1 Introduction

The risk of peritoneal disease is highlighted from the first reports of elective abdominal surgery. The fifth reported patient to ever undergo elective abdominal surgery died of 'peritoneal inflammation', and the eighth surgery was abandoned due to extensive peritoneal adhesions (Ellis 1996). Today, post-operative infection and adhesion formation remain common and expensive complications following abdominal surgery (Klevens, Edwards et al. 2007; Okabayashi, Ashrafian et al. 2013), especially following open abdominal surgery (Fleming, Kim et al. 2010; Howard, Datta et al. 2010; Schnuriger, Barmparas et al. 2010; Utsumi, Shimizu et al. ; Aimaq, Akopian et al. 2011; Suh, Jeong et al. 2012). Current quality management programmes, including cleanliness and antibiotic regimes, prevent only 20% of hospital acquired infections (Harbarth, Sax et al. 2003). Similarly, with regard to preventing post-operative adhesion formation, recent literature reviews recommend the use of meticulous surgical technique, but routine use of any technology that consistently reduces post-operative adhesions formation is not yet recommended (Alpay, Saed et al. 2008; Schnuriger, Barmparas et al. 2010). There have been calls for additional technologies to further reduce the incidence of both surgical site infection (SSI) (Yoshida, Nabeshima et al. 2007; Aimaq, Akopian et al. 2011) and post-operative adhesion formation (Alpay, Saed et al. 2008).

The use of a CO<sub>2</sub> gas diffuser and an active humidification system is a new therapeutic invention proposed to protect the peritoneal mesothelium and has thereby been hypothesised to contribute to the reduction of the risk of both SSI (Persson and van der Linden 2008) and post-operative adhesion formation (Persson and van der Linden 2009) following open abdominal surgery. The invention insufflates humidified-warm CO<sub>2</sub> into the peritoneal cavity during open abdominal surgery; however, further research is required to completely understand the mechanisms of action.

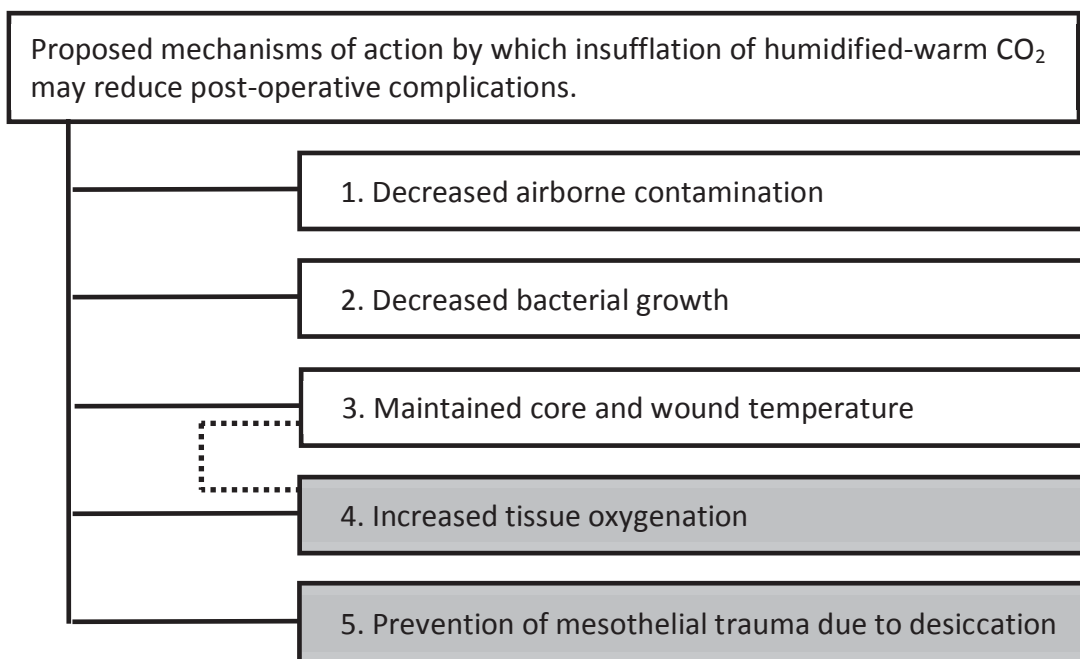
Insufflation of humidified-warm CO<sub>2</sub> optimises the gaseous environment of the peritoneal cavity during open abdominal surgery. Humidified-warm CO<sub>2</sub> is diffused into the open peritoneal cavity at a low velocity, yet at a flow rate high enough to create a local environment with a high concentration of CO<sub>2</sub> (Persson and Van der Linden 2003; Svenarud, Persson et al. 2003; Svenarud, Persson et al. 2003; Persson, Svenarud et al. 2004).

Insufflation of CO<sub>2</sub> into the peritoneal cavity is already common practise in laparoscopic surgery, in which the gas is used to create intra-peritoneal pressure and allow the surgeon space to see within the normally closed peritoneal cavity. The introduction of laparoscopic surgery has made important reductions in the risk of both post-operative adhesion formation (Schnuriger, Barmparas et al. 2010) and SSI (Fleming, Kim et al. 2010; Howard, Datta et al. 2010; Utsumi, Shimizu et al. 2010; Aimaq, Akopian et al. 2011; Suh, Jeong et al. 2012). Laparoscopy also presented the opportunity to easily optimise the gaseous exposure of the peritoneum. Modification of the temperature, humidity, and gas composition within the peritoneal cavity in

animal models of laparoscopy has reduced adhesion formation (Binda, Molinas et al. 2004; Binda, Molinas et al. 2006; Peng, Zheng et al. 2009; Corona, Binda et al. 2013), tumour implantation (Binda, Corona et al. 2014), and severity of intra-abdominal infection (Chatzimavroudis, Pavlidis et al. 2009). These results have recently been translated to a significant reduction in adhesion formation in humans (Koninckx, Corona et al. 2013). The striking success of insufflation of CO<sub>2</sub> into the peritoneal cavity during laparoscopic surgery provides a theoretical foundation for the introduction of insufflation of humidified-warm CO<sub>2</sub> into the peritoneal cavity during open abdominal surgery.

During open abdominal surgery, insufflated CO<sub>2</sub> will sit within the peritoneal cavity because it is heavier than air and because the Coanda effect causes the CO<sub>2</sub> to flow out of the wound attached to the adjacent surface (Cater and van der Linden 2014). The proposed mechanisms of action by which intra-abdominal insufflation of humidified CO<sub>2</sub> may reduce surgical complications are fivefold (Persson and van der Linden 2008; Persson and van der Linden 2009; Binda, Corona et al. 2014) and are summarised in Figure 1-1. The first three mechanisms of action in Figure 1-1 (boxes with no fill) have undergone preliminary investigation with promising results. Firstly, there is evidence that the CO<sub>2</sub> deflects nearly all airborne contamination (Persson and van der Linden 2004). Results confirm that CO<sub>2</sub> can act as an anti-biotic, reducing the growth of bacteria within the wound (Persson, Svenarud et al. 2005). In addition, human studies show increased core and wound temperature during open surgery (Frey, Svegby et al. 2010; Frey, Janson et al. 2012; Frey, Janson et al. 2012). The final two mechanisms of

action proposed for the use of humidified-warm CO<sub>2</sub> during open abdominal surgery, shown highlighted in Figure 1-1 (Persson and van der Linden 2008; Persson and van der Linden 2009), remain un-tested. There has been no evaluation of whether insufflation of humidified-warm CO<sub>2</sub> into the peritoneal cavity during open abdominal surgery can increase tissue oxygenation, or reduce peritoneal trauma due to desiccation.



**Figure 1-1: Proposed mechanisms of action for the prevention post-operative complications by insufflation of warm-humidified CO<sub>2</sub> into open surgical cavities.**

**1-3 (no fill) = some existing evidence; 4-5 (grey fill) = no evidence, mechanisms that will be the focus of this thesis. The dashed line between improved core and wound temperature and improved tissue oxygenation indicates that these two mechanisms overlap.**

Insufflation of humidified-warm CO<sub>2</sub> is used in preference to humidified-warm air for several reasons. Firstly, insufflation of CO<sub>2</sub> has been shown to reduce air-borne contamination of a surgical wound model compared to insufflation of clean air at the same flow rate, most likely because of the higher density of CO<sub>2</sub> (Persson and van der

Linden 2004). Insufflation of CO<sub>2</sub> is also significantly more successful than air insufflation at maintaining the temperature of a surgical wound model (Persson, Elmqvist et al. 2004). The superior performance of CO<sub>2</sub> to reduce heat loss and evaporation is maintained when the gases are humidified and warmed (Persson, Elmqvist et al. 2004). In addition CO<sub>2</sub> is also hypothesised to increase tissue oxygenation, as described in detail below, a physiological effect that is not expected with exposure to air (Persson and van der Linden 2008).

#### 1.1.1 Tissue oxygenation

An investigation is required to determine whether intra-operative insufflation of humidified-warm CO<sub>2</sub> into the open peritoneal cavity can increase sub-peritoneal tissue oxygen partial pressure (PtO<sub>2</sub>). Tissue oxygen partial pressure is a strong predictor of SSI (Hopf, Hunt et al. 1997; Greif, Akça et al. 2000), as oxygen pressure drives the production of bactericidal superoxide by phagocytes (Babior 1978; Babior 1978; Allen, Maguire et al. 1997). Maintenance of intra-operative PtO<sub>2</sub> is also important for wound healing including leak-free healing of colonic anastomoses (Sheridan, Lowndes et al. 1987). As such, increasing peri-operative PtO<sub>2</sub> is a common recommendation for the prevention of surgical complications (Sessler 2006; Hopf and Rollins 2007; Yoshida, Nabeshima et al. 2007; Hunt, Gimbel et al. 2008; Davis and Rivadeneira 2013). However, most methods to increase PtO<sub>2</sub> are applied systemically and therefore delivery of oxygen relies on adequate surgical site tissue perfusion, which is frequently suboptimal in surgical patients (Jonsson, Jensen et al. 1987; Hartmann, Jonsson et al. 1992; Hopf, Hunt et al. 1997). Inadequate perfusion to the

surgical site likely explains why the use of high inspired fraction of oxygen, the most studied method of increasing  $PtO_2$ , has conflicting impact on SSI (Al-Niaimi and Safdar 2009; Hunt and Hopf 2009; Brar, Brar et al. 2011; Meyhoff, Jorgensen et al. 2011; Akça, Kurz et al. 2013; Wadhwa, Kabon et al. 2014). Importantly, patients at high risk of SSI often have poor tissue perfusion due to factors such as smoking (Jensen, Goodson et al. 1991; Sørensen 2012) or obesity (Kabon, Nagele et al. 2004; Hager, Reddy et al. 2006; Govinda, Kasuya et al. 2010; Waisbren, Rosen et al. 2010; Anannamcharoen, Vachirasrisirikul et al. 2012). Insufflation of humidified-warm  $CO_2$  is a therapy applied locally to the surgical wound that may be able to overcome poor perfusion often observed in surgical patients.

Insufflation of humidified-warm  $CO_2$  into the open peritoneal cavity is hypothesised to increase sub-peritoneal  $PtO_2$  by both vasodilation and via the Bohr Effect (a right shift in the oxygen haemoglobin dissociation curve resulting in release of more oxygen from haemoglobin). Previous research shows that topical  $CO_2$  increases skin blood flow [42-44] and  $PtO_2$  [44]. Pressurised intra-abdominal  $CO_2$  in laparoscopy has been shown to increase sub-peritoneal  $PtO_2$  compared with open abdominal surgery in humans [45] and in a murine model [46]. However, the effect of insufflation of  $CO_2$  into the abdominal cavity at atmospheric pressure during open abdominal surgery on  $PtO_2$  has not yet been measured. In addition to increased  $PtO_2$  by exposure to  $CO_2$ , delivery of the  $CO_2$  both humidified and warm is hypothesised to further elevate  $PtO_2$  by reducing evaporative heat loss and thereby improving maintenance of tissue temperature. Local tissue warming has been shown to increase sub-cutaneous  $PtO_2$  in healthy volunteers

(Sheffield, Sessler et al. 1996; Ikeda, Tayefeh et al. 1998), likely by additional vasodilation and Bohr shift of the haemoglobin dissociation curve (Persson and van der Linden 2008). It is therefore expected that any increase in  $PtO_2$  will be achieved by a combination of the delivery of both  $CO_2$  and humidity/warmth to the peritoneal cavity. An investigation into whether an increase in  $PtO_2$  is achieved by the insufflation of humidified-warm  $CO_2$  into the open abdominal cavity has not yet been conducted.

#### 1.1.2 Peritoneal damage due to desiccation

Investigation is also required to determine whether insufflation of humidified-warm  $CO_2$  into the open peritoneal cavity can reduce damage to the peritoneal mesothelium caused by desiccation. The peritoneal mesothelium plays an essential role in the prevention of post-operative adhesion formation and peritoneal tumour implantation (Jayne 2007; Mutsaers and Wilkosz 2007). Following damage to the peritoneal mesothelium, as occurs during surgical incision, the presence of neighbouring mesothelial cells is essential to control the delicate balance between the deposition and breakdown of fibrin, to allow the mesothelium to heal adhesion free and avoid peritoneal tumour implantation (Gillett, James et al. 1994; Mutsaers and Wilkosz 2007; Hellebrekers and Kooistra 2011). Therefore, avoidance of inadvertent damage to the peritoneal mesothelium during abdominal surgery is important to avoid surgical complications.

Peritoneal desiccation is one possible cause of inadvertent loss of peritoneal mesothelium (Ryan, Grob  ty et al. 1971; Ryan, Grob  ty et al. 1973; Verger, Luger et al. 1983; Burns, Skinner et al. 1995; von Ruhland, Newman et al. 2003; Binda, Molinas et

al. 2006; Binda, Corona et al. 2014; Carpinteri, Sampurno et al. 2015). Consequently, prevention of desiccation during abdominal surgery is commonly recommended (DeWilde, Trew et al. 2007; Schnuriger, Barmparas et al. 2010; 2013). In laparoscopy, desiccation by insufflation of dry-cold CO<sub>2</sub> causes loss of peritoneal mesothelium (Volz, Koster et al. 1996). Furthermore, in laparoscopy, humidification of the insufflation gas can reduce the damage to the peritoneal mesothelium (Erikoglu, Yol et al. 2005; Peng, Zheng et al. 2009; Davey, Hayward et al. 2013) and has been found to be an important factor in reducing adhesion formation (Binda, Molinas et al. 2006; Peng, Zheng et al. 2009; Koninckx, Corona et al. 2013) and peritoneal tumour implantation (Binda, Corona et al. 2014; Carpinteri, Sampurno et al. 2015). However, during open abdominal surgery it remains unclear whether desiccation caused by exposure of the peritoneum to the desiccating environment of the operating room is sufficient to cause loss of peritoneal mesothelium. Furthermore, while insufflation of humidified-warm CO<sub>2</sub> into models of open surgical wounds shows reduction in evaporation from the surface of the wound (Persson, Elmquist et al. 2004; Persson and Van Der Linden 2005), there has been no investigation into whether this reduction in evaporation results in prevention of trauma to the mesothelium.

The lack of investigation of desiccation in open abdominal wounds is likely because exposure of the peritoneum to the operating room was previously believed to be unavoidable. Additionally, there are also several technical factors that make the controlled investigation of the topic difficult: in order to evaluate loss of mesothelium, tissue samples are optimally taken 12 hours after surgery (Koster, Spacek et al. 1999;

Volz, Koster et al. 1999; Volz, Koster et al. 1999); a strict protocol for handling tissue samples is essential as mesothelium is easily damaged (Eckmann, Holstein et al. 1985; von Ruhland, Newman et al. 2003); and exposure of the tissue to a controlled operating room environment and protection from trauma by other surgical tasks must be guaranteed. A systematic review is required to determine the current state of knowledge regarding the effect of gaseous exposure of the peritoneum during abdominal surgery on the peritoneal mesothelium. Furthermore, a new experimental animal model is required to be developed and used to advance our understanding of the degree of mesothelial damage and whether any such damage can be reduced by the insufflation of humidified-warm CO<sub>2</sub>.

#### 1.1.3 Summary and experimental objectives

Insufflation of humidified-warm CO<sub>2</sub> into the peritoneal cavity during open abdominal surgery has been proposed to reduce the risk of major complications following abdominal surgery. However, further research is required to completely understand the mechanisms of action of this new therapy. Therefore, the objectives of this research were:

- 1) To conduct a systematic review to determine the current state of knowledge regarding the effect of gaseous exposure of the peritoneum during abdominal surgery on morphology of the peritoneal mesothelium;
- 2) To develop an experimental animal model for examining the effect of gaseous exposure of the peritoneum during open abdominal surgery;

- 3) To investigate the effect of humidified-warm CO<sub>2</sub> on sub-peritoneal PtO<sub>2</sub>, and to elucidate the relative effect of dry versus humidified-warm CO<sub>2</sub>.
- 4) To test whether exposure of the peritoneum to the operating room environment during open abdominal surgery causes sufficient desiccation to result in loss of peritoneal mesothelium, and furthermore, whether any loss can be reduced by the insufflation of humidified-warm CO<sub>2</sub>;

## 1.2 Hypotheses

1. That insufflation of humidified-warm CO<sub>2</sub> into the peritoneal cavity during open abdominal surgery would increase sub-peritoneal tissue oxygen partial pressure compared with laparotomy without gas insufflation.
2. That insufflation of room temperature, dry CO<sub>2</sub> into the peritoneal cavity during open abdominal surgery would increase sub-peritoneal tissue oxygen partial pressure compared with laparotomy without gas insufflation.
3. That insufflation of humidified-warm CO<sub>2</sub> into the peritoneal cavity during open abdominal surgery would increase sub-peritoneal tissue oxygen partial pressure compared to insufflation of room temperature, dry CO<sub>2</sub>.
4. That exposure of the peritoneum to normal operating room ventilation would cause loss of peritoneal mesothelial cells compared with non-surgical control tissue.
5. That insufflation of humidified-warm CO<sub>2</sub> into the open peritoneal cavity would reduce loss of peritoneal mesothelial cells compared with laparotomy without gas insufflation.

### 1.3 Thesis structure

Chapter one of this thesis is an introduction that establishes the need and aims of the research, and introduces the topic areas of the subsequent chapters. Chapter two is an exhaustive narrative literature review of the topic. Chapter three is a systematic review that expands on an important background area of this thesis, the current state of knowledge regarding the effect of gaseous exposure of the peritoneum during abdominal surgery on morphology of the peritoneal mesothelium. Chapter four comprises details of the development of a rat model to study the effect of open abdominal surgery on the peritoneum. Chapter five and six then contain research that was conducted using the model. Chapter seven summarises the main research findings, limitations, recommendations for future research directions and provides an overall conclusion. A combined reference list for all chapters is then presented. Finally, an appendix includes guidelines developed to grade both scanning electron and light microscopic images of the peritoneal mesothelium.

## 2 Literature Review

### 2.1 Recognition of peritoneal exposure in early years of elective abdominal surgery

The first elective abdominal surgery is reported to have been conducted in 1809 by an American doctor Ephraim McDowell (Ellis 1996). Dr McDowell conducted an ovariectomy to successfully remove a large tumour from a 44 year old lady, who went on to live to the age of 78. Dr McDowell operated on eight women over the next two decades, by which time the medical community was only just beginning to believe his reports.

These first brief reports of abdominal surgery already highlight the consequences of peritoneal disease. McDowell reported that his fifth patient died of 'peritoneal inflammation' on the third post-operative day, and his eighth surgery was abandoned due to extensive peritoneal adhesions (Ellis 1996). While abdominal surgery was in its infancy, caution was used in determining the safety of opening the peritoneal cavity. In 1823 Dr James Blundell reported, following his experiments on rabbits, that moderate openings of the peritoneum were not fatal (Aveling 1890). Confidence in abdominal surgery quickly grew in the proceeding decades, to the great benefit of the community. However, in 1929, in a speech to the Clinical Congress of the American College of Surgeons, surgeons were criticised for taking for granted the tolerance of the peritoneum and abdominal viscera to surgical interference (Wilkie 1929). Surgeons were pleaded with to reduce the trauma of surgery, particularly by reducing tension placed on the peritoneum and underlying viscera (Wilkie 1929). Even in this early

account, irritation of the parietal peritoneum was reported as the most common cause of clinically observed abdominal pain, presumably referring to post-operative peritoneal adhesions (Wilkie 1929). Furthermore, the speech highlighted the importance of appreciating the role of the peritoneum in resistance to infection (Wilkie 1929). Today, post-operative infection and adhesion formation remain common and expensive complications following abdominal surgery (Klevens, Edwards et al. 2007; Okabayashi, Ashrafian et al. 2013), especially following open abdominal surgery (Fleming, Kim et al. 2010; Howard, Datta et al. 2010; Schnuriger, Barmparas et al. 2010; Utsumi, Shimizu et al. ; Aimaq, Akopian et al. 2011; Suh, Jeong et al. 2012). There has been a call for additional technologies to further reduce the incidence of both SSI (Yoshida, Nabeshima et al. 2007; Aimaq, Akopian et al. 2011) and post-operative adhesion formation (Alpay, Saed et al. 2008).

## 2.2 How the peritoneal mesothelium is exposed during abdominal surgery

During abdominal surgery the peritoneal cavity, normally only a potential space, is converted to an actual space to facilitate surgical access. This exposes the monolayer of mesothelial cells on the cavity side of the peritoneum, both parietal and visceral layers, to an abnormal environment, particularly including physical trauma and exposure to gases which may be cold, dry, contaminated by organisms and particles and under supra-atmospheric pressure. In addition to injury to the peritoneum caused by the necessary surgical procedures and tissue handling, exposure of the peritoneum to such an abnormal environment may also result in cellular injury and therefore, alter tissue function.

The type of exposure faced by the peritoneal mesothelium varies, depending on the mode of access to the peritoneal cavity. During laparoscopic surgery the mesothelium is exposed to a pressurised gas, most commonly carbon dioxide. Conversely, during open abdominal surgery (laparotomy) the mesothelium is exposed to the ambient environment of the operating room, including temperature, humidity and pressure. Compared with the native environment of the peritoneum, these operative environments are comparatively cold, dry, dirty and of abnormal gas composition. The ensuing inflammation of the mesothelium has been suggested to induce changes in peritoneal morphology that impact on cellular function and this is suspected to be the underlying cause of post-operative adhesion formation and peritoneal tumour implantation (Binda, Corona et al. 2014).

## 2.3 Intra-abdominal insufflation of humidified-warm CO<sub>2</sub> to protect the peritoneum against post-operative complications

### 2.3.1 Proposed mechanisms of action

Insufflation of humidified-warm CO<sub>2</sub> into the peritoneal cavity has been proposed as a therapy to reduce the risk of post-operative complications such as surgical site infection (Persson and van der Linden 2008); post-operative adhesion formation (Persson and van der Linden 2009); and peritoneal tumour implantation (Binda, Corona et al. 2014). The promising hypothesis that the therapy may decrease the risk of SSI was acknowledged with the 2008 David Horrobin Prize for medical theory (2009). The proposed mechanisms of action by which intra-abdominal insufflation of humidified CO<sub>2</sub> may reduce surgical complications are fivefold (Persson and van der

Linden 2008; Persson and van der Linden 2009; Binda, Corona et al. 2014) and are summarised in Figure 1-1. The focus of this thesis is the two mechanisms that have not been tested, highlighted in Figure 1-1.

### 2.3.2 Current evidence

The first three mechanisms of action in Figure 1-1 have undergone preliminary investigation with promising results. Firstly intra-abdominal insufflation of warm-humidified CO<sub>2</sub> is proposed to decrease airborne contamination. Within the operating room, the main external source of wound contamination is likely airborne from the surgical team leaning over the surgical wound and is exacerbated by the presence of overhead air filters that direct air flow towards the surgical cavity (Persson and Van Der Linden 2004). By directing airflow away from the surgical wound using a diffuser that sits within the cavity, there is a marked reduction in the number of particles that enter a wound cavity (Persson and Van Der Linden 2004). Conversely, contamination increases, due to turbulence, if an open ended tube is used instead of a gas diffuser (Persson and van der Linden 2004). When using a diffuser, wound bacterial contamination is further reduced by continual insufflation of CO<sub>2</sub> instead of air (Persson and van der Linden 2004). This is likely because CO<sub>2</sub> is heavier than air and tends to adhere to the wound surface by the Coanda effect (which is the tendency for a fluid to attach itself and flow around a nearby curved surface) (Cater and van der Linden 2014). The CO<sub>2</sub> thereby acts like a protective cushion, deflecting nearly all airborne particles (Persson and van der Linden 2004).

The second proposed mechanism of action is that intra-abdominal insufflation of warm-humidified CO<sub>2</sub> decreases bacterial growth. High concentrations of CO<sub>2</sub> are used in food packaging to inhibit the growth of both aerobic and anaerobic bacteria (Persson and van der Linden 2008). In a bench model at body temperature, carbon dioxide has been shown to reduce the growth of *Staphylococcus aureus* (Persson, Svenarud et al. 2005; Persson and van der Linden 2008).

Thirdly, intra-abdominal insufflation of warm-humidified CO<sub>2</sub> is proposed to assist in the maintenance of core and wound temperature, important for the maintenance of vasodilation and immune function (Sessler 2006). Two human studies found that intra-abdominal insufflation of humidified-warm CO<sub>2</sub> during open colon surgery maintains wound and core temperature closer to body temperature than does a control (undergoing surgery without gas insufflation) (Frey, Janson et al. 2012; Frey, Janson et al. 2012). Furthermore, insufflation of humidified warm CO<sub>2</sub> reduced the incidence of hypothermia from 62 % to 18 % when hypothermia was defined as <36.5 °C and from 18 % to zero when defined as <36.0 °C (Frey, Janson et al. 2012). Increased wound temperature during CO<sub>2</sub> insufflation was also achieved in a crossover trial in cardio-thoracic surgery (Frey, Svegby et al. 2010). The increase in patient core and wound temperature is likely due to CO<sub>2</sub> acting as a greenhouse gas trapping heat radiating from the patient's surface and also due to protection from evaporative heat loss caused by exposure of moist surfaces to ambient air with a low relative humidity.

The fourth proposed mechanism of action by which insufflation of humidified-warm CO<sub>2</sub> may reduce post-operative complications is via an increase in tissue oxygenation

(PtO<sub>2</sub>). To date, there has been no investigation of the effect of CO<sub>2</sub> insufflation during open abdominal surgery on PtO<sub>2</sub>. However, research in humans has shown a 1.2 °C increase in wound temperature within 2 minutes of commencement of CO<sub>2</sub> insufflation into an open wound (Frey, Svegby et al. 2010). It is unlikely that reduction in evaporative and radiative heat loss alone can explain such a rapid increase in temperature. The rapid temperature increase is likely due to an increase in blood perfusion to the wound, which if true would lead to a concomitant increase in oxygen delivery to the tissues. Two further human investigations, discussed earlier, have shown that insufflation of humidified-warm CO<sub>2</sub> increases the average wound temperature over the duration of a colo-rectal procedure by 1.2 °C (Frey, Janson et al. 2012) to 1.7 °C (Frey, Janson et al. 2012). In addition to vasodilation by exposure to CO<sub>2</sub>, maintained elevated wound temperature may cause further vasodilation and further increase oxygen delivery.

The final proposed mechanism of action by which insufflation of humidified-warm CO<sub>2</sub> may reduce post-operative complications is prevention of mesothelial trauma due to desiccation. Bench studies measuring the rate of evaporation within a wound model have shown that reduction in wound surface desiccation is achieved only when CO<sub>2</sub> gas is delivered with a gas diffuser; desiccation increases if an open ended tube is used (Persson and Van Der Linden 2005). Furthermore, diffusion of air, whether dry or humidified, does not change humidity or evaporation rate in the wound cavity (Persson, Elmqvist et al. 2004). This is likely due to rapid diffusion of the insufflated air into the surrounding environment. However, desiccation is dramatically reduced by

insufflation of CO<sub>2</sub> using a diffuser and the addition of humidification further reduces evaporation rate by > 90 % compared with without insufflation (Persson, Elmqvist et al. 2004). These results suggest that insufflation of humidified-warm CO<sub>2</sub> will decrease wound desiccation, however, it has not been investigated whether this translates to a reduction in mesothelial trauma.

Preliminary evidence exists for the first three mechanisms of action by which insufflation of humidified-warm CO<sub>2</sub> is proposed to reduce post-operative complications, (shown un-highlighted in Figure 1-1). However, there is a significant gap in the literature investigating the two final mechanisms of action, increased tissue oxygenation and prevention of mesothelial trauma due to desiccation, (shown highlighted in Figure 1-1). This thesis has investigated these remaining two untested mechanisms of action.

## 2.4 Role of peri-operative tissue oxygen partial pressure in the prevention of post-operative complications

Investigations into the relationship between peri-operative PtO<sub>2</sub> and post-operative complications, and their indicators, are summarised in Table 2-1.

### 2.4.1 Surgical site infection

#### 2.4.1.1 Burden of surgical site infection

The overall incidence of SSI following surgical procedures of all types has been estimated as 2 % (Graves, Nicholls et al. 2003; Kleven, Edwards et al. 2007). However, the risk of infection depends on the type of procedure. Colo-rectal procedures are

consistently reported as one of the procedures with the highest risk. The rate of in-hospital diagnosis of SSI in colo-rectal surgery is reported as 12.5 % in the Australian state of New South Wales (McLaws and Taylor 2003); 8.8 in the Australian state of Victoria (Russo, Bull et al. 2006); and 5.6 % in the USA (Edwards, Peterson et al. 2009) (note that the difference in rates may be explained by inconsistent reporting methods). Furthermore, SSI rates rise significantly when out-patient diagnoses are included (Humphreys 2009). Surgical site infections carry a large financial burden as the treatment cost for a patient with a SSI is approximately twice the cost of a patient without (Kirkland, Briggs et al. 1999; Broex, van Asselt et al. 2009; Varela, Wilson et al. 2010). The cost of SSI diagnosed in hospital equates to USD 47 billion in one year in the USA (Klebens, Edwards et al. 2007). In addition to the financial burden, in the USA in 2002, SSI was estimated to affect 274,098 patients and was associated with the deaths of an estimated 8205 patients (Klebens, Edwards et al. 2007). Current guidelines for the prevention of SSI focus on adherence to aseptic and 'clean and dirty' techniques; maintenance of normothermia and normoglycemia; and the use of prophylactic antibiotics (Mangram, Horan et al. 1999; Leaper, Fry et al. 2013). However, hospital compliance with guidelines does not accurately predict a low SSI rate (Leaper, Fry et al. 2013), with only approximately 20 % of hospital acquired infections preventable by quality management programmes (Harbarth, Sax et al. 2003). Surgical site infection remains a significant burden on the health system following abdominal surgery, and investigations are warranted into further approaches to reduce the rate of surgical site infection (Yoshida, Nabeshima et al. 2007; Aimaq, Akopian et al. 2011).

#### 2.4.1.2 Importance of peri-operative PtO<sub>2</sub> in the prevention of surgical site infection

Maintenance of high peri-operative PtO<sub>2</sub> is a common recommendation for the prevention of surgical site infection (Hopf and Rollins 2007; Yoshida, Nabeshima et al. 2007; National Collaborating Centre for Nursing and Supportive Care 2008).

Bactericidal function of leukocytes increases with oxygen availability both in-vitro and in experimental animal wounds (Hohn, MacKay et al. 1976). This is because the partial pressure of oxygen drives the production of superoxide by phagocytes (Babior 1978; Babior 1978; Allen, Maguire et al. 1997). Superoxide production is thought to result in microbial killing by facilitating both oxidative killing by reactive oxygen species and by creating a high pH environment within the phagosome that leads to liberation of phagosomal protease stores (Reeves, Lu et al. 2002; Roos and Winterbourn 2002; Roos, Van Bruggen et al. 2003; Segal 2005). The Michaelis Menten constant (Km) for oxygen consumption by human neutrophils is an oxygen tension of 80 mmHg (Allen, Maguire et al. 1997). This is well within the range of oxygen tension in surgical wounds, which is often in the range of 40-60 mmHg below the skin (Plattner, Akça et al. 2000; Buggy, Doherty et al. 2002; Kabon, Fleischmann et al. 2003; Bakri, Nagem et al. 2008) and likely approaches zero at the wound edge (Tsai, Johnson et al. 2003). Oxygen availability is therefore crucial in the immune response to infection (Hohn, MacKay et al. 1976; Roos, Van Bruggen et al. 2003).

The clinical relationship between low peri-operative PtO<sub>2</sub> and increased risk of SSI is largely based on the results of one clinical trial (Hopf, Hunt et al. 1997). That observational trial included 130 patients undergoing a major surgical procedure that

were considered at risk of SSI. Patients were considered at risk if they had at least one of the following four risk factors: an abdominal operation; an operation lasting 2 hours or more; notable wound contamination; three or more diagnoses on discharge from hospital. Tissue oxygen partial pressure ( $P_tO_2$ ) was measured in a surrogate wound in the upper arm 6 hours, 1 and 2 days after the surgical procedure and patients were followed for 30 days to record the rate of SSI. 24 patients developed a SSI (18 %). Arm- $P_tO_2$  was significantly lower in infected patients compared with uninfected patients. Furthermore, arm- $P_tO_2$  was inversely related to the risk of surgical site infection ( $r=0.91$ ,  $p<0.001$ ). Patients with arm- $P_tO_2$  of  $<50$  mmHg had a 43 % risk of SSI, while none of the patients with arm- $P_tO_2 \geq 90$  mmHg developed a SSI.

The relationship between tissue  $P_tO_2$  and SSI was supported by a later study of 500 patients undergoing elective colo-rectal surgery (Greif, Akça et al. 2000). The main conclusion of that study was that supplemental inspired oxygen, 80 %  $O_2$  administered during and for two hours after surgery, reduced the incidence of SSI from 11.2 % to 5.2 % ( $p=0.01$ ). The authors also measured subcutaneous  $P_tO_2$  in a subset of patients and found that average  $P_tO_2$  was approximately 50 mmHg higher in the group receiving supplemental inspired oxygen (Greif, Akça et al. 2000).

A further study concluded that peri-operative tissue oxygenation predicts SSI (Govinda, Kasuya et al. 2010). However, that study used an inferior measurement of tissue oxygenation and the results are less compelling (Govinda, Kasuya et al. 2010). In an attempt to find a less invasive method of measuring tissue oxygenation, the investigation used 'tissue oxygen saturation', a measure of haemoglobin oxygen

saturation in the volume of tissue illuminated by trans-cutaneous near-infrared light. Tissue oxygen saturation was measured in patients following colon resection both 75 hours and one day after surgery at three locations (arm, thenar eminence and abdominal incision). Of the six measurements, only that at the arm 75 hours after surgery was significantly different in patients with SSI compared with patients without SSI (Govinda, Kasuya et al. 2010). Tissue oxygen saturation is a limited measurement of  $PtO_2$  as it does not take into account perfusion rate and actual  $O_2$  delivery, this likely explains why it has less utility in predicting SSI.

#### 2.4.2 Importance of peri-operative $PtO_2$ in non-infected wound healing

##### 2.4.2.1 Collagen deposition

Wound strength is created by deposition of collagen by fibroblasts, a process that requires oxygen and is highly sensitive to wound hypoxia (Hopf and Rollins 2007). The relationship between oxygen tension and collagen synthesis in-vitro and in animal models has been clearly established (Hopf and Rollins 2007). Furthermore, the relationship between  $PtO_2$  and collagen deposition in wounds has been confirmed in surgical patients (Jonsson, Jensen et al. 1986; Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992).

Hartmann *et al.*, measured collagen deposition in patients undergoing abdominal surgery for cancer or inflammatory bowel disease (Hartmann, Jonsson et al. 1992). Half the patients were randomised to have usual fluid replacement dictated by clinical criteria (such as blood pressure, pulse rate and urine output). The other half underwent strict control of fluid replacement based on their  $PtO_2$  response to an

oxygen challenge, an indication of perfusion. Patients' whose  $PtO_2$  did not increase by at least 20 % upon administration of supplemental oxygen, the 'oxygen challenge', were deemed poorly perfused and were administered supplemental fluids until  $PtO_2$  increased by at least 20 % during oxygen challenge. Tissue oxygen partial pressure and collagen deposition were measured in all patients in the upper arm. Patients whose fluid replacement was dictated by  $PtO_2$  response to oxygen challenge had a significantly greater increase in  $PtO_2$  in response to oxygen challenge, and also significantly higher collagen deposition (Hartmann, Jonsson et al. 1992). This result supports earlier work, by collaborating authors, in which  $PtO_2$  following oxygen challenge predicted collagen deposition in two separate cohorts of patients (Jonsson, Jensen et al. 1986; Jonsson, Jensen et al. 1991) undergoing a range of surgical procedures. In addition, collagen deposition was independent of haematocrit, suggesting that  $PO_2$  is more important than haemoglobin bound oxygen (Jonsson, Jensen et al. 1986; Jonsson, Jensen et al. 1991).

There is a clear relationship between  $PtO_2$  in response to oxygen challenge and collagen deposition in surgical patients (Jonsson, Jensen et al. 1986; Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992). However, in the two studies that reported baseline  $PtO_2$ , that is  $PtO_2$  measured prior to oxygen challenge, both found that baseline  $PtO_2$  did not predict collagen deposition (Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992). The oxygen challenge assesses whether increased inspired oxygen is delivered to the wound, and is therefore a measure of wound perfusion. These studies illustrate that adequate perfusion is necessary for oxygen

delivery and confirm previous reports that assessment of perfusion is important when investigating the role of oxygen in wounds (Jonsson, Jensen et al. 1987; Hopf, Hunt et al. 1997).

A further study, that did not assess perfusion, was unable to confirm the relationship between high  $PtO_2$  and collagen deposition (Greif, Akça et al. 2000). Fifty six patients were randomised to receive either 30 or 80 % inspired fraction of oxygen during and for two hours following colorectal resection. Tissue oxygen partial pressure was measured in a surrogate wound in the upper arm, and both collagen and protein deposition were measured in a tube inserted parallel to the abdominal incision. Although the group receiving higher inspired fraction of oxygen had significantly higher  $PtO_2$  both intra-operatively (59 v 109 mmHg) and post-operatively (54 v 73 mmHg), and high inspired fraction of oxygen significantly reduced SSI, there was no difference in collagen or protein deposition measured seven days after surgery (Greif, Akça et al. 2000). There are three striking differences between this study (Greif, Akça et al. 2000) and the previous investigations (Jonsson, Jensen et al. 1986; Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992). Firstly, the authors only presented the average value of collagen deposition in each group, so it is unclear whether  $PtO_2$  and collagen deposition were related as a paired analysis. Secondly, the patients did not undergo an oxygen challenge prior to measurement of  $PtO_2$ . Therefore there was no measure of tissue perfusion. This is particularly important as  $PtO_2$  was measured in the upper arm, while collagen deposition was measured parallel to the wound. In contrast, two of the previous investigations measured the  $PtO_2$  and collagen in immediately adjacent

wounds in the upper arm and were therefore confident in relating the measurements (Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992) (note that the third investigation did not report the location of collagen deposition measurement (Jonsson, Jensen et al. 1986)).

In conclusion, collagen deposition is dependent on the presence of local, high oxygen pressure. In a clinical setting it is necessary to ensure that adequate perfusion of the wound is present to deliver the oxygen.

#### 2.4.2.2 Anastomotic leakage

Leakage of bowel contents from a surgical anastomosis is a serious complication of surgery to the gastro-intestinal tract, resulting in increased mortality and prolonged hospital stay (Buchs, Gervaz et al. 2008). The incidence of anastomotic leakage following colo-rectal surgery is at least 3-6 % (Buchs, Gervaz et al. 2008; Taflampas, Christodoulakis et al. 2009), with a mortality rate of 13 % (Buchs, Gervaz et al. 2008). It is described as the most feared complication specific to colo-rectal surgery (Buchs, Gervaz et al. 2008). Documented risk factors of anastomotic leak following colorectal surgery are: presence of systemic disease (American Society of Anaesthesiologists physical status classification system score  $\geq 3$ ); infra-peritoneal location of the anastomosis; and prolonged operating time (Buchs, Gervaz et al. 2008). Surgical technique is also important. The fundamental principles of a successful anastomosis are: adequate perfusion; avoidance of hypoxia; and lack of tension (Taflampas, Christodoulakis et al. 2009; Davis and Rivadeneira 2013). The presence of oxygen is essential for collagen synthesis (see above) and angiogenesis, and therefore strength,

in the healing wound (Hopf and Rollins 2007). Sheridan and co-workers measured peri-anastomotic  $PtO_2$  in patients undergoing colonic resection and anastomosis (Sheridan, Lowndes et al. 1987). Patients that experienced anastomotic leak had significantly lower bowel  $PtO_2$  (approximately 17 mmHg vs 37 mmHg,  $p < 0.01$ ), and bowel  $PtO_2 < 20$  mmHg was predictive of anastomotic leak ( $p < 0.001$ ) (Sheridan, Lowndes et al. 1987).

**Table 2-1: Summary of investigations that have investigated the effect of peri-operative tissue oxygen partial pressure on post-operative outcomes in humans**

First author, year, reference	N	Study design Procedure	Intervention	Primary outcome	Tissue O <sub>2</sub> method- Timing of placement, instrument & location.	Timing of O <sub>2</sub> reading	Results - control vs intervention (PtO <sub>2</sub> in mmHg) Mean±SD (unless stated)	Conclusion (comments)
Hopf et al., 1997 (Hopf, Hunt et al. 1997).	130	Prospective observational. Surgical procedures, anticipated SENIC >1.	None- observation al. All: Oxygen challenge of F <sub>I</sub> O <sub>2</sub> 40-60 %.	SSI (drainage of purulent material within 30 days of operation).	During surgery. PtO <sub>2</sub> via subcutaneous silicone tonometer & polaro-graphic electrode (biogenesis) (n=114) or optode (InnerSpace Medical) (n=16). Lateral upper arm.	Post op <6hrs & day 1, 2. Equilibration period ≥ 25 min.	PtO <sub>2</sub> during F <sub>I</sub> O <sub>2</sub> 40-60 % F <sub>I</sub> O <sub>2</sub> related to SSI (r=0.91). (PtO <sub>2</sub> baseline: r=0.66) SSI ↓ with ↑ PtO <sub>2</sub> up to 110 mmHg. Critical risk of SSI in PtO <sub>2</sub> range 0-40.	PtO <sub>2</sub> during oxygen challenge is a powerful predictor of SSI.
Sheridan, (Sheridan, Lowndes et al. 1987).	50	Prospective observational Colonic resection and anastomosis.	None – observation al.	Anastomotic leakage – radiologic exam.	Prior to intestinal mobilisation. Clark-type polarographic oxygen electrode. Intestinal serosa <10 mm from anastomosis and control site (ileum).	Before surgical mobilisation of the intestine and after construction of the anastomosis.	5 cases of anastomotic leakage. Anastomoses constructed in tissue with PtO <sub>2</sub> < 20 mmHg had increased risk of subsequent leakage p<0.001.	Peri-anastomotic PtO <sub>2</sub> is predictive of anastomotic leakage if <20 mmHg; 50 % of pre-resection value; 15 % of arterial P <sub>a</sub> O <sub>2</sub> or 40 % of a control site (ileum).

First author, year, reference	N	Study design Procedure	Intervention	Primary outcome	Tissue O <sub>2</sub> method- Timing of placement, instrument & location.	Timing of O <sub>2</sub> reading	Results - control vs intervention (PtO <sub>2</sub> in mmHg) Mean±SD (unless stated)	Conclusion (comments)
Greif et al., 2000 (Greif, Akça et al. 2000).	54 (subset of 500)	RCT. Open colorectal resection.	F <sub>I</sub> O <sub>2</sub> 30 vs 80 % intra-op & first 2 h of recovery.	Collagen and protein deposition in wound 7 d post-op (hydroxyproline and protein/mm in tube embedded next to incision).	After induction. PtO <sub>2</sub> via subcutaneous silicone tonometer & Licox™. Lateral upper arm (Chang, Goodson et al. 1983).	Intra-op to two hrs post-op. Stabilisation period not stated.	PtO <sub>2</sub> Intra-op: 59±15 vs 109±43 p<0.001. PtO <sub>2</sub> Post-op: 54±25 vs 73±25 p=0.02. Collagen deposition: 267±109 v 258±118 ng/mm p=0.38. Protein deposition: 163±74 v 153±97 p=0.31.	Supplemental O <sub>2</sub> ↑ P <sub>I</sub> O <sub>2</sub> , but did not change collagen or protein deposition next to the surgical incision.
Jonsson et al., 1991 (Jonsson, Jensen et al. 1991).	33	Prospective observational. Various surgical procedures.	All: Oxygen challenge of F <sub>I</sub> O <sub>2</sub> 60-70%. Well-perfused defined as PtO <sub>2</sub> increasing ≥ 20% in response to increased F <sub>I</sub> O <sub>2</sub> .	Collagen deposition upper arm, 5th and 7 <sup>th</sup> d (hydroxyproline /cm) Perfusion.	End of surgery. Silver/chloride and platinum needle electrons placed in a silastic catheter. Immediately adjacent to wounds.	2-6 h post-op then daily.	Both PtO <sub>2</sub> during F <sub>I</sub> O <sub>2</sub> of 60-70 % (p<0.01) and perfusion (p<0.001) predicted collagen deposition.	Collagen deposition was directly and significantly proportional to wound oxygen tension and measures of perfusion.

First author, year, reference	N	Study design Procedure	Intervention	Primary outcome	Tissue O <sub>2</sub> method- Timing of placement, instrument & location.	Timing of O <sub>2</sub> reading	Results - control vs intervention (PtO <sub>2</sub> in mmHg) Mean±SD (unless stated)	Conclusion (comments)
Hartmann et al., 1992 (Hartmann, Jonsson et al. 1992).	29	RCT. Abdominal surgery for cancer or inflammatory bowel disease.	Fluid replacement decided by clinical criteria or PtO <sub>2</sub> (< 20 % increase in PtO <sub>2</sub> in response to 60-70 % F <sub>I</sub> O <sub>2</sub> ).	Collagen deposition 5 and 7 d post-op at upper arm (hydroxyproline /cm in subcutaneous embedded tubes).	Beginning of surgery. Silicon catheter and PtO <sub>2</sub> monitor (Biogenesis, San Francisco). Upper arm.	2-4 h, and 1 & 2 d post-op.	PtO <sub>2</sub> increased during high F <sub>I</sub> O <sub>2</sub> in only 24 % of patients. Fluid replacement decided by PtO <sub>2</sub> ↑ the PtO <sub>2</sub> response to high F <sub>I</sub> O <sub>2</sub> (13 % vs 55% increase) and ↑collagen deposition at 7 days (0.49 vs 0.66 µg/cm, p<0.05).	Replacement of fluid according to PtO <sub>2</sub> rather than clinical criteria results in increased PtO <sub>2</sub> and accumulation of collagen in healing wounds. Paired PtO <sub>2</sub> /collagen data not presented.
Jonsson et al., 1986 (Jonsson, Jensen et al. 1986).	36	Prospective observational. General surgery procedures.	None, observational.	Collagen deposition 5 and 7 d post-op. (hydroxyproline /cm in subcutaneous embedded tubes). Location not stated.	Polarographic subcutaneous silastic tonometer. Upper arm. Mean PtO <sub>2</sub> & also determined if patients were "Well perfused" (>20 % ↑ in PtO <sub>2</sub> in response to F <sub>I</sub> O <sub>2</sub> 60-70 % following air breathing).	Operative day, and 1 & 2 d post-op.	Collagen deposition was positively related to PtO <sub>2</sub> and the perfusion score calculated from PtO <sub>2</sub> in response to oxygen breathing (r <sup>2</sup> = 0.56).	Smoking and increased age reduced perfusion and PtO <sub>2</sub> .

d= day(s); F<sub>I</sub>O<sub>2</sub> = Inspired oxygen fraction; h or hr(s) = hour(s); Intra-op = Intra-operative(y); min(s) = minute(s); Post-op = Post-operative(y); RCT = Randomised controlled trial; ↑ = Increase(s/ing); ↓ = Decrease(s/ing)

#### 2.4.3 Current methods to increase peri-operative PtO<sub>2</sub>

Table 2-1 summarises investigations that have investigated peri-operative methods to increase subcutaneous tissue oxygen partial pressure.

##### 2.4.3.1 Decreasing sympathetic vasoconstriction

Vasoconstriction and vasodilation of arterioles is under sympathetic control, with increased sympathetic activity causing vasoconstriction in most target tissues, with the exception of skeletal muscle, the heart, brain and the liver. Vasoconstriction will decrease perfusion and decrease the delivery of oxygen to cutaneous wounds.

Conversely, decreased sympathetic activity allows vasodilation and increased perfusion. Therefore interventions that decrease sympathetic vasoconstriction are expected to increase wound PtO<sub>2</sub> measured. As expected PtO<sub>2</sub> is increased by avoiding pain (Akça, Melischek et al. 1999), core and local cooling (Sheffield, Sessler et al. 1996), hypovolemia (Chang, Goodson et al. 1983; Hartmann, Jonsson et al. 1992; Arkiliç, Taguchi et al. 2003) and nicotine (Jonsson, Jensen et al. 1987; Jensen, Goodson et al. 1991). Furthermore, sympathetic blockade during surgery, by the use of spinal or epidural regional anaesthesia, causes an increase in PtO<sub>2</sub> of approximately 8-13 mmHg (Buggy, Doherty et al. 2002; Kabon, Fleischmann et al. 2003; Treschan, Taguchi et al. 2003). The effects of limiting sympathetic vasoconstriction by regional anaesthesia may have clinically important effect on SSI. In a large retrospective study, patients that received total hip or knee replacement under epidural or spinal anaesthesia, without general anaesthetic, were half as likely to suffer from SSI than patients that received general anaesthetic (Chang, Lin et al. 2010). This is likely explained by greater regional

vasodilation during combined regional and general anaesthetic, compared with general anaesthesia alone (Treschan, Taguchi et al. 2003).

#### 2.4.3.2 Supplemental inspired oxygen

Supplemental inspired oxygen, i.e. high inspired fraction of oxygen, is proposed to increase arterial partial pressure of oxygen ( $P_aO_2$ ) and subsequently increase  $PtO_2$  (Hopf and Holm 2008).

Several studies have concluded that increasing inspired fraction of oxygen is able to increase peri-operative  $PtO_2$  (Chang, Goodson Iii et al. 1983; Jonsson, Jensen et al. 1987; Greif, Akça et al. 2000; Kabon, Nagele et al. 2004; Kimberger, Fleischmann et al. 2007; Bakri, Nagem et al. 2008). However, results emphasise that adequate tissue perfusion is necessary for high inspired fraction of oxygen to increase  $PtO_2$ , and that tissue perfusion is not always normal in patients undergoing surgery. As discussed in section 2.4.1, Hopf *et al.* found that  $PtO_2$  measured following doubling patients' inspired oxygen fraction was more predictive of risk of SSI than the baseline  $PtO_2$  (Hopf, Hunt et al. 1997). They concluded that supplemental oxygen is only likely to be useful when it is combined with adequate perfusion. This hypothesis is supported by two earlier studies. Jonsson *et al.* measured  $PtO_2$  in the upper arm of patients following major abdominal or peripheral surgery (Jonsson, Jensen et al. 1987). Tissue oxygen partial pressure was measured while the patients breathed room air and 60-70%  $O_2$ . On average,  $PtO_2$  increased with high inspired fraction of oxygen. However, in 12 patients  $PtO_2$  did not respond despite an increase in  $P_aO_2$ . These patients were deemed malperfused and were given additional fluid replacement, after which  $PtO_2$

increased (Jonsson, Jensen et al. 1987). Similarly, Hartman *et al.* measured PtO<sub>2</sub> in the upper arm during supplemental oxygen breathing in 29 patients undergoing abdominal surgery for cancer or inflammatory bowel disease (Hartmann, Jonsson et al. 1992). Tissue oxygen partial pressure increased in only 24 % of patients in response to the increased inspired fraction of oxygen. However, following subsequent fluid administration, 93 % of patients responded to the oxygen challenge (Hartmann, Jonsson et al. 1992).

There have been several investigations into the effect of high inspired fraction of oxygen on post-operative complications. High inspired fraction of oxygen is recommended by a recent review to reduce the risk of bowel anastomotic leak based on two human trials (Davis and Rivadeneira 2013). However, the evidence for the effect of high inspired fraction of oxygen the risk of SSI remains conflicting (Al-Niaimi and Safdar 2009; Hunt and Hopf 2009; Brar, Brar et al. 2011; Meyhoff, Jorgensen et al. 2011; Wadhwa, Kabon et al. 2014). The inconsistent results may be explained by lack of control over tissue perfusion, as adequate tissue perfusion is necessary if PtO<sub>2</sub> is to respond to increased P<sub>a</sub>O<sub>2</sub> caused by high inspired fraction of oxygen. The routine use of high inspired fraction of oxygen remains controversial as research is still inconclusive as to whether oxygen fraction ≥ 60 % increases adverse events, including mortality (Wetterslev, Meyhoff Christian et al. 2015).

In conclusion, supplemental inspired oxygen is a promising therapy, however, if the oxygen is to be delivered to the tissue at risk of infection, the tissue must be well perfused, which is often not the case in surgical patients.

#### 2.4.3.3 Hypercapnia

Hypercapnia, increased  $P_a\text{CO}_2$ , is proposed to increase peri-operative  $\text{PtO}_2$  by:

- 1) Increasing cardiac output, following activation of central and peripheral chemoreceptors;
- 2) A right shift of the oxygen/haemoglobin dissociation curve; and/or
- 3) A direct vasodilatory effect of increased  $\text{PaCO}_2$  or decreased pH

Hypercapnia can be achieved during mechanical ventilation by increasing end tidal  $\text{CO}_2$ . This can be accomplished by eliminating soda lime, which normally scavenges  $\text{CO}_2$  from the expired air; by reducing the rate of fresh gas flow; allowing rebreathing of expiratory gases (Akca, Liem et al. 2003) and be hypoventilation.

Increased end tidal  $\text{CO}_2$  has been shown to increase  $\text{PtO}_2$  in the upper arm of healthy anaesthetised controls in a dose dependent manner (Akça, Doufas et al. 2002). In a randomised controlled trial in abdominal and orthopaedic surgical patients, raising end tidal  $\text{CO}_2$  from 30 to 45 mmHg increased  $\text{PtO}_2$  by approximately 15 mmHg (Akca, Liem et al. 2003). Similar results were seen in a randomised control trial in gastric bypass patients, in which raising end tidal  $\text{CO}_2$  from 35 to 50 mmHg increased  $\text{PtO}_2$  by approximately 15 mmHg (Hager, Reddy et al. 2006). A further trial to elucidate the mechanism of action by which hypercapnia increases  $\text{PtO}_2$  was conducted in patients undergoing cardio-pulmonary bypass (Akça, Sessler et al. 2006). Because they were undergoing cardio-pulmonary bypass, hypercapnia could not affect cardiac output. In a randomised cross-over design,  $P_a\text{CO}_2$  was alternated between 35 and 50 mmHg by

adding CO<sub>2</sub> to the oxygenator unit. Hypercapnia did not increase sub-cutaneous PtO<sub>2</sub>, suggesting that the mechanism of action by which hypercapnia increases sub-cutaneous PtO<sub>2</sub> in heart-beating patients is by regulation of cardiac output. However, hypercapnia did increase cerebral PtO<sub>2</sub>, suggesting that there are regional differences in how much the contractility of vascular smooth muscle is dependent on local CO<sub>2</sub> concentration (Akça, Sessler et al. 2006).

Despite the strong evidence that hypercapnia increases PtO<sub>2</sub>, a recent trial of 1206 patients randomised to receive P<sub>a</sub>CO<sub>2</sub> during colon surgery of either 35 or 50 mmHg, was unable to show a large enough relative reduction in SSI with hypercapnia to justify their continuation of the trial (despite an absolute reduction in infections, high SSI rates predicted very large numbers needed to reach significance) (Akça, Kurz et al. 2013). The authors believe that the likely reason that hypercapnia appeared to be ineffective was that hypercapnia was not able to increase PtO<sub>2</sub> by 25-30 mmHg as they had expected, possibly due to inadequate perfusion. This hypothesis could not be confirmed because neither wound PtO<sub>2</sub> nor perfusion were measured in the trial (Akça, Kurz et al. 2013).

In conclusion, hypercapnia increases PtO<sub>2</sub> in surgical patients by a combination of increased cardiac output, and local effects. The effect is likely dependent on the presence of adequate local perfusion.

#### 2.4.3.4 Topical O<sub>2</sub>

Application of O<sub>2</sub> to the surgical wound has been proposed to provide transdermal local delivery of oxygen to the wound. The hypothesis was tested in a study of 30 patients following open cardiac surgery procedures (Bakri, Nagem et al. 2008). All patients had two cannulae attached to the skin near the midline incision, which were carefully sealed under a transparent dressing. Depending on group allocation, the cannulae delivered O<sub>2</sub> at 6 ml/hr. to the wound, or were inactive. There was no difference in PtO<sub>2</sub> between groups, as measured at the sternal wound (Bakri, Nagem et al. 2008). The result suggests that the O<sub>2</sub> was unable to penetrate the skin. It is possible that the extensive wound dressing, necessary to protect the vulnerable surgical wound, may have limited the delivery of topical oxygen to the portion of the wound at the tips of two cannulae.

#### 2.4.3.5 Intra-abdominal CO<sub>2</sub>

Intra-abdominal CO<sub>2</sub> may increase PtO<sub>2</sub> via vasodilation caused by direct exposure to CO<sub>2</sub>. The local vasodilatory effect of topical CO<sub>2</sub> is illustrated by placing a hand in a water bath of known temperature. When the water is saturated with CO<sub>2</sub>, the hand becomes red and heat elimination to the bath increases by 40 % (Diji 1959). Increases in both blood flow, measured via laser Doppler, and tissue oxygenation have been reported following immersion of a foot in CO<sub>2</sub> enriched water (Hartmann, Bassenge et al. 1997). The use of intra-vital video microscopy in rats has enabled visualisation of the vasodilation of microvessels in the skin, included muscular arterioles, when exposed to topical CO<sub>2</sub> (Minamiyama and Yamamoto 2010). Increased blood flow is

also measured when the animal is locally denervated, suggesting a direct local effect of CO<sub>2</sub> (Ito, Moore et al. 1989). Combined with a measured drop in skin pH (Minamiyama and Yamamoto 2010), these results suggest that local CO<sub>2</sub> inhibits the contractility of vascular smooth muscle. In addition to this vasodilatory effect, decreased pH and increased PCO<sub>2</sub> caused by topical CO<sub>2</sub> may further increase PtO<sub>2</sub> by the Bohr Effect, a right shift of the oxygen-haemoglobin dissociation curve that results in unloading of more oxygen to tissues at a given PO<sub>2</sub>.

Insufflation of CO<sub>2</sub> into the abdominal cavity is common practise in laparoscopic surgery, in which the gas is used to create intra-peritoneal pressure and allow the surgeon space to see within the normally closed peritoneal cavity. Three studies have investigated the effect of CO<sub>2</sub> pneumoperitoneum on PtO<sub>2</sub>. Gianotti *et al.* randomised patients to either laparoscopic or open colectomy (Gianotti, Nespoli et al. 2011). They found higher PtO<sub>2</sub> in the colon wall of patients under laparoscopic surgery, both at the start and end of surgery. The results of this study are supported by an investigation in mice, in which the creation of CO<sub>2</sub> pneumoperitoneum increased sub-peritoneal PtO<sub>2</sub> (Bourdel, Matsuzaki et al. 2007). The increase in sub-peritoneal PtO<sub>2</sub> was not observed when pneumoperitoneum was created with air (Bourdel, Matsuzaki et al. 2007). P<sub>a</sub>CO<sub>2</sub> increased but P<sub>a</sub>O<sub>2</sub> did not change, supporting the hypothesis that intra-abdominal CO<sub>2</sub> affects PtO<sub>2</sub> independent of P<sub>a</sub>O<sub>2</sub>. However, an increase in PtO<sub>2</sub> during CO<sub>2</sub> pneumoperitoneum is challenged by an observational investigation that measured PtO<sub>2</sub> in a surrogate wound in the upper arm of 52 patients undergoing laparoscopic or open colonic surgery (Fleischmann, Kugener et al. 2007). Despite similar arm-PtO<sub>2</sub> at

the start of surgery, and controlled  $P_aO_2$  and end tidal  $CO_2$ , arm  $PtO_2$  was significantly lower at the end of surgery in patients undergoing laparoscopic compared to open surgery (Fleischmann, Kugener et al. 2007). The authors suggest that the decrease in arm- $PtO_2$  may be due to increased intra-abdominal pressure in the laparoscopic group causing increased systemic vascular resistance, and subsequently reduced peripheral blood flow. If this is the case, the use of epidural anaesthesia by Gianotti *et al.* may have limited the increase in vascular tone and may explain why they were able to observe an increase in colon wall  $PtO_2$  (Gianotti, Nespoli et al. 2011).

While intra-abdominal  $CO_2$  may increase local  $PtO_2$ , high intra-abdominal pressure during pneumoperitoneum can decrease local  $PtO_2$  by increasing local vascular resistance via compression. The  $PtO_2$  in the colon wall is decreased by  $CO_2$  pneumoperitoneum of 15 mmHg compared with pressure  $\leq 12$  mmHg (Gianotti, Nespoli et al. 2011). In a porcine model, helium pneumoperitoneum of 15 and 25 mmHg caused a decrease in  $PtO_2$  of the small bowel from 43 to 31 and 12 mmHg, respectively, despite maintenance of  $PtO_2$  of approximately 90 mmHg at the axillary (Bongard, Pianim et al. 1995). Although a drop in cardiac output was measured, the maintenance of axillary peripheral  $PtO_2$ , despite the drop in bowel  $PtO_2$ , suggests that an increase in local vascular resistance is the predominant cause of the decrease in bowel  $PtO_2$  (Bongard, Pianim et al. 1995). In further support of the effect of high intra-abdominal pressure, high pressure  $CO_2$  pneumoperitoneum decreased sub-peritoneal  $PtO_2$  and increased peritoneal hypoxia compared to low pressure in a murine model (Bourdel, Matsuzaki et al. 2007; Matsuzaki, Jardon et al. 2010).

The results of these experiments, investigating CO<sub>2</sub> applied topically to the skin and to the abdomen during CO<sub>2</sub> pneumoperitoneum, suggest that intra-abdominal CO<sub>2</sub> increases local PtO<sub>2</sub>.

#### 2.4.3.6 Local and core heating

Increased temperature may cause vasodilation and also result in additional right shift of the oxygen-haemoglobin dissociation curve. Local warming of the skin (Sheffield, Sessler et al. 1996; Ikeda, Tayefeh et al. 1998) and maintenance of core body temperature (Sheffield, Sessler et al. 1996) have been shown to increase subcutaneous PtO<sub>2</sub> in healthy volunteers.

#### 2.4.3.7 Avoidance of anaemia

A study in 25 healthy, awake volunteers induced anaemia by isovolemic haemodilution and found haemodilution does not affect PtO<sub>2</sub> (Hopf, Viele et al. 2000). The authors concluded that blood transfusion to improve healing is not supported. However, hypovolemia does decrease PtO<sub>2</sub>. A study in dogs showed that a controlled haemorrhage of approximately 20 % of blood volume caused a 50 % drop in PtO<sub>2</sub> that was reversed upon re-infusion (Gottrup, Firmin et al. 1987). These results suggest that maintenance of tissue perfusion is more important than total blood oxygen content.

#### 2.4.3.8 Obesity

Obesity decreases both baseline perioperative PtO<sub>2</sub> and also the PtO<sub>2</sub> response to increased inspired fraction of oxygen, suggesting that obesity decreases tissue perfusion (Kabon, Nagele et al. 2004; Fleischmann, Kurz et al. 2005). This is most likely

explained by is an increase in size of fat cells without increased blood flow, leading to hypo-perfusion of fat tissue observed in both dogs (Di Girolamo, Skinner Jr et al. 1971) and humans (Jansson, Larsson et al. 1992). As predicted by low  $PtO_2$ , obesity is consistently reported as a risk factor for SSI (Waisbren, Rosen et al. 2010; Anannamcharoen, Vachirasrisirikul et al. 2012; Akça, Kurz et al. 2013). Following colon surgery, obese patients with BMI (body mass index)  $> 35 \text{ kg m}^{-2}$  have more than twice the rate of SSI compared with patients with BMI  $18\text{-}25 \text{ kg m}^{-2}$  (Akça, Kurz et al. 2013).

**Table 2-2: Summary of investigations that have investigated peri-operative strategies to increase tissue oxygen partial pressure in humans**

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)
Articles that measured peri-operative PtO <sub>2</sub>							
Gianotti et al., 2011 (Gianotti, Nespoli et al. 2011).	40	RCT. Left sided colectomy including re-anastomosis.	RCT: Open abdominal vs laparoscopic surgery (intra-abdominal pressure 0-15 mmHg) All: F <sub>i</sub> O <sub>2</sub> to achieve PtO <sub>2</sub> of 150 mmHg.	Beginning of the operation prior to bowel manipulation Licox™ CC1P1 Inserted through abdominal wall into the right colon wall. Mean distance to anastomosis 40 cm.	Continuously during surgery & daily for 1 hour until post-op day 6.	PtO <sub>2</sub> higher for laparoscopic group at 0, 15, 30, 180 minutes of surgery and post-op day 1, 2, 3 (p<0.05). End of operation: 53.1 ±4.7 vs 57.7 ± 7.9.	PtO <sub>2</sub> is ↑ in laparoscopic vs. open abdominal surgery. Intra-abdominal pressure of 15 mmHg ↓ PtO <sub>2</sub> compared with 10 & 12 mmHg. Mesenteric traction transiently ↓ PtO <sub>2</sub> .
Bakri et al, 2008 (Bakri, Nagem et al. 2008).	30	RCT + randomised crossover trial. CABG /or valve repair with CPB.	<u>RCT</u> : topical O <sub>2</sub> vs standard care. <u>All</u> : F <sub>i</sub> O <sub>2</sub> 60 % for post-op 1h then then <u>Crossover</u> : 20 % vs 50 % F <sub>i</sub> O <sub>2</sub> .	Pre wound closure Subcutaneous silicone tonometer & Licox™ At sternal wound, 0.5 mm below skin.	1h after admission to ICU, 1st & 2nd post-op mornings.	Mean (95 % confidence interval): <u>Topical O<sub>2</sub></u> : 25 (15-42) vs 26(16-42) p=0.88. <u>F<sub>i</sub>O<sub>2</sub></u> 20 vs 50 %: 23 (16-34) vs 27 (19-39) p<0.001.	Post-op high inspired O <sub>2</sub> ↑ PtO <sub>2</sub> & P <sub>a</sub> O <sub>2</sub> . Topical O <sub>2</sub> did not.
Fleischmann, 2007 (Fleischmann, Kugener et al. 2007).	52	Prospective observational. Elective open vs. laparoscopic left-sided colonic surgery.	Open vs. laparoscopic. All: F <sub>i</sub> O <sub>2</sub> to achieve P <sub>a</sub> O <sub>2</sub> 150 mmHg and ETCO <sub>2</sub> of 40 mmHg.	After induction. Subcutaneous silicone tonometer & Licox™. Lateral upper arm.	Every 10-min intra-op.	Start: 65.8±17.2 vs. 63.7±13.6 (p=0.714). End: 53.4±12.9 vs. 45.5±11.6 (p=0.012).	Laparoscopic colonic surgery decreases PtO <sub>2</sub> more than open colonic surgery. Suggest mechanism is increased intra-abdominal pressure.

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)
Hager et al., 2006 (Hager, Reddy et al. 2006).	30	RCT. Open gastric bypass. BMI > 50.	ETCO <sub>2</sub> 35 vs 50mmHg. All: FIO <sub>2</sub> 80 %.	After induction. Subcutaneous silicone tonometer & Licox™. Lateral upper arm.	Every 10-min intra-op.	56±13 vs 78±31 (p=0.029).	Intra-op mild hypercapnia ↑ PtO <sub>2</sub> in the obese.
Akca et al., 2006 (Akça, Sessler et al. 2006).	10	Randomised crossover trial. Surgery including cardio-pulmonary bypass.	Alternate exposure to 30 min PaCO <sub>2</sub> of 35 & 50 mm Hg during cardiopulmonary bypass. All: FIO <sub>2</sub> 85 %.	After induction. Subcutaneous silicone tonometer & Licox™ and FOXY™ fluorescence system (Ocean Optics Inc., Dunedin, FL, USA). Lateral upper arm.	Every 30-min intra-op.	122±39 vs 119±47, P=0.335 (Licox™) (Licox™ and FOXY™ measurement related r=0.92).	Hypercapnia probably ↑ peripheral PtO <sub>2</sub> by ↑ cardiac output, impossible during bypass, not by local vasodilation.
Fleischmann, 2005 (Fleischmann, Kurz et al. 2005).	35	Prospective observational study. Laparoscopy. Obese: Gastric banding Non-obese: fundoplication or cholecystectomy.	Grouped into 'Obese' (BMI ≥ 40 kg/m <sup>2</sup> ) and 'non-obese' (BMI <30 kg/m <sup>2</sup> ). All: FIO <sub>2</sub> to achieve P <sub>a</sub> O <sub>2</sub> 150 mmHg and PaCO <sub>2</sub> of 40 mmHg.	After induction. Subcutaneous silicone tonometer & Licox™. Lateral upper arm.	Every 5-min intra-op.	Average intra-op 41 ± 10 vs 57 ± 15 (p<0.001) FIO <sub>2</sub> required to maintain P <sub>a</sub> O <sub>2</sub> of 150 mmHg: 51 ± 13 % vs 40 ± 7 % (p=0.007).	Obesity ↓ PtO <sub>2</sub> and requires ↑ FIO <sub>2</sub> to reach P <sub>a</sub> O <sub>2</sub> of 150 mmHg.

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)																				
Kabon et al., 2004 (Kabon, Nagele et al. 2004).	46	Randomised cross over trial. Major abdominal surgery.	Crossover: Intra-op F <sub>I</sub> O <sub>2</sub> for 1 hr to achieve PaO <sub>2</sub> of 150 or 300 mmHg in random order. All: Post-op F <sub>I</sub> O <sub>2</sub> O <sub>2</sub> to achieve PaO <sub>2</sub> 120.	Subcutaneous silicone tonometer & Licox™ After induction: Lateral upper arm. End of surgery: 2–3 cm lateral & parallel to wound.	Arm: Intra-op, PACU, 1st post day. Wound: PACU & 1 <sup>st</sup> post op day.	PtO <sub>2</sub> : mean (25,75%) <table><tr><td>BMI &gt;30</td><td>BMI &lt;30</td></tr><tr><td>PaO<sub>2</sub></td><td></td></tr><tr><td>150</td><td>300</td></tr><tr><td>36*</td><td>47*# 57</td></tr><tr><td>30, (39, 55)</td><td>53, (56, 72) 92)</td></tr><tr><td>Intra-op (Arm)</td><td></td></tr><tr><td>Arm</td><td>Wound</td></tr><tr><td>43*</td><td>54 42*</td></tr><tr><td>(37, (47, 54)</td><td>(36, (49, 60) 68)</td></tr><tr><td>PACU</td><td></td></tr></table> * p<0.05 compared to non-obese; # compared to P <sub>a</sub> O <sub>2</sub> 150 mmHg. Post-op day1: No difference.	BMI >30	BMI <30	PaO <sub>2</sub>		150	300	36*	47*# 57	30, (39, 55)	53, (56, 72) 92)	Intra-op (Arm)		Arm	Wound	43*	54 42*	(37, (47, 54)	(36, (49, 60) 68)	PACU		PtO <sub>2</sub> is low in the obese, especially during surgery, even with supplemental F <sub>I</sub> O <sub>2</sub> . PtO <sub>2</sub> at arm not statistically different to wound.
BMI >30	BMI <30																										
PaO <sub>2</sub>																											
150	300																										
36*	47*# 57																										
30, (39, 55)	53, (56, 72) 92)																										
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PACU																											
Kabon, 2003 (Kabon, Fleischmann et al. 2003).	30	RCT. Liver resection; gastrectomy; or pancreaticoduodenectomy for cancer or pancreatitis.	General anaesthesia vs combined general and epidural anaesthesia (T4). All: F <sub>I</sub> O <sub>2</sub> 30 %.	After induction. Subcutaneous silicone tonometer & Licox™. Lateral upper arm. (Forearm-fingertip skin-temperature gradient.)	15 min intervals throughout surgery.	42.1±8.6 vs 54.3 ±7.4 (p=0.0002) (Forearm-finger skin gradient: 2.9±4.1 vs 1.6 ±3.4 °C, p=0.897).	Supplemental epidural anaesthesia increases PtO <sub>2</sub> during major abdominal surgery.																				

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)
Arkilic, 2003 (Arkilic, Taguchi et al. 2003).	56	RCT. Colon resection.	Conservative (8 mLkg <sup>-1</sup> h <sup>-1</sup> ) vs aggressive (16 to 18 mLkg <sup>-1</sup> h <sup>-1</sup> ) fluid management. All: F <sub>i</sub> O <sub>2</sub> 40 %.	After induction. Licox™. Upper arm. (Forearm-fingertip skin-temperature gradient.)	Every 15 min 30-180 min after induction and 0-60 min post-op.	Intra-op: 67±18 vs 81±26 (p=0.03) Post-op: 59±15 vs 77±26 (p=0.009). Forearm-fingertip skin temp: 3.6±2.8 °C vs 1.6±2.4 °C p=0.004.	Supplemental perioperative fluid administration significantly increases PtO <sub>2</sub> and perfusion.
Akca et al., 2003 (Akca, Liem et al. 2003).	20	RCT. Abdominal or orthopaedic surgery expected ≥ 2 h.	Intra-operative ETCO <sub>2</sub> 30 vs 45 mmHg. All: F <sub>i</sub> O <sub>2</sub> 40 %.	After induction. Subcutaneous silicone tonometer & Licox™. Lateral aspect upper arm. (Transcutaneous PtO <sub>2</sub> .)	Continuously intra-op.	62.9 ± 14.0 vs 88.81 ± 18.98 (p=0.014) (Trans-cutaneous PtO <sub>2</sub> : no difference between groups p=0.161).	Mild intra-op hypercapnia ↑ PtO <sub>2</sub> .
Buggy, 2002 (Buggy, Doherty et al. 2002).	32	RCT. Major abdominal surgery.	General anaesthesia with post-op iv analgesia vs combined general and epidural anaesthesia with post-op epidural analgesia.	End of surgery. Subcutaneous silicone tonometer & Licox™. Subcutaneous tissue of the wound along longitudinal axis.	Continuously for 24 h.	Mean 50.7 ±15 vs 64.4 ±14.	Epidural anaesthesia and post-operative analgesia increases wound PtO <sub>2</sub> compared with general anaesthesia and intravenous morphine analgesia.
Braga, 2001 (Braga, Gianotti et al. 2001).	257	RCT Resection for upper GI cancer.	Total para-enteral nutrition vs early enteral nutrition.	During surgery. Through abdominal wall into the thickness of the cecum wall. Licox™.	Immediately after laparotomy until the end of surgery, and daily for 7 d.	PtO <sub>2</sub> ↓ during surgery, similar between groups until day 5-7 p<0.01.	Early enteral nutrition ↑ gut PtO <sub>2</sub> following surgery.

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)
Plattner et al., 2000 (Plattner, Akça et al. 2000).	40	RCT Abdominal surgery incision ≥ 20 cm long.	Gauze & elastic adhesive vs warm bandage system that applies no pressure. Subgroup (n=10): Crossover between bandages, with warming off, i.e. simply a pressure-free bandage.	On completion of surgery. Subcutaneous silicone tonometer & Licox™ 2 to 3 cm lateral to incision.	Baseline: within 30 min post-op, bandage on, before heating. Recovery: 2 h post-op. 1st post-op day: 10-min intervals for 30 mins.	Baseline: 80±31 vs 110±40 p<0.05. Recovery: 85±34 vs 116±40 p=0.05. Post-op day1: 65±22 vs 82±30 p=0.05. Subgroup: Relieving pressure- ↑12±4 (p=0.005); Adding pressure- ↓13±3 (p<0.001).	PtO <sub>2</sub> was ~ 30 mmHg higher in patients with a heated bandage compared with conventional gauze and elastic. Part of this benefit is due to the pressure and contact free nature of the heated bandage system.
Greif et al., 2000 (Greif, Akça et al. 2000).	54 (sub set of 500)	RCT Open colorectal resection.	FiO <sub>2</sub> : 30 vs 80 % during surgery and for two h post-op.	After induction. Subcutaneous silicone tonometer & Licox™. Lateral upper arm. (SSI.)	Intra-op to two hrs post-op.	Intra-op: 59±15 vs 109±43 p<0.001. Post-op: 54±25 vs 73±25 p=0.02. (SSI: 11vs5% p=0.01).	Supplemental O <sub>2</sub> ↑ PtO <sub>2</sub> & is a practical method of reducing surgical-wound infections.
Akca et al, 1999 (Akça, Melischek et al. 1999).	30	RCT. Elective knee surgery.	Intra-articular saline (placebo) vs intra-articular lidocaine. All: Patient controlled intravenous opioid.	Timing not stated. Subcutaneous silicone tonometer & Licox™. Lateral upper arm. (Pain.)	From 1h after surgery, every 10 mins for 40 min.	86±15 vs 111±33 (p=0.016). (Pain 40 ± 17 vs 11 ± 10, on visual analogue scale out of 100.)	Poorly controlled surgical pain reduces P <sub>i</sub> O <sub>2</sub> .
Jacobi, 1996 (Jacobi, Zieven et al. 1996).	20	Prospective observational. Esophagogastr oostomy and gastric substitution.	Measurement of PtO <sub>2</sub> during different stages of surgical manipulation of the stomach.	Prior to gastric incision. Licox™ Stomach submucosa, apex of the gastric fundus close to the projected resection line.	Following midline incision, throughout surgery and post-op every 12 h for 4 d.	PtO <sub>2</sub> ↓ throughout surgery. Baseline vs after resection of the lesser curvature: 54.6 ± 10.9 vs 25.5 ±9 (p<0.01).	Surgical manipulation ↓ PtO <sub>2</sub> transiently.

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)
Bongard, 1995 (Bongard, Pianim et al. 1995).	6	Cross-over porcine model. Pneumoperitoneum only.	Intra-abdominal pressure 0 mmHg vs Helium pneumoperitoneum 15 mmHg vs 25mmHg.	Noncumulative fluorescence quenching optode (InnerSpace, California). Small bowel mucosa and subcutaneous at the axillary fold.	Every 15 min through-out surgery.	Bowel: 0 mmHg: 43 ±12 15 mmHg: 31 ± 12 25 mmHg: 12 ± 8 p<0.003 Axillary ~ 91 throughout. Cardiac output ↓.	Increased intra-abdominal pressure ↓ bowel PtO <sub>2</sub> mainly due to increased visceral vascular resistance.
Jonsson et al., 1987 (Jonsson, Jensen et al. 1987).	46	Prospective observational. Major abdominal or peripheral surgery (mastectomy, extremity, thyroidectomy).	Room air to FiO <sub>2</sub> 60-70 %, then saline infusion if PtO <sub>2</sub> did not increase by 20 %, despite ↑ P <sub>a</sub> O <sub>2</sub> (non-responder).	End of surgery. "Reference electrode" & sheathed platinum-needle electrode (760, Diamond Electrode, Inc.) in opposite ends of silicone tonometer. Lateral upper arm.	Post-op every 5 min.	Mean±SEM Room air vs FiO <sub>2</sub> 60-70 % day 0: 47±7 vs 80±27 p<0.05. Before vs after infusion (non-responders only): 48±8 vs 71±24 p<0.001.	Overall ↑ FiO <sub>2</sub> led to ↑ PtO <sub>2</sub> . 10/12 'non-responders' had ↑ PtO <sub>2</sub> when fluid was additionally infused, indicating they were poor perfused.
Chang et al., 1983 (Chang, Goodson lii et al. 1983).	33	Prospective observational. Mastectomy, abdominal surgery, vascular cardiac operations and non-operative controls.	Different levels of post-op F <sub>i</sub> O <sub>2</sub> . Supplemental fluid infusion as deemed clinically necessary (n=10).	Silicone tonometer & Clark-type electrode (Microsystem Denmark). Lateral arm. In mastectomy patients (n=11): 2 <sup>nd</sup> electrode under the most caudal portion inferior skin flap.	3-8 hrs post-op & daily to the fifth post-op day.	P <sub>t</sub> O <sub>2</sub> at wound ~10 mmHg less than at arm at same F <sub>i</sub> O <sub>2</sub> (p<0.05). P <sub>t</sub> O <sub>2</sub> ↑ with ↑F <sub>i</sub> O <sub>2</sub> at both sites (p<0.05) for each surgery type. Supplemental fluid ↑ P <sub>t</sub> O <sub>2</sub> in 19/22 measurements.	Tissue hypoxia is common following surgery & lessened over several days. PaO <sub>2</sub> even above sat of Haemoglobin ↑ PtO <sub>2</sub> . Fluid infusion often elevated P <sub>t</sub> O <sub>2</sub> .

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)												
Articles that measured PtO <sub>2</sub> in healthy volunteers																			
Treschan et al., 2003 (Treschan, Taguchi et al. 2003).	15	Randomised cross over. Healthy volunteers, anaesthesia only.	No anaesthesia vs 1. General anaesthesia. 2. Epidural anaesthesia (T10). 3. Combined general & epidural anaesthesia. All: F <sub>I</sub> O <sub>2</sub> 30 %.	Prior to anaesthesia. Subcutaneous silicone tonometer & Licox™. Lateral upper arm and lateral left thigh. (Forearm-fingertip and calf-toe skin temperature gradient.)	Continuously throughout.	Baseline: Arm- 57±11; Thigh: 54 ± 8. General anaesthetic: no difference. Epidural: ↑ PtO <sub>2</sub> by ~9 at thigh compared to arm. (Epidural: caused vasodilation of the legs, not the forearm.)	Epidural anaesthesia increased PtO <sub>2</sub> by a small amount, both with and without general anaesthesia.												
Akca et al., 2002 (Akça, Doufas et al. 2002).	10	Randomised cross-over trial. Anaesthesia only in healthy volunteers.	ETCO <sub>2</sub> : 20, 30, 40, 50, 60 mmHg. 45 min each PCO <sub>2</sub> . All: F <sub>I</sub> O <sub>2</sub> 30 %.	After induction. Subcutaneous silicone tonometer & Licox™ 5-6 cm lateral upper arm.	Intra-op at end of each period of ETCO <sub>2</sub> .	<table><tr><th>ETCO<sub>2</sub></th><th>PtO<sub>2</sub></th></tr><tr><td>20</td><td>51.9±9.9</td></tr><tr><td>30</td><td>57.8±11.2</td></tr><tr><td>40</td><td>65.2±14.5</td></tr><tr><td>50</td><td>74.0±12.3</td></tr><tr><td>60</td><td>82.4±18.6</td></tr></table> Linear regression: PtO <sub>2</sub> = 0.77 P <sub>a</sub> CO <sub>2</sub> + 35.42 (p<0.0001).	ETCO <sub>2</sub>	PtO <sub>2</sub>	20	51.9±9.9	30	57.8±11.2	40	65.2±14.5	50	74.0±12.3	60	82.4±18.6	PtO <sub>2</sub> ↑ with P <sub>a</sub> CO <sub>2</sub> in healthy, anaesthetised controls.
ETCO <sub>2</sub>	PtO <sub>2</sub>																		
20	51.9±9.9																		
30	57.8±11.2																		
40	65.2±14.5																		
50	74.0±12.3																		
60	82.4±18.6																		
Hopf et al., 2000 (Hopf, Viele et al. 2000).	25	Cohort + RCT. Healthy volunteers (un- anaesthetised)	All: Isovolemic haemodilution to haemoglobin 50 g/l. RCT: Perfusion control prior to haemodilution (by pre hydration & forced air warmer) vs. haemodilution only.	>30 min prior to intervention. Subcutaneous silicone tonometer & Licox™. Lateral upper arm.	Continuously.	Baseline: Perfusion control vs dilution only: 65.3±10.6 vs. 56.0±11.0. After haemodilution: No change in PtO <sub>2</sub> , with or without perfusion control.	Anaemia alone is not sufficient to reduce P <sub>t</sub> O <sub>2</sub> , and therefore transfusion solely to improve healing is not supported.												

First author, year, reference	N	Study design; Procedure	Intervention	PtO <sub>2</sub> method- Timing of placement, instrument & location. (Secondary measure)	Timing of O <sub>2</sub> reading	PtO <sub>2</sub> result - control vs intervention (mmHg) Mean±SD (unless stated)	Main O <sub>2</sub> conclusion (comments)										
Ikeda, 1998 (Ikeda, Tayefeh et al. 1998).	18	a): Randomised Crossover. b): Crossover. Healthy volunteers.	Heated bandage on anterior thigh. a): Bandage at 38, 42 or 46 °C for 2 h (n=8). b): 1 week each heated (38°C) then unheated bandage (n=10).	Prior to intervention. Subcutaneous silicone tonometer & Licox™. Thigh, under bandage. (Collagen deposition 8 days.)	Every 5 min.	50 % ↑PtO <sub>2</sub> irrespective of bandage temperature (p<0.05). PtO <sub>2</sub> remained elevated for 3 h after heating. (No difference in collagen deposition.)	Radiant heating at 38 °C significantly increases subcutaneous PtO <sub>2</sub> .										
Sheffield et al., 1996 (Sheffield, Sessler et al. 1996).	5	Cross over, set order. Healthy volunteers (un- anaesthetised)	All: F <sub>I</sub> O <sub>2</sub> to achieve PaO <sub>2</sub> of 325 mmHg <u>1-2</u> : Core cooled (shivering)+/- one arm warmed <u>3-4</u> : Core warmed (sweating) +/- one arm cooled.	Day before investigation. Fluorescent (InnerSpace Medical, Irvine, CA) (Jonsson, Jensen et al. 1991) Both lateral upper arms, depth 0.5 to 1.0 cm. (Laser Doppler flowmetry.)	Continuously.	<table><tr><td>Euthermic</td><td>116±29</td></tr><tr><td>Shivering</td><td>70±15*</td></tr><tr><td>Shivering &amp; arm warmed</td><td>113±12<sub>+</sub></td></tr><tr><td>Sweating</td><td>158±55</td></tr><tr><td>Sweating &amp; arm cooled</td><td>153±75</td></tr></table> <p>*p&lt;0.05 vs euthermic +p&lt;0.05 vs other arm (Doppler flowmetry measurements changed in line with PtO<sub>2</sub>.)</p>	Euthermic	116±29	Shivering	70±15*	Shivering & arm warmed	113±12 <sub>+</sub>	Sweating	158±55	Sweating & arm cooled	153±75	Hypothermia induced vasoconstriction ↓ PtO <sub>2</sub> ~40 mmHg and so may increase infection. Change is reversed by local heating.
Euthermic	116±29																
Shivering	70±15*																
Shivering & arm warmed	113±12 <sub>+</sub>																
Sweating	158±55																
Sweating & arm cooled	153±75																

ETCO<sub>2</sub> = End tidal partial pressure CO<sub>2</sub> ; RCT = Randomised controlled trial; BMI = Body mass index; FiO<sub>2</sub> = Inspired oxygen fraction; h or hr(s) = hour(s); ICU = Intensive care unit; Intra-op = Intra-operative(y); min(s) = minute(s); PACU = Post anaesthetic care unit; PCA = Patient controlled anaesthesia; Post-op = Post-operative(y); ↑ = Increase(s/ing); ↓ = Decrease(s/ing)

## 2.5 Role of the peritoneal mesothelium in the prevention of post-operative complications

### 2.5.1 Overview of the function of the peritoneal mesothelium

The peritoneum is a selectively permeable barrier that lines the abdominal wall (parietal peritoneum) and the outside of most of the abdominal organs (visceral peritoneum). It is an extensive organ with a surface area similar in magnitude to that of the skin. Structurally, the peritoneum is comprised of a cobblestone-like monolayer of mesothelial cells above underlying basement membrane and areolar connective tissue. The mesothelial cells of the parietal and visceral peritoneum are separated by peritoneal fluid secreted by the mesothelial cells. The mesothelial cells are held together by specialised junctions, most commonly tight junctions, with gap junctions also present (Michailova and Usunoff 2006). The mesothelial layer provides a physical protective barrier that prevents invasion of pathogens (Mutsaers and Wilkosz 2007). Peritoneal fluid secreted by the mesothelial cells is also suspected of providing physical separation from toxins created by the activity of lymphocytes within the peritoneal cavity (Mutsaers and Wilkosz 2007).

In addition to providing a physical barrier, which is similar to other epithelial layers, the mesothelium also plays an important active role in immune responses (Mutsaers and Wilkosz 2007). Along with peritoneal macrophages resident within the peritoneal cavity, mesothelial cells activate an inflammatory response by secreting a variety of chemokines and adhesion molecules that stimulate infiltration of leukocytes across the mesothelial monolayer (Topley 1995; Mutsaers and Wilkosz 2007). There is also

evidence that mesothelial cells assist in the innate immune response by presenting antigens and also by secreting reactive oxygen species in response to bacteria and chemokines (Mutsaers and Wilkosz 2007). Inflammatory cell clearance most likely occurs via stomata, openings in the mesothelial layer that lead directly to the lymphatic system (Mutsaers and Wilkosz 2007).

Finally, the fluid secreted by the mesothelium also reduces friction between intra-abdominal organs that aids in the prevention of adhesion of peritoneal surfaces, both normally and in response to injury (Mutsaers 2002). Friction is further reduced by the binding of the fluid in microvilli on the surface of the cell (Mutsaers, Prêle et al. 2007).

## 2.5.2 Post-operative adhesion formation

### 2.5.2.1 Burden of post-operative adhesion formation

Post-operative adhesion formation is a common and expensive problem that remains largely unsolved (Wilson 2007). Large database reviews show that 5 % of patients will have at least one readmission directly related to adhesions within five years of colorectal surgery, excluding appendectomies (Parker, Wilson et al. 2005). Following open gynaecological procedures there are between 0.6 and 2.9 readmissions directly related to adhesions per 100 initial procedures, depending on the type of surgery (Lower, Hawthorn et al. 2000). Over a ten-year period in the UK, an estimated 908 million Euros is spent on adhesion-related readmissions due to lower abdominal surgery (Wilson 2007). Adhesions are a leading cause of infertility, bowel obstruction and difficult re-operation (Diamond and Freeman 2001).

Recent literature reviews recommend the use of meticulous surgical technique, but there is no recommended routine use of any technology that consistently reduces post-operative adhesions formation (Alpay, Saed et al. 2008; Schnuriger, Barmparas et al. 2010; 2013). Nevertheless, the prevention of post-operative adhesion formation is very important, since surgery is the only treatment for peritoneal adhesions yet surgical division commonly leads to adhesion re-formation (Holmdahl 1999). There has been a call to investigate additional technologies to further reduce the incidence of post-operative adhesion formation (Alpay, Saed et al. 2008).

#### 2.5.2.2 Role of peritoneal mesothelium in the prevention of post-operative adhesion formation

One of the primary functions of the peritoneal mesothelium is to allow adhesion-free, low friction movement of intra-abdominal organs. This is achieved by secretion of fluid, including glycosaminoglycans and the surfactant phosphatidylcholine, and by trapping the surfactant in surface microvilli (Mutsaers, Prêle et al. 2007). Peritoneal trauma causes disruption of the low friction surface between the visceral and parietal peritoneum and can be caused by mechanical or thermal injury, infection, ischemia, desiccation or foreign body reaction (2013). This disruption of the two contacting surfaces of the mesothelial lining is the first step in post-operative adhesion formation (Haney and Doty 1994; Holmdahl 1999). The cascade following peritoneal trauma results in the formation of fibrin deposits. Breakdown of the fibrin requires the conversion of plasminogen to plasmin, and necessitates delicate control over plasminogen activators and inhibitors by local remaining mesothelial cells (Hellebrekers and Kooistra 2011). Mesothelial cells secrete both of the two

plasminogen activators utilised in this process (tissue plasminogen activator and urokinase plasminogen activator) and also the plasminogen activator inhibitors (1 and 2) (Mutsaers, Prêle et al. 2007; Hellebrekers and Kooistra 2011). If adequate fibrinolysis occurs, the mesothelial layer heals adhesion free. However, if there is inadequate fibrinolysis, fibrin deposition persists, capillary ingrowth ensues, and peritoneal adhesions are created.

Therefore, healthy mesothelial cells must be present to control the delicate balance between the deposition and breakdown of fibrin in order for the mesothelium to heal adhesion free. Research suggests that the mesothelium can heal without adhesion following a surgical incision in which the mesothelium is otherwise left intact (Gillett, James et al. 1994). However, if the incision is accompanied by denudation of the surrounding area of mesothelium by abrasion, post-operative adhesions form (Gillett, James et al. 1994). This emphasises the importance of reducing inadvertent loss of mesothelial cells during surgical exposure of the peritoneum in order to reduce the risk of post-operative adhesion formation.

### 2.5.3 Intra-peritoneal tumour implantation

#### 2.5.3.1 Burden of intra-peritoneal tumour implantation

Peritoneal implantation of tumour cells is non-curative and therefore a devastating progression of cancer metastases (Jayne 2007). Despite this, there is little data on the actual incidence of peritoneal carcinomatosis. Recently, data from the compulsory Stockholm County Council Registry in Sweden was reviewed to report on the incidence and prevalence of peritoneal carcinomatosis in all patients diagnosed with colo-rectal

cancer between 1995 and 2007, with follow up to 2010 (Segelman, Granath et al. 2012). 4.3 % of patients were diagnosed with peritoneal metastasis at the time of initial diagnosis of colo-rectal cancer, or within 30 days after diagnosis. A further, 4.2 % of patients developed peritoneal metastases within the follow up period. A total of 8.3 % of colo-rectal cancer patients were diagnosed with peritoneal metastases (Segelman, Granath et al. 2012). It is unclear how many of these diagnoses of peritoneal metastases followed a previous surgical procedure in which damage to the peritoneum may have been induced. However, it is likely that nearly all patients had surgical resection of their colo-rectal cancer upon initial diagnosis. This data is supported by a second review of 2406 colo-rectal cancer patients from a German database (Kerscher, Chua et al. 2013). Peritoneal metastases were detected in 4.5 % of patients at the time of initial diagnosis of colo-rectal cancer, and a further 5.9 % developed peritoneal metastases at later follow up (total 10.6 %). This data shows that peritoneal metastasis is common in colo-rectal cancer patients.

#### 2.5.3.2 Role of peritoneal mesothelium in the prevention of post-operative intra-peritoneal tumour implantation

Tumour cells proceed through several steps in the process of peritoneal tumour metastasis: entrance to the peritoneal cavity; adherence to the mesothelium; penetration of the mesothelium; invasion of the underlying connective tissue; and finally induction of angiogenesis (Jayne 2007; Mutsaers and Wilkosz 2007). Of these steps, the mesothelium appears to have the most potential to interfere with the initial adherence. Indeed it has been shown that tumour cells adhere preferentially to areas where the mesothelium is disrupted, exposing the underlying basement membrane, in

in-vitro human (Kiyasu, Kaneshima et al. 1981); in animal studies (Buck 1973; Koster, Volz et al. 1998; Van Den Tol, Van Rossen et al. 1998; Aoki, Shimura et al. 1999; Volz, Koster et al. 1999); and in tissue culture (Yu, Kuebler et al. 2010) investigations. Furthermore, when tumour cells are injected into the abdomen under conditions in which changes to the morphology of mesothelial layer have been observed, the peritoneum has increased permeability and increased implantation of tumour cells (Volz, Koster et al. 1999; Yu, Kuebler et al. 2010). When the mesothelium is intact, tumour cells are capable of adhering to the mesothelial surface, likely via adhesion molecules expressed on cancer cells, and induce mesothelial cell apoptosis (Jayne 2007; Mutsaers and Wilkosz 2007). However, mesothelial cells secrete hyaluronan that is able to bind to tumour cell adhesion molecules, inhibiting their adhesion (Mutsaers and Wilkosz 2007). Importantly, hyaluronan is present both as free hyaluronan in the peritoneal fluid and in a pericellular coat on the surface of the mesothelium. It is the free hyaluronan that is able to inhibit tumour adherence. If free hyaluronan is lost, as may occur following peritoneal lavage or evaporation of peritoneal fluid, binding to peri-cellular hyaluronan can aid in the adherence of tumour cells to the mesothelium (reviewed by Mutsaers and Wilkosz (Mutsaers and Wilkosz 2007)). Furthermore, inflammation of the mesothelium likely contributes to adhesion of tumour cells via increased expression of adhesion molecules on the mesothelial cell surface (Groothuis, Koks et al. 1999; Mutsaers and Wilkosz 2007).

Research in two independent mouse models of laparoscopic surgery has shown that preventing desiccation of the peritoneal mesothelium, by humidification of insufflation

CO<sub>2</sub>, reduces intra-peritoneal tumour dissemination (Binda, Corona et al. 2014; Carpinteri, Sampurno et al. 2015). The authors suggested that by reducing loss of peritoneal mesothelium caused by desiccation, the mesothelium remains a more intact barrier to tumour implantation. It is also possible that prevention of evaporation of peritoneal fluid, and therefore reduced interference with free hyaluronan, may also contribute to the finding.

#### 2.5.4 Expression of COX-2 expression in post-operative adhesion formation and tumourigenesis

Cyclooxygenase 2 isoform (COX-2) is an enzyme induced during a normal inflammatory response and it synthesizes prostaglandin precursors from fatty acids liberated from cell membranes by the action of phospholipase. It therefore is a primary step in the prostaglandin inflammatory pathway (Menter, Schilsky et al. 2010). Over-expression of cyclooxygenase 2 (COX-2) has been implicated in post-operative adhesion formation (Alpay, Saed et al. 2008) and administration of a COX-2 inhibitor reduces adhesion formation following laparotomy in a rat model (Guvenal, Cetin et al. 2001). COX-2 also appears to play a role in cancer progression (Greenhough, Smartt et al. 2009; Menter, Schilsky et al. 2010). Expression of COX-2 in tissue adjacent to tumour tissue is correlated with cancer recurrence and poor survival in human cancer patients (Lin, Lin et al. 2013) and COX inhibitors dramatically improve colo-rectal cancer prevention (reviewed by Smalley and DuBois (Smalley and DuBois 1997)). The relationship between COX-2 and cancer recurrence may be due to COX-2 stimulated increase in adhesion molecule expression and inhibition of apoptosis (Tsuji and DuBois 1995) and increase in production of angiogenic factors (Tsuji, Kawano et al. 1998). In a

laparoscopic mouse model, reducing inflammation by humidifying and warming the insufflation gas reduced both COX-2 expression and intra-peritoneal tumour implantation (Carpinteri, Sampurno et al. 2015). Therefore COX-2 expression may be a marker of mesothelial damage.

#### 2.5.5 Current evidence on disruption of the peritoneal mesothelium caused by surgical exposure

A systematic review of the effect of abdominal surgery on disruption of the peritoneal mesothelium is presented in Chapter 4. That systematic review concludes, firstly, that laparoscopic surgery can cause desquamation of areas of the mesothelial layer of the peritoneum. The degree of disruption appears to be reduced at lower pneumoperitoneum pressures and by the reduction of desiccation via warming and humidification of the insufflation gas. Secondly, a significant gap in current knowledge exists in the understanding of the effect of laparotomy on morphology of the peritoneal mesothelium, including whether desiccation of the peritoneum during laparotomy is sufficient to damage the peritoneal mesothelium.

Desquamation of mesothelial cells is the most important change reported following abdominal surgery. Loss of parts of the mesothelium leaves the abdominal cavity vulnerable during the time required for re-epithelisation. The loss of mesothelium appears to peak 12 hours after surgery, at least in murine models of laparoscopic surgery (Koster, Spacek et al. 1999; Volz, Koster et al. 1999). In addition to desquamation, morphologic changes including bulging and retraction of mesothelial cells were commonly reported following abdominal surgery, see Chapter 4, which is

more difficult to interpret. Cellular bulging and retraction likely represents change from flat to smaller diameter, cuboidal mesothelial cells that are characterised by increased organelles and a more metabolically active state (Mutsaers, Whitaker et al. 1996; Mutsaers 2002; Mutsaers, Whitaker et al. 2002; Mutsaers 2004). Activated mesothelial cells may contribute to peritoneal metastases both through increased expression of adhesion molecules and by exposure of the basement membrane around retracted cells (Mutsaers and Wilkosz 2007). Research has shown that this change in cell shape does not indicate that cellular death is inevitable. Retraction and elongation of mesothelial cells in a monolayer culture exposed to menstrual effluent was not associated with apoptosis or necrosis (Demir Weusten, Groothuis et al. 2000). In addition, in response to aseptic peritonitis, induced by intra-peritoneal injection of starch, mesothelial cells retracted but still retained cytoplasmic bridges between cells (Kaufman, Rostovshchikov et al. 1983). Inspection of the nucleus of those cells suggested that the retraction was not attributable to cell death (Kaufman, Rostovshchikov et al. 1983).

Conversely, other research shows that mesothelial cells do not always recover from this change in cell shape. Bulging and retraction of cells can be an indication of cellular necrosis or apoptosis, respectively (Saraste 1999; Krysko, Vanden Berghe et al. 2008). Following experimental bacterial peritonitis, retraction of the mesothelium was followed by cell death (Kaufman, Rostovshchikov et al. 1983). In addition, intra-abdominal pressure during laparoscopic surgery increases both mesothelial cell retraction (Du, Yu et al. 2011) and the proportion of mesothelial cells undergoing

apoptosis (Tosun, Samli et al. 2007). Therefore, neither the presence of mesothelial cells on the surface of the peritoneum, nor the presence of bulged, retracted cells, can rule out eventual mesothelial cell death.

#### 2.5.5.1 Detection of late stages of cell death by DNA fragmentation

Previous research suggests that abdominal surgery induces apoptosis in mesothelial cells (Tosun, Samli et al. 2007). At the time of peak mesothelial cell loss, it is likely that if remaining mesothelial cells are undergoing cell death they will be in the late stages of the process. During the late stages of apoptosis DNA is cleaved into small fragments (Csizmadia and Csizmadia 2009; Loo 2011). This fragmentation can be measured using a TdT mediated dUTP-biotin nick end labelling (TUNEL) assay in which the enzyme Terminal Deoxynucleotidyl transferase (TdT) attaches to the 3'-hydroxyl termini of fragmented genomic DNA (Csizmadia and Csizmadia 2009; Loo 2011). TUNEL staining will detect DNA damage that may be caused by apoptosis or necrosis (Charriaut-Marlangue and Ben-Ari 1995; Loo 2011). However, DNA fragmentation may also be detected during DNA repair and gene transcription (Loo 2011). Several articles have shown that it is possible to detect DNA fragmentation in paraffin embedded tissue sections (Csizmadia and Csizmadia 2009; Loo 2011). A TUNEL assay will not quantify apoptosis, rather help to confirm that cells still adherent to the peritoneal surface are not undergoing apoptosis. Counting of apoptotic cells would underestimate apoptosis due to the short life of apoptotic cells (Saraste 1999; Santamaría, Benito-Martin et al. 2009). In addition, apoptotic mesothelial cells are expected to become detached from the peritoneal surface (Marchi, Liu et al. 2000), itself a process of cell death (Saraste

1999; Krysko, Vanden Berghe et al. 2008). Detection of DNA fragmentation in mesothelial cells that remain adherent to the peritoneal surface following abdominal surgery may therefore be complementary to the measurement of mesothelial cell loss.

#### 2.5.5.2 Detection of sub-mesothelial oedema by measurement of sub-mesothelial thickness

Sub-mesothelial thickness is measured as the average thickness of the sub-mesothelial connective tissue measured perpendicular to the peritoneal surface, as defined and illustrated by Williams *et al.* (2002) (Williams, Craig et al. 2002). Two distinct areas within the sub-mesothelial connective tissue can be identified (Williams, Craig et al. 2002). The superficial 'compact zone' is characterised by mature fibrous tissue containing collagen and scattered elastin fibres. The deeper 'loose zone' is characterised by more loosely spaced collagen fibres, leukocytes, small blood and lymphatic vessels, nerves and adipose tissue (Williams, Craig et al. 2002). As the current research is interested in inflammation, the total area including both the compact and loose zone is more relevant than just the compact zone, which is significant to the measurement of peritoneal fibrosis (Williams, Craig et al. 2002).

Measurements of sub-mesothelial thickness and area vary between sections that are cut perpendicular to the orientation of the underlying muscle fibres, and sections cut parallel to the underlying muscle fibres, although whether the difference is statistically significant is unclear (Duman, Sen et al. 2001; Duman and Şen 2009). It is prudent to ensure that all slides are cut the same way, with respect to orientation in relation to the direction of the underlying muscle fibres. Sub-mesothelial thickness within a given

section, measured as the mean of the maximum and minimum thickness, does appear to be more consistent when the section is cut parallel to the underlying muscle fibres (Duman, Sen et al. 2001; Duman and Şen 2009).

#### 2.5.5.3 Detection of mesothelial defects by fluorescein application

Topical fluorescein is used in the detection and monitoring of corneal epithelial defects, as it binds to areas with disrupted epithelia and the underlying tissue, but not to intact epithelia (Lekhanont, Jongkhajornpong et al. 2013). A fluorescein solution is administered into the eye and any excess is washed. An abrasion of the corneal epithelium is then visible, under blue light, as a green fluorescent streak. Fluorescein may therefore have utility in visualising disruption to the peritoneal mesothelium by a similar mechanism.

#### 2.5.6 Current evidence on the role of desiccation during laparotomy on loss of peritoneal mesothelium

While there is evidence that desiccation contributes to loss of peritoneal mesothelium during laparoscopy, there is currently insufficient evidence to judge the extent of desiccation during laparotomy, see Chapter 4. However, it is clear that desiccation can damage the peritoneum. Microscopic peritoneal injury has been found following desiccation of the cecum with a “gentle stream” (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973) and 30 L/min (Burns, Skinner et al. 1995) of dry, compressed air for 5 min. Gently drying the mesothelium with a blow dryer for 10-20 min has been shown to denude the peritoneal surface and also cause changes in peritoneal transport similar to that observed following repeated peritoneal dialysis that

resulted in peritonitis (Verger, Luger et al. 1983). Furthermore, folding back of the peritoneum to allow the mesothelium to dry naturally for 5 min has been shown to alter to the appearance and density of microvilli and, after 15 min, to cause irregular cell morphology and create areas devoid of microvilli (von Ruhland, Newman et al. 2003). Desiccation has been commonly used to induce mesothelial damage experimentally (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973; Burns, Skinner et al. 1995; Seeger, Kaelin et al. 1997), and prevention of tissue desiccation is a common recommendation for the prevention of post-operative adhesion formation caused by mesothelial damage (DeWilde, Trew et al. 2007). It is therefore likely that exposure of the peritoneal mesothelium to the air of the operating room will cause damage. However, such an investigation has not been conducted, perhaps because peritoneal desiccation in open abdominal surgery had previously been considered unavoidable. An investigation into the effect on the peritoneal mesothelium of exposure to the air of the operating room must be conducted before strategies to prevent any such damage can be investigated.

#### 2.5.7 Current methods to reduce desiccation of the peritoneal mesothelium during surgery

Prevention of desiccation during abdominal surgery is commonly recommended (DeWilde, Trew et al. 2007; Pados, Venetis et al. 2010; Schnuriger, Barmparas et al. 2010; De Wilde, Brölmann et al. 2012; 2013), but description of exactly how to prevent desiccation is often vague. Expert consensus positions for gynaecological surgery recommend reducing mesothelial desiccation by limiting heat and light, using frequent irrigation and aspiration, and by minimising the use of dry towels or sponges (DeWilde,

Trew et al. 2007; De Wilde, Brölmann et al. 2012). Other guidelines recommend avoiding peritoneal desiccation but do not give advice as to how to do this (Schnuriger, Barmparas et al. 2010; The Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society of Reproductive Surgeons 2013).

Many guidelines recommend extensive lavage of the peritoneum during surgery (Alpay, Saed et al. 2008; De Wilde, Brölmann et al. 2012; Ouaïssi, Gaujoux et al. 2012; Correia 2014). However, criticism is growing against the unnecessary use of intra-peritoneal lavage, as it may increase the risk of post-operative complications by disrupting the peritoneal mesothelium and diluting the host defence (Mutsaers and Wilkosz 2007). Lavage may wash away fibrinolytic activity necessary to avoid post-operative adhesion formation (Hellebrekers and Kooistra 2011). In addition to reducing desiccation, lavage has been used according to the theory that it is necessary to wash contamination from the abdominal wound and cavity prior to wound closure (National Collaborating Centre for Women's and Children's Health and commissioned by the National Institute for Health and Clinical Excellence 2008). However, meta-analysis suggests that neither wound nor peritoneal lavage are effective in reducing the risk of surgical site infection (National Collaborating Centre for Women's and Children's Health and commissioned by the National Institute for Health and Clinical Excellence 2008).

In addition to intra-peritoneal lavage, wet gauze is often used to keep tissue moist.

However, pressing and rubbing the peritoneal surface causes loss of mesothelial cells

(von Ruhland, Newman et al. 2003). Mechanical trauma from gauze causes loss of peritoneal mesothelium that increases the risk of post-operative adhesion formation (Van Den Tol, Van Stun et al. 1997) and peritoneal implantation of tumour cells (Van Den Tol, Van Rossen et al. 1998). While prevention of desiccation during abdominal surgery is commonly recommended, there is no method of preventing intra-operative desiccation that is also atraumatic to the peritoneal mesothelium.

## 2.6 Previous rodent models to study the effect of gaseous exposure of the peritoneum during open abdominal surgery

While numerous well controlled animal models have been developed to study the effect of gaseous exposure during laparoscopy on the peritoneum (Binda, Molinas et al. 2006; Matsuzaki, Jardon et al. 2010), no such models exist for open abdominal surgery. This may be due to the previous belief that exposure of the peritoneum to the desiccating environment of the operating room could not be avoided. However, now that a promising therapy exists to protect the peritoneum during open abdominal surgery, a controlled animal model is required in order to study the effect of laparotomy on the peritoneum. Several studies have used rodents models to investigate the effect of laparotomy on morphology of the peritoneal mesothelium in rodents (Ryan, Grobety et al. 1973; Janik, Apkarian et al. 1982; Phillips and Dudley 1984; Koster, Volz et al. 1998; Bloechle, Kluth et al. 1999; Suematsu, Hirabayashi et al. 2001; Rosario, Ribeiro Jr et al. 2006; Lopes, De Oliveira et al. 2007). All of these models are limited by lack of control of exposure of the peritoneum to the desiccating environment of the operating room. The models either do not describe the laparotomy incision, or they describe the length of the incision only, suggesting that very little

exposure of the peritoneum was achieved. Furthermore, control of the operating room environment itself is not described and the models lack mechanical ventilation. In addition, methods to protect the mesothelium during tissue collection and processing are poorly described despite evidence that mesothelial damage occurs easily during tissue handling (Eckmann, Holstein et al. 1985; von Ruhland, Newman et al. 2003).

In addition to animal models used to investigate peritoneal morphology following laparotomy, relevant rodent models have also been developed to study the effect of peritoneal dialysis (Gotloib, Wajsbrot et al. 1995; Hekking, Aalders et al. 1998; Duman and Şen 2009). As peritoneal dialysis involves only minimal access, the models do not include exposure of the peritoneum to the operating room environment. However, very useful information has been published about the best way to handle the peritoneum to reduce artefact when studying peritoneal morphological changes following peritoneal dialysis (Duman, Sen et al. 2001; von Ruhland, Newman et al. 2003; Duman and Şen 2009) and these should be used to guide a model of the effects of laparotomy.

In addition to models of peritoneal dialysis, animal models developed to investigate adhesion formation following laparotomy may also provide useful examples for developing a rodent model for the current project. Adhesion formation following laparotomy has been studied in a mouse model that made an attempt to expose the peritoneum to the air within the operating room (Matsuzaki, Canis et al. 2007). In this model a 3 cm laparotomy incision was made and two sutures were applied 'to expose the peritoneum to the air for 60 min'. Seven days after surgery, laparotomy was

performed and showed an increase in adhesion formation along the mid-line incision. Retraction of the abdominal wall with two sutures is a promising idea for the current project, however it is likely that this design only exposed peritoneum along the mid-line and does not achieve sufficient peritoneal exposure for the current project. In addition to mid-line incision only, a rat uterine horn model has also been used to investigate adhesion formation following laparotomy (Guvenal, Cetin et al. 2001; Guvenal, Yanar et al. 2010; Erdemoglu, Seçkin et al. 2012; Çağlar, Yavuzcan et al. 2014; Karatas, Ozlu et al. 2014). In that model following 3-4 cm midline incision, cautery injuries were made on the uterine horns, and 2-3 weeks later, adhesion formation was measured at the uterine horns. The method of retraction or exposure to the operating room was not described, and again exposure of the peritoneum was insufficient for the current project.

One of the aforementioned adhesion models was also used to measure tissue oxygen partial pressure ( $PtO_2$ ) (Matsuzaki, Canis et al. 2007). Of particular relevance to the current project, the results showed that mechanical ventilation is necessary in order to avoid peritoneal hypoxia during insufflation of  $CO_2$  into the abdominal cavity, at least under the pressure of pneumoperitoneum (Bourdel, Matsuzaki et al. 2007; Matsuzaki, Jardon et al. 2010). In the model the  $PtO_2$  probe was inserted into the retroperitoneal space. It is preferable in the current model to insert the tissue oxygen probe in tissue that is directly exposed to the gaseous environment within the abdominal cavity, as this may provide more sensitive measurement of changes in  $PtO_2$ .

### **3 Abdominal Surgery and Disruption of the Peritoneal**

#### **Mesothelium: A Systematic Review**

##### **3.1 Introduction**

Post-operative adhesion formation is a common and expensive problem that remains largely unsolved (Wilson 2007). Disruption of the two contacting surfaces of the mesothelial lining of the peritoneum is described as the first step in post-operative adhesion formation (Haney and Doty 1994; Holmdahl 1999). Furthermore, adhesion formation requires loss of mesothelial cells in addition to the surgical incision (Gillett, James et al. 1994). Mesothelial disruption is multifactorial and can be induced by exposing the peritoneal surface to: rubbing (Gillett, James et al. 1994; Burns, Skinner et al. 1995); ischemia (Matsuzaki, Jardon et al. 2010); desiccation (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973; Burns, Skinner et al. 1995); and lavage solutions (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973; Lopes, De Oliveira et al. 2007). Despite understanding that many factors contribute to the disruption of the mesothelium, most proposed interventions to prevent adhesion formation focus on manipulation of mesothelial repair (Holmdahl 1999; Schnuriger, Barmparas et al. 2010). There is a relative lack of research investigating prevention of the initial iatrogenic disruption to the mesothelium during surgery. This may, in part, be explained by an absence of information about the morphological disruption to the peritoneal mesothelium that occurs post abdominal surgery, particularly open abdominal surgery. Identification of the specific morphological

damage that occurs will allow design and testing of interventions, tailored to prevent the mesothelial disruption and associated adhesion formation.

Therefore, the aim of this systematic review was to identify and summarise what is currently known about the consequences of abdominal surgery (laparotomy and laparoscopy) on the morphological integrity of the peritoneal mesothelium. The hypotheses were that 1) exposure of the visceral and parietal peritoneum to a gaseous environment during surgery will cause loss of continuity of the mesothelium, due to both retraction and loss of mesothelial cells; 2) the disruption will be associated with increased exposure (longer duration of surgery; larger area of surgical access; increased pneumoperitoneum gas flow rate, pressure and desiccation). Outcomes from this review may allow researchers to pursue new ways to prevent post-operative adhesion formation, by targeting the prevention of disruption to the mesothelium. This is the first systematic review of this topic.

### 3.2 Materials and methods

This study conforms to the PRISMA statement for preferred reporting items for systematic reviews (Moher, Liberati et al. 2009). A protocol for this systematic review has not been published.

### 3.2.1 Eligibility criteria

Investigations of any design that carried out microscopic morphological analysis of peritoneal tissue biopsies, collected antemortem, following laparotomy or laparoscopy, of any duration. Investigations must have involved either incision of the abdominal wall for open abdominal surgery or the generation of a pneumoperitoneum. No restriction was placed on the type of insufflation gas, gas flow rate or pressure used. Included investigations had to assess at least one of the following morphological features: the presence/absence of mesothelial cells; mesothelial cell size or shape; visibility of intercellular clefts; visibility of basement membrane or basal lamina; microvilli appearance.

Investigations were excluded if the peritoneum examined was reported to have been exposed to deliberate experimental removal of the peritoneal mesothelium, peritoneal dialysis, any adhesion prevention drug or mesh/prosthesis or any other intra-abdominal mesh/prosthesis. Furthermore, analysis of diseased peritoneal tissue was excluded. Therefore investigations involving the structure of intra-abdominal adhesions, the structure of peritoneal tumours, peritonitis and intra-peritoneal endometriosis were all excluded.

### 3.2.2 Search methods for identification of investigations:

An electronic database search was conducted using SciVerse Scopus, which includes 100 % Medline and Embase coverage, and a wider range of journals than PubMed and Web of

Science (Jain 2005; Falagas, Pitsouni et al. 2008). No language or date restrictions were used. The titles, abstracts and key words were screened to identify whether the articles met the inclusion criteria. Full-text copies of identified articles were then reviewed for a final decision for inclusion. In addition, the titles and abstracts of articles in the reference lists of all included trials, and several review articles (Neuhaus and Watson 2004; Canis, Matsuzaki et al. 2007; van der Wal and Jeekel 2007; Brokelman, Lensvelt et al. 2010; Sammour, Kahokehr et al. 2010), were searched for additional trials that met the inclusion criteria.

### 3.2.3 Full search strategy

TITLE-ABS-KEY(surgery OR surgical OR laparotom\* OR laparoscop\* OR peritoneoscop\* OR celioscop\* OR pneumo-peritoneum OR pneumoperitoneum OR "pneumo peritoneum")

AND TITLE-ABS-KEY(mesotheli\* OR peritone\*)

AND TITLE-ABS-KEY(microscop\* OR SEM OR TEM OR EM OR histolog\*)

AND TITLE-ABS-KEY(morpholog\* OR integrity OR injur\* OR change\* OR structur\* OR damage OR alteration\*)

### 3.2.4 Data extraction

Two investigators (JM - Jean Marshall and AR - Angelique Riepsamen) independently carried out the identification of investigations and data extraction. The search strategies,

eligibility criteria and data extraction forms were piloted before use. The two investigators compared identified investigations and extracted data at multiple meetings. Any discrepancies were discussed and resolved. A list of the data that was extracted is shown in Figure 3-1. Data related to risk of bias was collected to help explain any disparity in results and assess the strength of evidence, as recommended by published guidelines (Moher, Liberati et al. 2009).

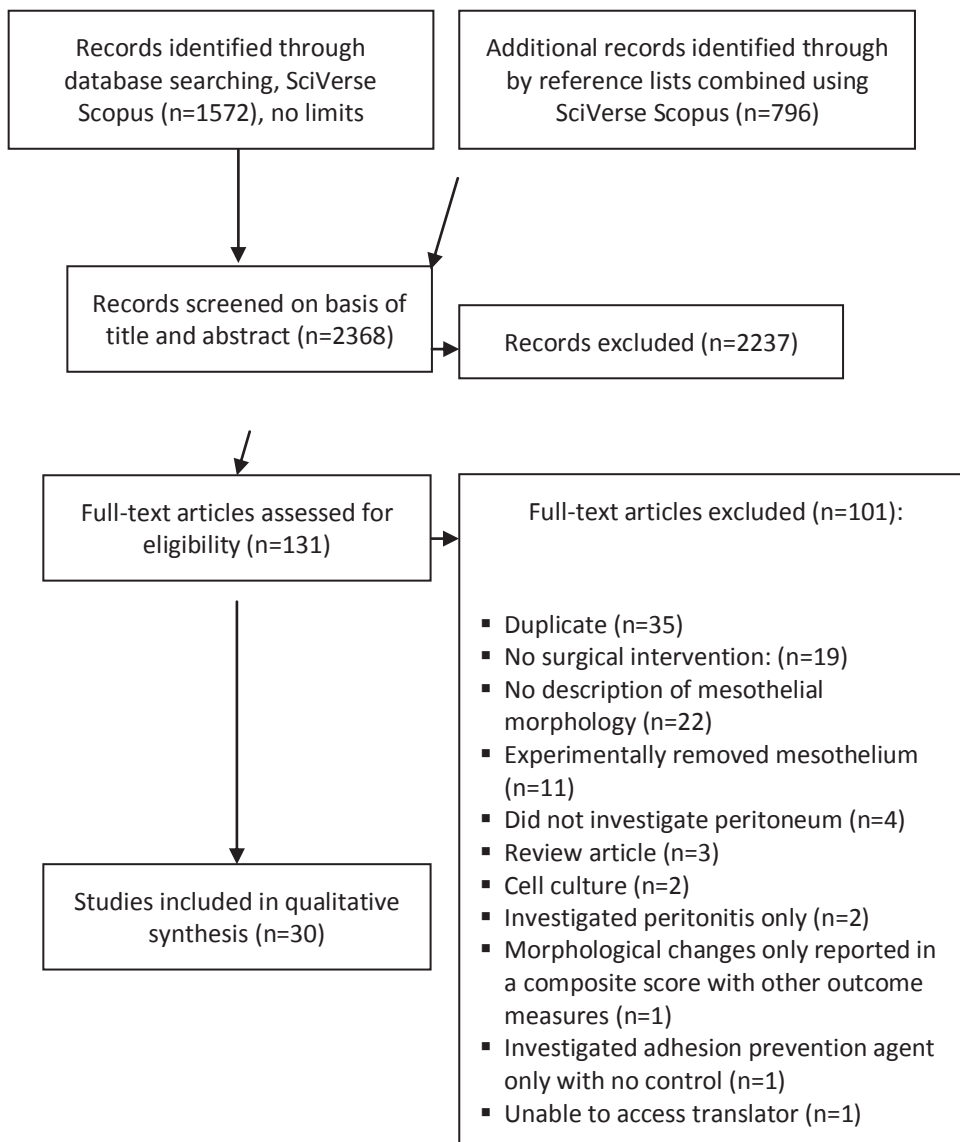
Methodology data extracted
<ul style="list-style-type: none"> <li>• Investigation design</li> <li>• Subject species strain, sex, age &amp; weight</li> <li>• Sample size</li> <li>• Intervention description</li> <li>• Intervention duration</li> <li>• Pneumoperitoneum pressure</li> <li>• Length of incision (s)</li> <li>• Control condition</li> <li>• Tissue biopsy location, timing &amp; collection method</li> <li>• Method of tissue fixation &amp; processing</li> <li>• Method of biopsy imaging</li> <li>• Method used to rate mesothelial morphology</li> </ul>
Results data extracted
<ul style="list-style-type: none"> <li>• Mesothelial morphology including size, shape, &amp; existence</li> <li>• Microvilli morphology including density, existence &amp; shape</li> <li>• Exposure of basement membrane</li> <li>• Statistical results &amp; p-values, when reported</li> <li>• Biopsy results at different time points were extracted separately</li> </ul>
Risk of bias data extracted
<ul style="list-style-type: none"> <li>• Randomisation of subjects</li> <li>• Blinding</li> <li>• Randomisation of portion of the microscopy image selected for analysis</li> <li>• Use of an objective rating method</li> <li>• Rating criteria – descriptive or quantitative</li> <li>• Full reporting of results</li> <li>• Control of air-conditioning during laparotomy, if applicable</li> <li>• Control of peritoneal exposure during laparotomy, if applicable</li> <li>• Provision of ventilatory support during general anaesthesia</li> <li>• Replacement of fluid</li> <li>• Timing of biopsy following surgical intervention</li> <li>• Number of assessors</li> <li>• Inclusion of an appropriate control group</li> </ul>

**Figure 3-1: List of data extracted from included investigations**

### 3.3 Results

#### 3.3.1 Data identification

The electronic database search was conducted by JM on 9<sup>th</sup> February 2012 and updated on 10<sup>th</sup> November 2015. Data identification and extraction was then carried out in duplicate by JM and AR. Data retrieval results are summarised in the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram, Figure 3-2. Four included investigations published in German (Eckmann, Holstein et al. 1985; Jonecko 1990; Schaeff, Paolucci et al. 1998; Koster, Spacek et al. 1999) and two articles published in Chinese (Liu and Hou 2006; Zhao, Li et al. 2010) were translated by a researcher fluent in the respective language. One additional article published in Chinese (Zhang, Wu et al. 2012) was identified during the 2015 database search, but could not be translated due to lack of access to a fluent translator and was therefore excluded.



**Figure 3-2: PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow diagram of data identification**

### 3.3.2 Risk of bias

The risk of bias within the studies reviewed is summarised in Table 3-1. Reporting of assessor blinding within the investigations was rare. All but one of the 30 included investigations (Du, Yu et al. 2011) used subjective morphology rating methods and none

used a mesothelial cell specific stain. Only two investigations reported using more than one assessor to review peritoneal microscopic images (Suematsu, Hirabayashi et al. 2001; Papparella, Nino et al. 2014) and none reported on the reliability or repeatability of the assessment. Eight investigations fully reported results; most included no more than a generalised description of group results. Of the human investigations, one of the six investigations reported randomisation of participants (Liu and Hou 2006). In addition, only three animal investigations reported ventilating the animals (Glew, Campher et al. 2004; Matsuzaki, Jardon et al. 2010; Carpinteri, Sampurno et al. 2015) and three reported replacement of lost fluids (Glew, Campher et al. 2004; Erikoglu, Yol et al. 2005; Du, Yu et al. 2011). Results did not appear to differ between investigations that employed more or less methods to reduce bias.

**Table 3-1: Summary of Assessment of Risk of Bias. Methods taken to reduce bias are highlighted in grey.**

#	1 <sup>st</sup> Author, Year	Randomisation	Blinding of tissue collector	Standard or randomised choice of image portion for analysis	All results reported	Rating method: <u>Descriptive/</u> <u>Quantitative/</u> <u>Semi-Quantitative</u>
Human laparotomy only						
1.	Eckmann, 1985 (Eckmann, Holstein et al. 1985)	N	N	NS	N	D
2.	Jonecko, 1990 (Jonecko 1990)	N	N	NS	N	D
3.	Slater, 1989 (Slater, Raftery et al. 1989)	N	N	NS	N	D
Human laparoscopy and laparotomy						
4.	Liu, 2006 (Liu and Hou 2006)	Y	NS	NS	N	D
5.	Schaeff, 1998 (Schaeff, Paolucci et al. 1998)	NS	NS	NS	N	D
6.	Zhao, 2010 (Zhao, Li et al. 2010)	N	Microscopist & assessor	Y	Y	SQ
Animal laparotomy only						
7.	Haney, 1992 (Haney and Doty 1992)	NS	NS	NS	N	D
8.	Janik 1982 (Janik, Apkarian et al. 1982)	NS	NS	NS	N	D
9.	Lopes, 2007 (Lopes, De Oliveira et al. 2007)	NS	NS	NS	Y	SQ
10.	Phillips, 1984 (Phillips and Dudley 1984)	NS	NS	NS	N	D
11.	Ryan, 1973 (Ryan, Grobety et al. 1973)	NS	NS	NS	N	D
Animal laparotomy and laparoscopy						
12.	Bloechle, 1999 (Bloechle, Kluth et al. 1999)	Y	Microscopist & assessor	NS <sup>b</sup>	Y	D
13.	Rosario, 2006 (Rosario, Ribeiro Jr et al. 2006)	NS	NS	Y	Y	SQ
14.	Suematsu, 2001 (Suematsu, Hirabayashi et al. 2001)	NS	Assessor	NS <sup>b</sup>	N <sup>a</sup>	SQ <sup>d</sup>
Animal laparoscopy only						
15.	Davey, 2013 (Davey, Hayward et al. 2013)	NS	Y	NS	Y	D
16.	Du, 2011 (Du, Yu et al. 2011)	Y	NS	Y	Y	Q
17.	Erikoglu, 2005 (Erikoglu, Yol et al. 2005)	NS	NS	NS	N <sup>a</sup>	SQ

#	1 <sup>st</sup> Author, Year	Randomisation	Blinding of tissue collector	Standard or randomised choice of image portion for analysis	All results reported	Rating method: Descriptive/ Quantitative/ Semi-Quantitative
18.	Glew, 2004 (Glew, Campher et al. 2004)	Y	NS	NS	N	D
19.	Hazebroek, 2002 (Hazebroek, Schreve et al. 2002)	Y	NS	NS <sup>b</sup>	N	D
20.	Koster, 1999 (Koster, Spacek et al. 1999)	NS	NS	NS	N	D <sup>c</sup>
21.	Matsuzaki, 2010 (Matsuzaki, Jardon et al. 2010)	Y	NS	NS	N	D
22.	Papparella, 2014 (Papparella, Nino et al. 2014)	Y	Y	NS	N	D
23.	Papparella, 2007 (Papparella, Noviello et al. 2007)	Y	NS	NS	N	D
24.	Peng, 2009 (Peng, Zheng et al. 2009)	Y	Assessor	Y	N	D
25.	Volz, 1999 (Volz, Koster et al. 1999)	Y	NS	NS	N	D <sup>c</sup>
Animal laparoscopy with tumour cell injection						
26.	Carpinteri, 2015 (Carpinteri, 2015 #745)	NS	NS	NS	Y	Q
27.	Hirabayashi, 2002 (Hirabayashi, Yamaguchi et al. 2002)	NS	NS	NS	N	D
28.	Koster, 1998 (Koster, Volz et al. 1998)	NS	NS	NS	N	D
29.	Ordemann, 2004 (Ordemann, Jakob et al. 2004)	Y	NS	Y	Y	SQ <sup>e</sup>
30.	Volz, 1999 (Volz, Koster et al. 1999)	NS	NS	NS	N	D <sup>c</sup>

NS = not stated; <sup>a1</sup> Table of summarised results for each group, but no statistics or individual results; <sup>b1</sup> Described that multiple areas were analysed, but did not describe how the portion of image to be analysed was chosen; <sup>c1</sup> Feature had to occur in entire group; <sup>d1</sup> Grade assigned by 3 or more observers was used; <sup>e1</sup> Feature had to occur in ≥2 quadrants

### 3.3.3 Effect of laparotomy on mesothelial morphology

The investigation characteristics and results of the six human and nine animal investigations that reported morphology of the mesothelium following laparotomy is shown in Table 3-2. Overall there were conflicting results regarding whether laparotomy causes morphological changes to the peritoneum. Importantly, there was poor reporting of study design aspects related to peritoneal exposure, illustrated by a lack of reporting of surgery duration (Table 3-2). Changes in mesothelial morphology were reported in seven investigations. Of those, two human (Eckmann, Holstein et al. 1985; Zhao, Li et al. 2010) and three animal (Ryan, Grobety et al. 1973; Suematsu, Hirabayashi et al. 2001; Lopes, De Oliveira et al. 2007) investigations reported mesothelial cell desquamation following laparotomy. Investigations that collected visceral peritoneum were just as likely to report changes in mesothelial morphology as investigations that collected parietal peritoneum.

**Table 3-2: Summary of Investigations of Morphological Changes of the Peritoneal Mesothelium following Laparotomy**

	1 <sup>st</sup> Author, Year	Species V(isceral) / P(arietal)	Biopsy timing	Intervention v control (number of subjects) <sup>c</sup>	Surgery duration (min)	Imaging method	Mesothelium morphology result summary			
							Following laparotomy			Summary
							Bulging	Retraction	Desquamation	
Human										
1.	Eckmann, 1985 (Eckmann, Holstein et al. 1985)	Human P	During LT	Various LT procedures - greater omentum moved anteriorly to facilitate tissue collection (5) v no control	NS	SEM TEM LM	NS	NS	Y	LT: Even the most careful handling of the large omentum caused loss of individual MC & tearing away of areas of the MC layer & BM.
2.	Jonecko, 1990 (Jonecko 1990)	Human P	During LT	LT (total 18 also including cases or peritonitis v no control. Surgery purpose NS	NS	SEM TEM LM	N	N	N	LT: Peritoneum was dominated by large flat MC & had areas of small spheroid MC.
3.	Liu, 2006 <sup>f</sup> (Liu and Hou 2006)	Human P	0, 30, 90, & 120 min into LT	LT (20) v LS (20) Myomectomy or hysterectomy	NS	TEM SEM	N	Y	N	LT: Inter cellular clefts occasionally after 90 min & significant at 120 min. No change <90 min LS: MC desquamation.
4.	Schaeff, 1998 <sup>f</sup> (Schaeff, Paolucci et al. 1998)	Human NS	0, 1, 2 & 3 h into LT	LT (3) v gasless LS (3) v LS (3) Surgery purpose NS	NS	EM TEM	N	N	N	LT & gasless LS: Confluent flat MC, dense microvilli. LS: MC separation, rounding & loss of contact with BM.
5.	Slater, 1989 (Slater, Raftery et al. 1989)	Human V & P	During LT	Excision of kidneys for donation (5) v no control	NS	TEM	N	N	N	Confluent MC

6.	Zhao, 2010 <sup>†</sup> (Zhao, Li et al. 2010)	Human V	During LT or LS	LT (23) v LS (27) Resection of colorectal cancer	183	LM SEM	Y	Y	Y	LT: MC oedema, retraction, separation & exposure of BM. More peritoneal surface injury compared to LS. Composite peritoneal injury score 34(LT) v 13(LS) (p<0.01)
Animal										
7.	Bloechle, 1999 <sup>†</sup> (Bloechle, Kluth et al. 1999)	Rat P	LT start, ab puncture start, 2&12 h	6-8 mm LT followed after 12 h by abdominal puncture (24) – with gastric perforation (12) or without (12)	NS	SEM	NS	Y	N	Without perforation: Intact layer, dense microvilli. Perforation: MC broke into groups. Shrinking & vanished microvilli at 12 h.
8.	Haney, 1992 (Haney and Doty 1992)	Mouse P	NS (results at 3 h & 3 d described)	LT - Dorsal incision (29) <sup>b,e</sup>	NS	SEM TEM	N	N	N	LT: Flat MC with indistinct MC borders & scattered microvilli.
9.	Janik, 1982 (Janik, Apkarian et al. 1982)	Rabbit V	Immediately after LT	LT length NS & rubbing 5 times with latex gloved fingers or gauze sponge v LT only (6 total)	NS	SEM	N	N	N	LT & rubbing (finger or glove): loss of the collagen latticework resting on top of intestinal MC. Flattened MC compared to rolling shape in control.
10.	Lopes, 2007 (Lopes, De Oliveira et al. 2007)	Rat P	24 h Control: end of LT	4 cm LT & for 1-2 minutes instillation of 0.9 % NaCl at 50°C (10), 37°C (10) or 0°C (10) v 4 cm LT only (20)	2	LM TEM	NS	NS	Y	Sight to moderate destruction of MC seen in all groups including 90 % of LT only animals (p>0.05 <sup>h</sup> ).
11.	Phillips, 1984 (Phillips and Dudley 1984)	Rat V & P	24,48,72 h & 1 month	LT length NS & peritoneal lavage: low & high concentration of antibiotic v 0.9 % saline. With & without handling of the peritoneum (100 total)	NS	SEM	NS	NS	N	High concentration lavage: complete loss of microvilli at 24 & 48 h. Partial recovery 72 h, normal at 1 month. Saline or low concentration lavage: No loss of microvilli. All handling groups: Early loss of microvilli, normal at 1 month.
12.	Rosario, 2006 <sup>†</sup> (Rosario, Ribeiro Jr et al. 2006)	Mice P	2 & 24 h	LT 2 cm (10) v anaesthesia only (10)	30	SEM	N	N	N	LT: Reduction in microvilli presence only (p<0.05 <sup>h</sup> ) Control: Confluent layer of MC, no intercellular clefts, 'unchanged' microvilli.

13.	Ryan, 1973 (Ryan, Grobety et al. 1973)	Rat V	0h & up to 2 months	LT length NS 5 min drying with a gentle stream of condensed air or 30 min wetting with isotonic saline v control condition not described (NS)	5 min Or 30 min	LM TEM	N	N	Y	Drying: Extensive MC loss at 0h & complete loss at 4-24 h. Regeneration complete at 7 d. Wetting: Complete loss of MC at 30 min. Regeneration not described. Control: Thin, flat confluent MC layer
14.	Suematsu, 2001 <sup>f</sup> (Suematsu, Hirabayashi et al. 2001)	Mouse V	0, 24, 72 h Control: 0 h	LT (9) with abdomen covered with wet gauze v anaesthesia only (3)	30	SEM	NS	NS	Y	LT: Complete MC destruction Control: No detachment or bulging of MC, or intercellular clefts. Delayed samples not taken in control group.
Animal laparotomy with intra-peritoneal tumour cell injection (IPC)										
15.	Koster, 1998 <sup>f</sup> (Koster, Volz et al. 1998)	Mouse P	1, 2, 6, 12, 24, 48, 72, 96 h	LT length NS & IPC (47) v IPC only (47)	NS	SEM	N	N	N	LT with IPC & control: No observation of MC retraction, cuboidal shape or BM exposure.

BM=basement membrane; d= day(s); EM=scanning electron microscopy; IPC=intraperitoneal cancer cell injection; h=hour(s); LM=light microscopy; LS=laparoscopy;  
LT=laparotomy; MC=mesothelial cell(s); min=minute(s); NS=not stated; n/a =not applicable; TEM=transmission electron microscopy; v=versus; V=visceral  
peritoneum; P=parietal peritoneum.

a This is a control group of an investigation of gastric perforation; b These are control groups an investigation of adhesion barriers; c Number in parenthesis is  
number of subjects; e Ventral excision & excision of peritoneum also described, but results not reported; f Assessed LT & LS; g Peritoneal injury created on  
abdominal wall & one uterine horn, or both uterine horns with uterine horns loosely sutured together. Injury created by excision, scraping, incision or  
electrocautery; h compared with control group

### 3.3.4 Effect of laparoscopy on mesothelial morphology

The investigation characteristics and results of the three human and 19 animal investigations that assessed the effect of laparoscopy on the morphology of the mesothelial layer of the peritoneum are shown in Table 3-3. The majority of investigations reported that pneumoperitoneum for laparoscopy surgery causes morphological changes to the peritoneal mesothelium including mesothelial desquamation. Meta-analysis was not possible due to the lack of quantification of changes.

All three human investigations reported morphological changes to the mesothelium. Mesothelial cell desquamation was reported following gynaecological procedures (Liu and Hou 2006) and un-specified abdominal surgery (Schaeff, Paolucci et al. 1998). A further human study reported peritoneal surface changes following resection of colo-rectal cancer, however, it is not clear if any mesothelial desquamation was observed as a composite score of several morphological features was utilised (Zhao, Li et al. 2010). All tissue samples were taken during the surgical procedure.

Mesothelial changes reported in human investigations were supported by morphological changes were seen following laparoscopy in animals. Of 19 animal investigations, 17 found morphological changes following CO<sub>2</sub> pneumoperitoneum. Retraction and bulging of mesothelial cells was commonly reported. Most investigations found desquamation of mesothelial cells following CO<sub>2</sub> pneumoperitoneum, at times massive, leaving large denuded areas of peritoneum (Koster, Volz et al. 1998; Koster, Spacek et al. 1999; Volz,

Koster et al. 1999; Volz, Koster et al. 1999; Erikoglu, Yol et al. 2005; Peng, Zheng et al. 2009; Matsuzaki, Jardon et al. 2010; Du, Yu et al. 2011; Papparella, Nino et al. 2014). Conversely, one investigation found no change in morphology (Hirabayashi, Yamaguchi et al. 2002), and another found that pneumoperitoneum served to detach mesothelial cells only when followed by peritonitis induced by gastric perforation induced peritonitis (Bloechle, Kluth et al. 1999). This may be explained by collection of biopsies directly after completion of pneumoperitoneum, which may not allow time for changes in morphology to manifest (Koster, Spacek et al. 1999; Volz, Koster et al. 1999; Volz, Koster et al. 1999; Carpinteri, Sampurno et al. 2015). In addition, one animal investigation found morphological changes in only 16 % of animals and no desquamation of mesothelial cells (Ordemann, Jakob et al. 2004).

With regard to the question of whether mesothelial disruption is associated with increased exposure of the peritoneum, animal investigations reported desquamation of mesothelial cells and exposure of the basement membrane increased with pneumoperitoneum duration (Suematsu, Hirabayashi et al. 2001; Peng, Zheng et al. 2009; Du, Yu et al. 2011), pressure (Suematsu, Hirabayashi et al. 2001; Matsuzaki, Jardon et al. 2010; Du, Yu et al. 2011), gas flow rate (Du, Yu et al. 2011) and desiccation (Erikoglu, Yol et al. 2005; Peng, Zheng et al. 2009). In addition, two human investigations collected peritoneal biopsies at various times during surgery and reported that desquamation of mesothelial cells increased with surgery duration (Schaeff, Paolucci et al. 1998; Liu and Hou 2006). There was poor reporting of the length of surgical incision, and therefore no

data was available to test whether the size of area of surgical access is related to mesothelial disruption.

**Table 3-3: Summary of Investigations of Morphological Changes of the Peritoneal Mesothelium following Laparoscopy**

#	1 <sup>st</sup> Author, Year	Species V(isceral)/ P(arietal)	Biopsy timing <sup>b</sup>	Intervention v control (number of subjects) <sup>c</sup>	Surgery duration (min)	pp Parameters			Imaging method	Mesothelium morphology result summary				
						Insufflation gas	Pressure (mmHg)	Gas flow rate (L/min)		Following laparoscopy			Summary (p values presented when reported)	
										Bulging	Retraction	Desquam- ation		
Human														
16.	Liu, 2006 <sup>d</sup> (Liu and Hou 2006)	Human P	0,30,90 & 120 min into LS	LS (20) v LT (20) Myomectomy or hysterectomy	NS	CO <sub>2</sub>	14	NS	TEM SEM	Y	Y	Y	Y	LS: MC desquamation worsened with duration. Bulging evident immediately after pp. LT: Intercellular clefts, no MC desquamation.
17.	Schaeff, 1998 <sup>d</sup> (Schaeff, Paolucci et al. 1998)	Human NS	0,1,2 & 3h into LS	LS (3) v gasless LS (3) v LT (3) Surgery purpose NS	NS	CO <sub>2</sub>	12	NS	EM TEM	Y	Y	Y	Y	LS: MC separation, rounding & loss of contact with BM, worsening with surgery duration. Gasless LS & LT: Confluent layer of flat MC, dense microvilli.
18.	Zhao, 2010 <sup>f</sup> (Zhao, Li et al. 2010)	Human V	During LS	LS (27) v LT (23) Resection of colorectal cancer	151	CO <sub>2</sub>	10 - 12	NS	SEM LM	Y	Y	Y	N	LS: MC swelling, widening of intercellular space, BM not visible. Less peritoneal surface injury compared to LT. Composite peritoneal injury score 13(LS) v 34(LT) (p<0.01)

Animal												
19.	Bloechle, 1999 <sup>a</sup> (Bloechle, Kluth et al. 1999)	Rat P	LT start, pp start, 2 & 12 h	LT 6-8 mm duration NS, followed after 12 h by pp with (12) or without (12) gastrotomy	60 (pp)	CO <sub>2</sub>	4	NS	SEM	N	Y	With gastrotomy: MC detachment, disintegration at 12 h.  Without gastrotomy: Intact mesothelium. MC stretched with flattening of microvilli & mesothelial folds. Recovered after 12 h.
20.	Davey, 2013 (Davey, Hayward et al. 2013)	Rat	12 h	Anaesthesia only (3) v cold-dry pp (5) v warm-dry pp (5) vs humidified-warm (5)	120	CO <sub>2</sub>	5	0.05	LM SEM	Y	Y	Humidified-warm pp protected the MC against damage observed following cold-dry and warm-dry pp.
21.	Du, 2011 (Du, Yu et al. 2011)	Rat P	12 h	Pp (45) v anaesthesia only (5)	1, 2 or 3 h	CO <sub>2</sub> or He	5 or 8	1, 2 or 3	SEM	Y	Y	Pp: Fraction of BM exposed increases with pp pressure, duration & gas flow (p<0.05). Diameter of MC cells decreases with increasing pressure, duration & gas flow (p<0.05). Changes not related to gas type (p>0.05). Control: Confluent layer of MC, dense microvilli.
22.	Erikoglu, 2005 (Erikoglu, Yol et al. 2005)	Rat NS	12 h	Humidified pp (10) v dry pp (10) v anaesthesia only (10)	120	CO <sub>2</sub>	10	0.003 #	SEM	Y	Y	Cold dry CO <sub>2</sub> pp: BM clearly seen due to massive desquamation of MC.  Warm, humidified CO <sub>2</sub> pp & control: Marked MC bulging, no detachment or BM exposure.
23.	Glew, 2004 (Glew, Campher et al. 2004)	Piglet V & P	5 h	Dry pp (5) v humidified pp (5)	50	CO <sub>2</sub>	8	1	LM	N S	N S	MC absence in both groups - authors noted this may be artefact.

24.	Hazebroek, 2002 (Hazebroek, Schreve et al. 2002)	Rat P	0,2&24 h	Humidified (warm 12; cold 12) v dry (warm 12; cold 12) pp v manual abdominal wall lifting (12) v “no anaesthesia” control (4)	120	CO <sub>2</sub>	6	0.3	SEM LM	Y	Y	N	Pp groups & abdominal wall lifting: Exposure of BM. Reduced microvilli. No inter-group difference in intercellular cleft size or distribution of microvilli Control: Confluent, flat MC layer with abundant microvilli. IP pentobarbital in all groups except control.
25.	Koster, 1999 (Koster, Spacek et al. 1999)	Mouse P	1,2,6,12, 24,48,72 & 96 h	Pp (32) v ‘control for artefact’ (4)	30	CO <sub>2</sub>	6	NS	SEM	Y	Y	Y	CO <sub>2</sub> pp: MC separation, clustering & cubic shape. Peaked at 12 h. Large areas of BM exposed. Microvilli increased 36-96 h. Control: Intact MC, thick microvilli.
26.	Matsuzaki, 2010 (Matsuzaki, Jardon et al. 2010)	Mouse P	End of pp/anaesthesia	Pp (15) v anaesthesia only (5)	60	CO <sub>2</sub>	2-15	2	LM	N S	N S	Y	Pp at 15 mmHg: Few MC detectable due to MC detachment. Pp < 15 mmHg: MC detachment not observed. Control: MC present, no further description.
27.	Papparella, 2007 (Papparella, Noviello et al. 2007)	Rat V & P	2 & 24 h	Pp (50) v anaesthesia only (10)	30	CO <sub>2</sub> or air	10 - 12	1	LM	Y	N S	Y	CO <sub>2</sub> pp: Cubic shaped MC. Air pp: Patchy loss of MC. More marked changes than CO <sub>2</sub> pp V&P results not reported separately. Control: Confluent layer of MC.
28.	(Papparella, Nino et al. 2014)	Rat V & P	24 h	CO <sub>2</sub> pp (High (16) v Low (16) pressure) v Air pp (High (8) v Low (8) pressure) v anaesthesia only control (8)	30	CO <sub>2</sub> or air	10 or 6	0.5	LM	N	N	Y	MC loss was seen following both CO <sub>2</sub> and air pp, but no anaesthesia only controls. Low pressure pp caused minor peritoneal injury compared to high pressure pp.

29.	Peng, 2009 (Peng, Zheng et al. 2009)	Rat P	6, 24, 48, & 96 h	Warm, humidified pp (60) v cold, dry pp (60) v anaesthesia only (5)	3, 4, or 5 h	CO <sub>2</sub>	9	0.3	SEM LM	Y	Y	Y	Both pp groups: MC Desquamation increased with pp duration. Dry pp: Massive MC desquamation. Humidified pp: Less changes & mesothelium recovered more quickly. Control: Confluent layer of MC.
30.	Rosario, 2006 <sup>d</sup> (Rosario, Ribeiro Jr et al. 2006)	Mouse P	2 & 24 h	Pp (20) v anaesthesia only (10)	30	CO <sub>2</sub> or air	8	NS	SEM	Y	Y	N S	CO <sub>2</sub> pp: MC bulging, retraction; intercellular clefts; exposure of large areas of BM; decreased microvilli (p<0.05 <sup>e</sup> ). Air pp: Not associated with intercellular clefts or exposure of BM. Decreased microvilli (p<0.05 <sup>e</sup> ) MC bulging less than CO <sub>2</sub> pp (p<0.05). Control: Confluent layer of MC, no intercellular clefts, 'unchanged' microvilli.
31.	Suematsu, 2001 <sup>d</sup> (Suematsu, Hirabayashi et al. 2001)	Mouse V	0, 24, 72 h <sup>a</sup>	Pp (33) v anaesthesia only (3)	30 or 60	CO <sub>2</sub> , air or He	5 or 10	NS	SEM	Y	Y	N	All pp groups: Larger intercellular clefts with high pp pressure. CO <sub>2</sub> & He pp: Bulging & retraction, MC recovered faster in CO <sub>2</sub> group than He. Air pp: Bulging only, no retraction. Control: No detachment or bulging of MC; no intercellular clefts.

32.	Volz, 1999 (Volz, Koster et al. 1999)	Mouse P	During, 1,2,6,12, 24,48,72 & 96 h Control: 1,2,3,4 d	CO <sub>2</sub> pp (36) v "short" anaesthesia (4)	30	CO <sub>2</sub>	6	NS	SEM	Y	Y	Y	Pp: MC bulging, retraction & desquamation, peaking at 12 h. Large portions of BM uncovered. Carpet of microvilli was nearly unchanged. Regeneration extensive after 4 d. Control & during pp: Confluent, flat layer of MC. No BM exposure or intercellular clefts.
Animal laparoscopy with intra-peritoneal cancer cell injection (IPC)													
33.	(Carpinteri, Sampurno et al. 2015)	Mouse P	2, 8, 24, 48 h & 7 d	All IPC: Anaesthesia only (15) v cold-dry pp (15) v humidified-warm pp (15)	60	CO <sub>2</sub>	2	0.014 - 0.052	SEM	Y	Y	N	IPC & pp: Less MC rounding & retracting, & shortening of microvilli following cold-dry pp compared to humidified-warm pp and anaesthesia only (p<0.01).
34.	Hirabayashi, 2002 (Hirabayashi, Yamaguchi et al. 2002)	Mouse P (port sites)	0 h, 3, 8 d	IPC & pp (15) v no control	20	CO <sub>2</sub>	4-6	NS	SEM LM	N S	N S	Y	IPC & pp: Peritoneum peeled away at port sites & port-site metastasis occurred. Abdominal peritoneum neighbouring the port sites intact in all examinations.
35.	Koster, 1998 <sup>d</sup> (Koster, Volz et al. 1998)	Mouse P	1,2,6,12, 24,48,72, 96 h	Pp & IPC (47) v IPC only (47)	60	CO <sub>2</sub>	8	NS	SEM	Y	Y	Y	Pp & IPC: MC retraction & cuboidal shape. BM exposed with large portions denuded. IPC only: None of the above changes observed.
36.	Ordemann, 2004 (Ordemann, Jakob et al. 2004)	Rat P	2,12,24, 48, & 96h Control: 2 h	Pp & IPC (50) v anaesthesia & IPC (5)	30	CO <sub>2</sub> of He	15	0.25	SEM	Y	Y	N	CO <sub>2</sub> & He pp: Peritoneum not disturbed in 84 % of animals in each group. 16 % of animals showed bulging or separation of MC (p<0.01 <sup>a</sup> ) Control: Confluent sheet of flat MC, microvilli.

37.	Volz, 1999 (Volz, Koster et al. 1999)	Mouse P	Before & 1,2,6,12, 24,48,72, & 96 h Control: 1,2,3,4 d	Pp & IPC (36) v IPC only (36) v artefact control (4)	30	CO <sub>2</sub>	6	NS	SEM	Y	Y	Y	CO <sub>2</sub> pp & IPC: Diffuse uncovering of BM. IPC only: Some MC desquamation observed, no bulging or retraction. No changes in microvilli in either group. Control: Confluent layer flat MC, dense microvilli.
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BM=basement membrane; EM=scanning electron microscopy; He=Helium; IP=intraperitoneal; IPC=intraperitoneal cancer cell injection; h=hour(s); LM=light microscopy; LS=laparoscopy; LT=laparotomy; MC=mesothelial cells; min=minute(s); NS=not stated; P=parietal peritoneum; pp=pneumoperitoneum;

TEM=transmission electron microscopy; v= versus; V=visceral peritoneum

# Abdomen re-insufflated every 15 minutes, flow rate calculated from assumed IP volume of 50ml.

a Samples only taken at t=0 for control, high pressure (10 mmHg) & extended duration (60min) groups; b All biopsy times are taken from the end of the surgery unless otherwise stated; c Number in parenthesis is number of subjects; d Assessed LT & LS; e Compared with control group

### 3.4 Discussion

This systematic review summarised what is currently known about the consequences of abdominal surgery on the morphological integrity of the peritoneal mesothelium. The morphological characteristics reviewed were mesothelial size, shape, & existence; and exposure of the basement membrane. Evidence was found to support the hypothesis that exposure of the peritoneum to a gaseous surgical environment during laparoscopic surgery can cause loss of continuity of the mesothelium, due to both retraction and loss of mesothelial cells. However, the data regarding exposure during laparotomy is less clear. Evidence was also found to support the second hypothesis, that the severity of disruption to the mesothelium is associated with extent of exposure (longer duration, increased pneumoperitoneum gas flow rate and pressure). However, no data was found to test the hypothesis that the severity of disruption is associated with the size of area of surgical access.

#### 3.4.1 Changes in mesothelial morphology following laparotomy

The results of laparotomy investigations are inconsistent, likely due to poor control over investigation conditions. Most notably, there was a lack of reporting of surgical exposure of the peritoneal tissue biopsied. Tissue exposure is important as mesothelial desquamation has been reported following exposure of the peritoneum to non-physiological fluids (Ryan, Grobety et al. 1973; Lopes, De Oliveira et al. 2007), drying (Ryan, Grobety et al. 1973) and delicate handling (Phillips and Dudley 1984) during laparotomy. Factors that influence tissue exposure were poorly reported, including length of incision; duration of surgery; handling of the peritoneum prior to biopsy; and

timing of tissue collection. None of the investigations reported the control of operating room environmental conditions surrounding the open abdominal cavity, including lack of control of air flow, humidity, temperature and overhead heating. Further investigation with tighter control over peritoneal exposure is warranted.

#### 3.4.2 Changes in mesothelial morphology following laparoscopy

Both animal and human investigations included in this review have shown that laparoscopic surgery causes morphologic changes and desquamation of the mesothelial layer of the peritoneum. The extent of mesothelial disruption in human biopsies may have been under-estimated by the collection of biopsies during surgery. Several time-course investigations in mice have shown that changes in peritoneal morphology peak about 12 hours after surgical intervention (Koster, Spacek et al. 1999; Volz, Koster et al. 1999; Volz, Koster et al. 1999). Conversely, the changes seen in animal biopsies may be influenced by the use of exaggerated intra-abdominal pressure and/or gas flow rate in all of the animal models of laparoscopy; recommended to be 5 mmHg (Bloechle, Emmermann et al. 1995; Avital, Itah et al. 2009) and 40 mL/min (Sammour, Mittal et al. 2011), respectively, for a rat model. Furthermore, there was a lack of mechanical ventilation in all but three investigations (Glew, Campher et al. 2004; Matsuzaki, Jardon et al. 2010; Carpinteri, Sampurno et al. 2015). Mechanical ventilation has been shown to reverse peritoneal hypoxia and subsequently reduce adhesion formation in both rabbit (Mynbaev, Adamyan et al. 2009) and mouse (Bourdel, Matsuzaki et al. 2007; Matsuzaki, Jardon et al. 2010) models. Any further

investigation should utilise more appropriate pressure, gas flow and mechanical ventilation.

Manipulation of pneumoperitoneum gas flow, pressure and duration in animal investigations consistently showed that desquamation of mesothelial cells and exposure of the basement membrane worsened with increasing surgical exposure of the peritoneum. These results were supported by human investigation showing that desquamation of mesothelial cells increased with duration of surgery (Schaeff, Paolucci et al. 1998; Liu and Hou 2006). However, as all human biopsies were taken during surgery in that investigation, the increased desquamation seen at the later time points may be confounded by the delay in visibility of morphological changes (Liu and Hou 2006). The importance of high intra-abdominal pressure as a factor in disruption of the peritoneum during laparoscopy is supported by two other investigations that found high pressure pneumoperitoneum increases mesothelial cell apoptosis (Tosun, Samli et al. 2007) and reduces immunological function (Volz, Koster et al. 1996). Furthermore, one of the animal investigations included in the current review reported an increase in inflammatory infiltrate at higher pneumoperitoneum pressure, although they did not report whether higher pressure also increased the observed loss of mesothelium (Papparella, Nino et al. 2014). The specific use of CO<sub>2</sub> gas may not be responsible for the observed mesothelial damage as damage was also seen when pneumoperitoneum is created with by He or air (Suematsu, Hirabayashi et al. 2001; Papparella, Noviello et al. 2007; Du, Yu et al. 2011; Papparella, Nino et al. 2014). This review found evidence

exists to support recommendations for the use of low intra-abdominal pressure and gas flow during laparoscopy, and for limiting the duration of surgery when possible.

Prevention of peritoneal desiccation is a common recommendation to minimise post-operative adhesion formation (DeWilde, Trew et al. 2007; Schnuriger, Barmparas et al. 2010; Koninckx, Ussia et al. 2012; The Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society of Reproductive Surgeons 2013). Some findings from this review suggest that warming and humidification of insufflation gas during laparoscopy may prevent peritoneal disruption due to desiccation. Three animal investigations found that warming and humidifying the insufflation gas reduces both desquamation of the mesothelium and changes in morphology (Erikoglu, Yol et al. 2005; Peng, Zheng et al. 2009; Davey, Hayward et al. 2013). Two further investigations found the basement membrane became exposed regardless of humidification of the insufflation gas (Hazebroek, Schreve et al. 2002; Glew, Campher et al. 2004). Exposure of the basement membrane in one of these investigations was confounded by the use of intra-peritoneal anaesthetic (pentobarbital) that was not administered to the control group (Hazebroek, Schreve et al. 2002). Pentobarbital is a known histopathologic tissue changes including peritoneal hyperaemia and accumulation of blood in the peritoneal cavity (Iwarsson and Reh binder 1993; Artwohl, Brown et al. 2006; Grieves, Dick Jr et al. 2008). The second investigation did not have an anaesthesia only control group and so could not rule out that their results were due to artefact (Glew, Campher et al. 2004).

### 3.4.3 Areas of future research

The findings of this systematic literature review suggest that the degree of peritoneal damage following laparotomy is not yet fully understood. This is important as specific damage to the peritoneum is the necessary first step in adhesion formation (Gillett, James et al. 1994; Haney and Doty 1994; Holmdahl 1999), and the risk of adhesion formation following laparotomy remains high (Schnuriger, Barmparas et al. 2010). Disruption to the peritoneum has also been implicated in intra-abdominal infections (Yao, Platell et al. 2003), tumour dissemination (Jayne 2007; Binda, Corona et al. 2014) and post-operative abdominal pain (Eckmann, Holstein et al. 1985). Furthermore, Kahokehr and colleagues (Kahokehr, Sammour et al. 2011) suggest that decreased peritoneal damage may reduce postoperative fatigue that follows abdominal surgery.

Further research is required to clarify the effect of laparotomy on disruption to the morphological integrity of the mesothelium, ensuring control over surgical exposure of the area of the peritoneum to be biopsied. The identification of factors that cause disruption to the mesothelium may allow tailored interventions to be designed and tested, to reduce mesothelial disruption and associated post-operative complications.

It is recommended that such research should attempt to address the sources of bias identified in the current review. Further research should ensure that peritoneal biopsies are collected at the time of peak inflammation (reported in mice as 12 hours following the surgical intervention (Koster, Spacek et al. 1999; Volz, Koster et al. 1999; Volz, Koster et al. 1999)), or take advantage of early markers of peritoneal damage such as the appearance of apoptotic bodies and necrotic nuclei (Tarhan, Barut et al.

2013). Subjects should be ventilated in order to avoid peritoneal hypoxia (Bourdel, Matsuzaki et al. 2007; Mynbaev, Adamyan et al. 2009; Matsuzaki, Jardon et al. 2010). The addition of mesothelium specific staining, such as cytokeratin 18 staining (Marchi, Liu et al. 2000), would also improve the objectivity of image analysis. Inflammatory status of subjects prior to surgery should also be considered (Hellebrekers and Kooistra 2011).

### 3.5 Conclusions

1. A significant gap in current knowledge exists in the understanding of the effect of gaseous exposure of the peritoneum during laparotomy on morphology of the peritoneal mesothelium.
2. Gaseous exposure during laparoscopic surgery can cause desquamation of the mesothelial layer of the peritoneum. The degree of disruption appears to be reduced at lower pneumoperitoneum pressures and by using humidified-warm insufflation gas.

### 3.6 Acknowledgements

Thank you to Martin Engel and Meng Mao for translation of German and Chinese articles, respectively. Also, thank-you to Antonio Lopez Lorca for translation of a Portuguese article that did not ultimately meet the inclusion criteria.

## **4 Development of a rat model to study the effect of gaseous exposure of the peritoneum during open abdominal surgery**

### **4.1 Justification for the need for an animal model**

Investigation of morphological changes to the peritoneum following abdominal surgery has been attempted in humans (Eckmann, Holstein et al. 1985; Schaeff, Paolucci et al. 1998; Liu and Hou 2006; Zhao, Li et al. 2010), however, the results are inconsistent. One of the key criticism of these investigations is that the peritoneal tissue samples are necessarily collected during surgery, despite animal research showing that the peritoneal mesothelium undergoes characteristic morphological changes following surgery and that the loss of mesothelium appears to peak 12 hours after surgery (Koster, Spacek et al. 1999; Volz, Koster et al. 1999). Collection of peritoneal tissue 12 hours after surgery requires the abdominal cavity to be opened for a second time. This second surgery cannot be justified in humans. The relevance of results from a carefully designed animal model is supported by a growing body of evidence successfully translating the results of rodent models of the effect of surgery on the peritoneal mesothelium to humans (Matsuzaki, Botchorishvili et al. 2011; Matsuzaki, Jardon et al. 2012; Koninckx, Corona et al. 2013).

In addition to animal models, tissue culture has also been used to study morphological changes to the peritoneum (Demir Weusten, Groothuis et al. 2000). However, a tissue culture model is not appropriate for the current investigation of the effect of desiccation during open abdominal surgery on mesothelial morphological. It is possible

that the mesothelium may be able to compensate for the degree of desiccation caused during surgery. The mesothelium needs to be associated with its vascular system to allow any such compensation to occur. Furthermore, during insufflation of CO<sub>2</sub>, tissue oxygen partial pressure is hypothesised to increase by a combination of increased perfusion and increased release of oxygen from haemoglobin. Both of these mechanisms of action occur in the tissue vasculature and therefore could not be measured in a tissue culture model.

## 4.2 Key requirements of an in-vivo model to study the effect of open abdominal surgery on the peritoneum

### 4.2.1 Area of peritoneum exposed

Sufficient peritoneal tissue needed to be exposed to the ambient gaseous environment of the peritoneal cavity to ensure that enough tissue could be collected for microscopic analysis. In addition, mesothelial cells of the delicate mesothelium are easily brushed off following even gentle touching of the membrane (von Ruhland, Newman et al. 2003). Tension also damages the mesothelium and lowers sub-peritoneal PtO<sub>2</sub> (Bourdel, Matsuzaki et al. 2007; Matsuzaki, Canis et al. 2007). Therefore, the model developed a surgical technique that ensured the peritoneum to be collected had not been touched and tension was minimised. Allowance was made to exclude mesothelium damaged during the incision by leaving an exclusion zone of 5 mm from the laparotomy incision. Three 5 mm<sup>2</sup> tissue samples were required for each of three different microscopic analyses and duplicates as backups, a total of 18 x 5 mm<sup>2</sup> samples, totally 90 mm<sup>2</sup>.

Furthermore, the model needed to permit exposure of a 30 mm length of peritoneum in order to insert the tissue oxygen partial pressure (PtO<sub>2</sub>) probe. Licox™ probes are regarded as the gold standard for tissue oxygen monitoring (Govinda, Kasuya et al. 2010). The Licox™ CC1P1 oxygen probe used in the current model (discussed further in section 4.10) requires the distal 30 mm within of the probe to be within the tissue to ensure that both the oxygen sensing and temperature sensing portions of the probe are embedded during measurement. This portion of the peritoneum must remain exposed and undisturbed for the duration of the surgery. Previous research investigating sub-peritoneal tissue oxygenation during laparoscopy found sufficient tissue to embed the probe in the retro-peritoneum of mice (Bourdel, Matsuzaki et al. 2007). However, the retroperitoneal space is inappropriate for the current model as the probe would not be directly underlying peritoneum that was exposed to the ambient gaseous environment of the peritoneal cavity.

#### 4.2.2 Recovery following surgery

Previous research has shown that the peritoneal mesothelium undergoes characteristic morphological changes following surgery and that the loss of mesothelium appears to peak 12 hours after surgery (Koster, Spacek et al. 1999; Volz, Koster et al. 1999). Therefore, in order to study the extent of mesothelial cell loss, the peritoneal tissue samples collected from the current model will be collected following euthanasia 12 hours after surgery. Careful consideration of animal welfare is required to ensure that the rats recovery comfortably during the time from the end of surgery to euthanasia. This requires consideration of adequate analgesia; recovery from

general anaesthesia including spontaneous breathing following mechanical ventilation and; avoidance of wound rupture.

#### 4.2.3 Mechanical ventilation

Previous research has shown that insufflation of CO<sub>2</sub> into the abdominal cavity for laparoscopy in mice that are not mechanically ventilated leads to hypercapnia and relatively low sub-peritoneal tissue oxygen partial pressure, which was reversed when the mice were mechanically ventilated (Bourdel, Matsuzaki et al. 2007; Du, Yu et al. 2011). Therefore, mechanical ventilation aids the animal to expire any absorbed CO<sub>2</sub>, and maintain normal P<sub>a</sub>CO<sub>2</sub> during CO<sub>2</sub> insufflation. Furthermore, in the intervention group the inspired gases must be isolated from surgical environment as the rat placed in a container of CO<sub>2</sub> gas would otherwise asphyxiate.

#### 4.2.4 Air flow rate

Previous research has shown that exposing the peritoneal to a flow of dry air from 'gentle' to 30 L/min can result in loss of peritoneal mesothelium (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973; Burns, Skinner et al. 1995). However, what is not clear is whether the flow rate of air in a normal air conditioned operating room is high enough to damage the peritoneal mesothelium. Therefore, the model included exposure of the peritoneal mesothelium to a flow rate of air that mimics that within an air-conditioned operating room.

#### 4.2.5 Intra-abdominal CO<sub>2</sub> environment

##### 4.2.5.1 CO<sub>2</sub> concentration

The purpose of this model was in part to investigate the effect of insufflation of CO<sub>2</sub> into the abdominal cavity. Therefore it was essential that the model enabled the creation of an intra-abdominal environment containing a >90 % concentration of CO<sub>2</sub>.

##### 4.2.5.2 Temperature

The intra-abdominal environment was maintained as close to body temperature as possible to minimise radiative heat loss.

##### 4.2.5.3 Humidity

The intra-abdominal environment was maintained close to 100 % relative humidity to minimise evaporative heat loss.

##### 4.2.5.4 Tissue oxygen partial pressure

Tissue oxygen partial pressure (PtO<sub>2</sub>) is one of the primary outcomes that this model was used to measure.

##### 4.2.5.5 Protection of the mesothelium during tissue fixation and processing

Because the peritoneal mesothelium is the thickness of a single layer of cells and very delicate, there is a high risk of experimental artefact caused by damage to the peritoneum inflicted during tissue collection or processing (von Ruhland, Newman et al. 2003). Therefore, the model developed optimised protocols for the collection,

fixation and processing of the peritoneal tissue to minimise the chance of collateral mesothelial damage, un-related to surgical exposure.

#### 4.3 Choice of animal species, strain and sex

Mammalian mesothelium is essentially similar, regardless of species (Michailova and Usunoff 2006). Several different species have been used to investigate peritoneal mesothelium; however, most research has been conducted in rats and mice (Michailova and Usunoff 2006). With respect to the current project, the University of Wollongong has a rodent animal facility housing both rats and mice, with little capacity for larger animals. Furthermore, a larger animal model was not financially viable within the project budget. Comparing mice and rats, the physical size of a rat allowed for a large enough incision to expose sufficient peritoneum for both collection for microscopic analysis, and to measure  $PtO_2$  during surgery. Both sexes and several different breeds of rat have been used in previous investigations of the peritoneal morphology with success and no notable differences; Female (Bloechle, Kluth et al. 1999) and male Wistar rats 275 g (Peng, Zheng et al. 2009) and 100-200 g (Ryan, Grobety et al. 1973); male Sprague-Dawley rats 250 – 290g (Erikoglu, Yol et al. 2005); and male BD-IX rats, 2 months old, 250 g (Ordemann, Jakob et al. 2004). Female Wistar rats were chosen for this project as it was identified that there was an opportunity for the sharing of ovarian tissue. Therefore the choice of female animals meant the animal tissue, following euthanasia, was more likely to be used in another scientific study.

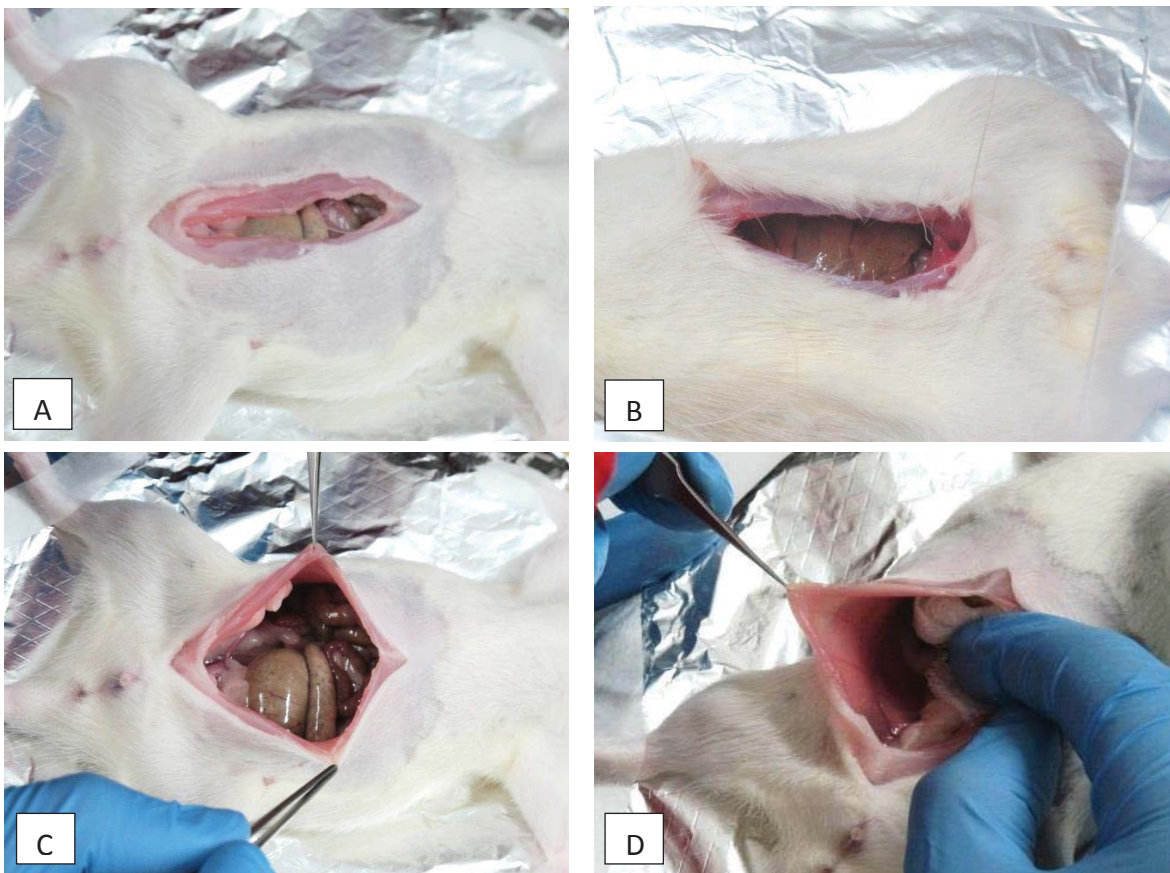
#### 4.4 Development of laparotomy technique

According to the requirements outlined in section 4.1, a laparotomy technique was required to be developed that achieved intra-operative exposure of at least 90 mm<sup>2</sup> area of un-handled peritoneum, including a length of 30 mm; minimised trauma to the peritoneum; and closed the abdomen following surgery in a manner that minimised pain from wound tension or bowel obstruction, and avoided wound rupture prior to euthanasia 12 hours after surgery. In addition, the wound needed to be closed quickly, so as to minimise the total surgery time and the risk that the rat would not recover from general anaesthesia. The advantages and disadvantages of different incision and retraction designs, and closure methods are described below.

##### 4.4.1 Midline incision only – inadequate exposure

A basic mid-line incision along the linea alba with no other incision was trialled. The abdominal wall tended to recede slightly laterally along the line of the incision, see Figure 4-1, resulting in exposure of only a small area of visceral peritoneum of the loops of the bowel. It would be difficult to collect the visceral peritoneum of the bowel for analysis as movement of the bowel following surgery would likely make it impossible to identify the portions of the bowel that had been exposed. No parietal peritoneum was exposed. To increase exposure, the incision was retracted by placing sutures through the skin, see Figure 4-1. The sutures were clamped and draped across an overhanging box. Exposure was still minimal and deemed insufficient. Exposure of the parietal peritoneum of the anterior abdominal wall could only be created by stretching the peritoneum both anteriorly and laterally. However, the peritoneum

remains at angle to the wound opening and so will be protected from air flow within the operating room. Although a midline incision alone results in an incision of minimal length and therefore simple closure, this method provided in-adequate exposure of the parietal peritoneum and significant stretch had to be applied that will likely damage the mesothelium (Du, Yu et al. 2011).



**Figure 4-1: Midline incisions with different types of retraction in rats previously euthanized.**

**Image A:** There was slight lateral recession of the abdominal wall exposing only a small area of visceral peritoneum and no parietal peritoneum. **Image B:** Suture material was placed through the skin; the suture was clamped and draped across an overhanging box. **Image C:** Bilateral retraction of the peritoneum both laterally and anteriorly. **Image D:** Increased exposure of the peritoneum by retracting intra-abdominal organs.

#### 4.4.2 135° Flap incision

The 135 ° flap incision shown in Figure 4-2 also provided inadequate exposure of the peritoneum. The large triangle in Figure 4-2 illustrates the portion of parietal peritoneum that was available to fold back to be exposed to the operating environment. The smaller, white triangle shows the very small area of tissue that remained free of manipulation and therefore available for analysis.



Figure 4-2: 135 ° flap incision in a euthanized rat.

(Note the extra incision in this image picture was made before the midline incision in order to gain un-exposed peritoneum for microscopy protocol development) The triangle in the right image shows the area of exposed parietal peritoneum that was available for collection. The smaller, filled triangle illustrates the portion of the tissue sample that was a minimum distance from the incision that it could be used for analysis.

#### 4.4.3 90 ° Flap – Adopted in the current model

After trialling several different shaped flaps, a 90 ° flap incision, see Figure 4-3, gave the best exposure of the parietal peritoneum on the anterior abdominal wall and also covering the psoas muscle on the posterior wall. This incision design exposed a triangular area of un-handled parietal peritoneum, with a base approximately 40 mm long, allowing a 5 mm margin along the incision line. The area was greater than the 90 mm<sup>2</sup> requirement and included a length longer than 30 mm. Adherence of the skin to

the underlying tissue was sufficient to allow the peritoneum to be held open by the weight of an arterial clamp clamped to the skin, Figure 4-3. This minimised trauma to the peritoneum due to both stretching and handling. It was possible to protect the bowel from desiccation by gently moving it under the right abdominal wall using a cotton bud. Care was taken to avoid twisting the bowel, which could result in bowel obstruction following surgery.

To maximise exposure of the peritoneum, the incision needed to come as low as possible on the pelvic wall. However, care was be taken to halt the skin incision approximately 10 mm below the level of the xiphoid process. If the skin incision was too close to the ribcage the skin tended to recede over the rib cage and became difficult to suture closed, Figure 4-3. For ease of suturing, peritoneal incisions were made shorter than the skin incisions; experiments showed that approximately 2-3 mm was ideal. Exposure of the peritoneum on the posterior abdominal wall could be improved by moving the left hind leg cranially and holding it in place with tape across the paw, see Figure 4-3.

#### 4.4.4 Exposure of the spleen

The spleen provides visceral peritoneum that could be easily exposed and resected, see Figure 4-3. The asymmetrical cross-section of the spleen ensured that the surface that was exposed during the operation could easily be identified and correctly analysed. Identification of the exposed surface was therefore much easier than the bowel. In addition collection of the spleen was simple as the entire organ can be removed, compared with the bowel where faecal contamination would be

unavoidable. The spleen was exposed by manipulating the mesentery, and therefore the entire exposed peritoneal surface of the spleen was free of trauma and available for analysis.



Figure 4-3: 90 ° midline incision.

**Left: Sutured 90 ° midline incision in a euthanized rat. Right: Example of a 90 ° incision in a euthanized rat with exposure of parietal peritoneum and visceral peritoneum of the spleen. The left hind leg was moved cranially and held in place with tape across the paw (dashed hollow black arrow) to improve exposure of the peritoneum on the posterior abdominal wall. The dashed line outlines the exposed and un-handled area of the abdominal wall used for analysis. Note that the abdominal wall has receded over the ribcage (solid black arrow), which would have made wound closure difficult. Care must be taken to halt the skin incision approximately 10 mm below the level of the xiphoid process.**

The laparotomy incision consists of both a deep layer (muscle/peritoneum) and a superficial layer (skin). The two layers must be closed separately. With regard to the muscle/peritoneal layer, both running and individual stitches were trialled. Running sutures provided a faster closure time compared with individual stitches and satisfactorily closed the muscle/peritoneal layer of the wound as long as good tension was maintained on the stitches, see Figure 4-4.

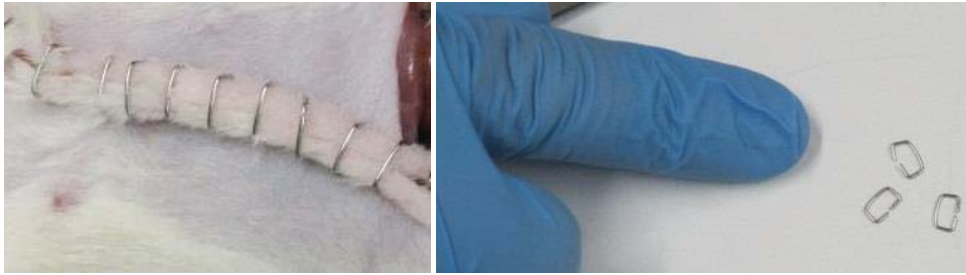
With regard to the superficial skin layer, both suturing and stapling of the skin wound were considered. Suturing the wound achieved adequate closure but was much more time consuming than stapling. However, while the first staple gun trialled (Appose ULC

35 S, Tyco Healthcare) achieved a neat and tight staple line, see Figure 4-5, there was approximately a 3 mm gap between the staple and the skin. This is a design feature of the staples, which are designed for easy removal following human surgery. In a rat trial this gap posed an easy way for the rat to pull at and potentially remove the staples, resulting in wound rupture. A staple gun custom designed for use with rats was later sourced, and this achieved a staple line that sat very close to the surface of the skin. A bandage was then wrapped around the staple line to reduce the likelihood of the rat opening the staples. The placement of the bandage was adjusted caudally after the bandage on the first recovered rat was placed too cranial, hindering movement of the rat's front legs.

In summary, in the current model comfortable recovery of the rat between surgery and euthanasia was achieved by closing the peritoneal/muscle incision with running sutures, using a rat-specific staple gun to close the skin and placing a bandage over the wound being careful not to interfere with the front legs.



**Figure 4-4: Running sutures successfully closed the peritoneal/muscle layer on a euthanized rat.**



**Figure 4-5: Stapling of the skin wound on a euthanized rat**

**The staples used in this image are too large, resulting in a gap between the staple and the skin that the rat could use to pull at the staple.**

## **4.5 Anaesthesia, analgesia and maintenance of core body temperature**

### **4.5.1 Choice of anaesthesia**

Isoflurane was chosen as the anaesthetic agent for this rat model for several reasons. An intra-peritoneal anaesthetic agent would not be appropriate for this investigation due to the chance of an effect on the peritoneum that may have confounded results. An inhaled anaesthetic agent was convenient for the current model as the rat was mechanically ventilated and therefore the anaesthesia can easily be administered with the ventilation gases. Chronic exposure to waste isoflurane has been shown to have several health concerns (Smith and Bolon 2003). Therefore elimination of isoflurane from the operative environment is warranted. In absence of an active exhaust system, the waste gas can be passed through a charcoal or soda lime canister. Research has shown that the EnviroPure™ brand of canister is an effective scavenger of isoflurane (Smith and Bolon 2003).

### **4.5.2 Analgesia**

The laparotomy incision in the current model was large and had the potential to cause significant pain in experiments in which the rat recovered from general anaesthesia.

This pain is best minimised by the appropriate use of pre-emptive analgesic agent(s) (Davis 2008; National Health and Medical Research Council 2008). Published research has shown that laparotomy induced pain in rats is significantly, and comparatively, alleviated by either opioids (0.5 mg/kg buprenorphine) or non-steroidal anti-inflammatory drugs (2.5-10 mg/kg Carprofen or Ketoprofen or 1-2 mg/kg Meloxicam) (Roughan and Flecknell 2001; Roughan and Flecknell 2003; Roughan and Flecknell 2004). However, opioids are Schedule 8 controlled drugs that are difficult to purchase and require tight controls due to its potential for abuse and addiction. Non-steroidal anti-inflammatory drugs are not Schedule 8 drugs and therefore are simpler to purchase, store and administer. Opioids also have higher risk of CNS depression resulting in hypothermia, bradycardia and respiratory depression, than non-steroidal anti-inflammatory drugs (Davis 2008). Meloxicam is the most easily sourced non-steroidal anti-inflammatory drug for animal use. Meloxicam also has the advantage of a 24 hour dosing interval, compared with 2-4 hourly dosage required with many opioids (National Health and Medical Research Council 2008), suggesting that one dose will provide sufficient analgesia until the time of euthanasia 12 hours after surgery. In conclusion, the current model administered 1 mg/kg subcutaneous meloxicam immediately after the induction of general anaesthesia. A further dose of meloxicam was administered if there were any signs of pain during the post-operative monitoring period, including back arching, fall/stagger, writhe and poor gait (Roughan and Flecknell 2003).

#### 4.5.3 Maintenance of core body temperature

Hypothermia greatly increases the risk of inadvertent death during general anaesthesia (Hanusch, Hoeger et al. 2007; Davis 2008). Maintenance of core body temperature during surgery can be very difficult. In the current model, the most effective way to maintain core body temperature was to use several different methods. To reduce heat loss from peripheral vasodilation during induction of general anaesthesia, rats were held in a warm blanket under a heating lamp for 10 minutes prior to anaesthesia. During anaesthesia the rats were warmed with a Small Animal Far Infra-Red Warming Pad (Kent Scientific Corporation, Connecticut, USA). This heating pad is specifically designed for warming rodents during surgery, without the need for any other external heating. It has several heating settings, allowing optimization of the rat's temperature. Core body temperature was monitored with a rectal thermometer (Surgipak Flexible Digital Thermometer, Vega Technologies Inc., Taipei, Taiwan). Fluids were warmed under a heat lamp prior to administration. Furthermore, a custom made sock was wrapped around the rat's tail to reduce convective heat loss from the tail, an area of high blood flow. An overhead heat lamp was available for use if the rat's temperature was falling to below 36 °C, but was rarely required when all the above measures were employed.

#### 4.6 Endotracheal intubation for mechanical ventilation

##### 4.6.1 Visualisation of the entrance to the trachea

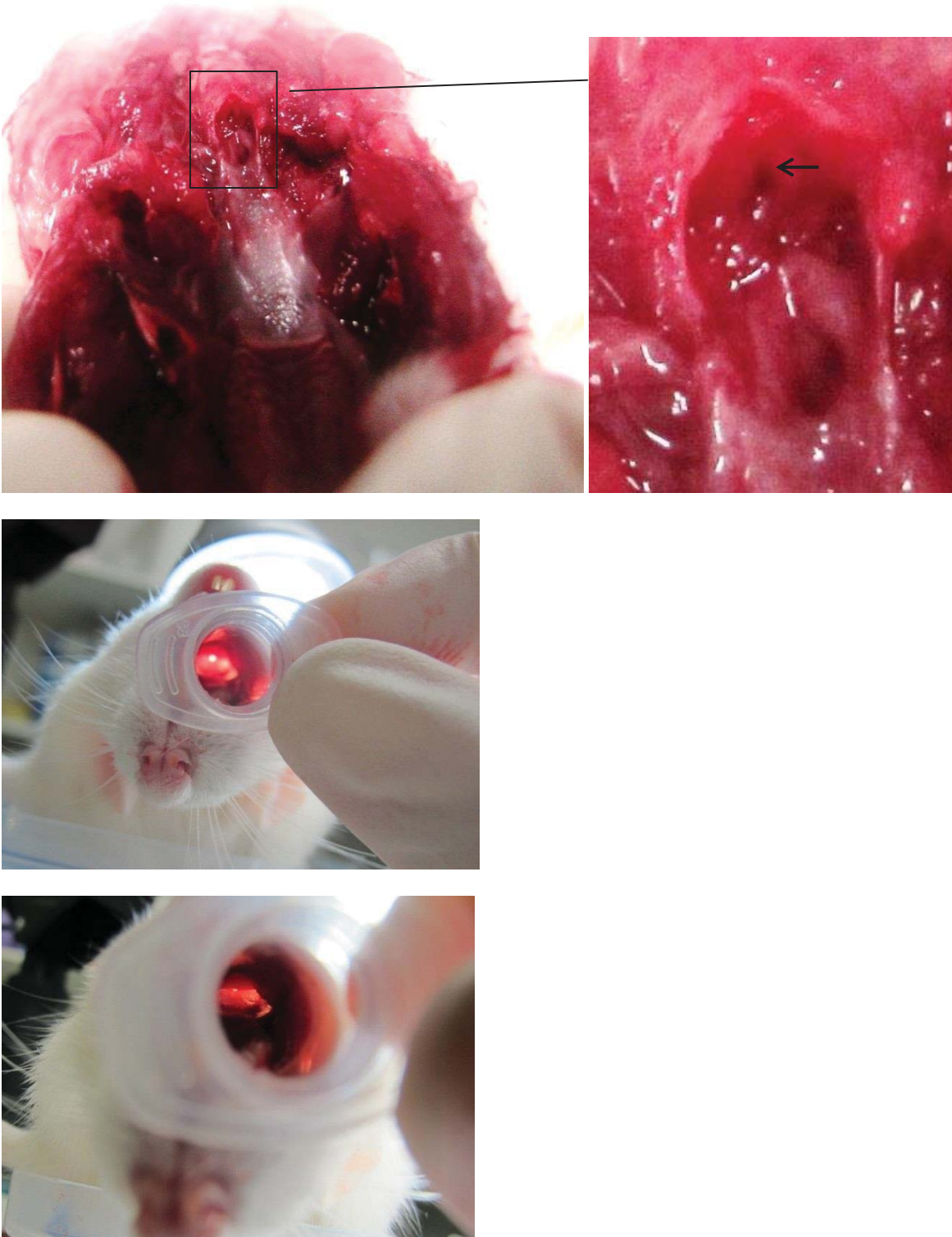
The most critical step in endotracheal intubation is visualisation of the entrance to the trachea. Blind intubation can result in iatrogenic injury (Cheong, Lee et al. 2010). A

‘facilitated’ blind method using a bent stylet and ventilator has been described, but still resulted in an 18 % rate of oesophageal intubation (Cheong, Lee et al. 2010). To visualise the entrance to the trachea many published intubation techniques require expensive optical equipment, most commonly an endoscope (Fuentes, Hanly et al. 2004; Gulati, Verma et al. 2011) or a stereo microscope (Hamacher, Arras et al. 2008). Use of video-endoscopy requires not only an endoscope, but also a camera and monitor (Fuentes, Hanly et al. 2004). This equipment is often described as readily available (Van Dongen, Remie et al. 1990), but our experience is that video-endoscopic equipment is often not available in research laboratories and is expensive to purchase. This equipment was not available to the current model.

#### 4.6.2 Transcutaneous Illumination of the trachea

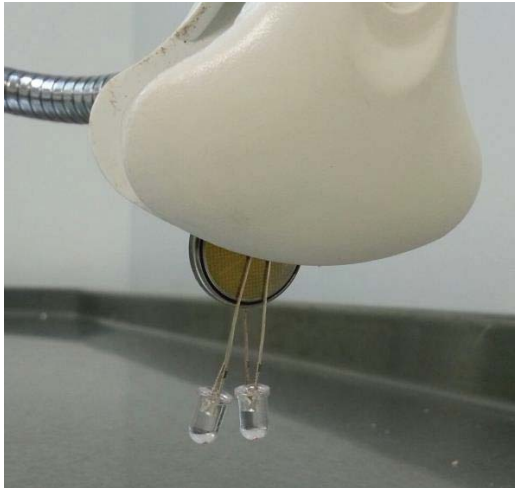
An alternative to video-endoscopic visualisation of the trachea is trans-illumination of the trachea through the skin, allowing the trachea to be visualised as a bright hole between the vocal cords (Jou, Tsai et al. 2000). This is a cheaper and simpler method (Jou, Tsai et al. 2000), although difficult in mice (Hamacher, Arras et al. 2008), likely due to the smaller relative size of the trachea compared with rats. Trans-illumination techniques require the researcher to look through the mouth and manipulate the airway to achieve a direct line of sight to the trachea. The light source required for sufficient transcutaneous illumination of the trachea has previously been described as “powerful” (Van Dongen, Remie et al. 1990) and as “a simple table lamp” (Jou, Tsai et al. 2000). In the current model a white light emitting diode table lamp only provided enough light if the skin was resected, which would not be feasible in a live rat. An

improved light source was built from two red light emitting diodes and worked very well, see Figure 4-6. The red colour penetrated more successfully through the skin layer. The stylet within the intubation tube can be used to gently move the epiglottis and confirm vocal cord visualisation. Inexpensive LEDs manufactured with resistors inbuilt can readily be purchased from electronic stores, so that soldering of resistors is not required. Trans-illumination of the trachea has been recently criticised, due to the improvement in certainty of tube placement when a stereomicroscope was used to intubate mice (Hamacher, Arras et al. 2008). This is likely due to the relatively small size of the trachea in mice compared with rats. In addition, the authors believed that the light was difficult to position (Hamacher, Arras et al. 2008). In the current model the LED light source was attached to a double ended flexible clamp that was attached to the edge of the surgical table. With this setup positioning of the narrow band of light over the trachea was very simple, see Figure 4-7. In addition visualisation of the trachea during the local anaesthetic step, described in more detail in section 4.6.5, was used to ensure that the light has been positioned correctly prior to intubation. The same authors also recommended the use of a guide wire to improve visualisation during intubation of mice (Hamacher, Arras et al. 2008). The guide wire was reported used to overcome the 'final blindness' prior to insertion of the tube into the trachea of mice. In the current model the anatomy of rats was large enough that a 16 G catheter could be placed under direct vision, without the need for a guide wire.



**Figure 4-6: Anatomical visualisation for oesophageal intubation.**

**Upper images: visualisation of the trachea, vocal cords and oesophagus by dissection. The picture on the right shows a close up view of the image within the square on the picture on the left. The arrow indicates the dark entrance to the trachea. Lower images: Visualisation of the bright entrance to the trachea with a simple oro-pharyngeal wedge and a red LED light directed transcutaneously.**



**Figure 4-7: Simple red LED light made with a double ended clamp, battery and LED with in built resistors**

#### 4.6.3 Upper airway manipulation with an oropharyngeal wedge

Further aiding in visualisation of the entrance to the trachea, another critical step in endotracheal intubation is manipulation of the upper airway to allow direct line of sight through the mouth to the trachea. The upper airway straightens easily when the rat is placed in a supine position with its head tilted backwards. The use of an oropharyngeal wedge is a simple, published method using a trimmed syringe plunger as an intubation wedge to visualise the vocal cords (Jou, Tsai et al. 2000), see Figure 4-6. In the current model, the technique was used to successfully intubate several euthanized rats. To allow easy orientation, the oro-pharyngeal wedge should be cut so

that the tip of the wedge is centred. The wedge used in Figure 4-6 was off centre and therefore the handles of the wedge are not parallel with the work bench. The authors of the oropharyngeal wedge intubation technique state that they place the bottom of the wedge on top of the rat's tongue (Jou, Tsai et al. 2000). Experiments with the current model found that the tongue tended to move with the wedge, folding into the back of the mouth and obscuring view of the vocal cords and trachea. Previous publications have suggested the need to retract the tongue with a penetrating towel clamp (Fuentes, Hanly et al. 2004) or a mosquito clamp (Cheong, Lee et al. 2010). However the current model agreed with a subsequent publication that showed the tongue can be simply and quickly retracting using a cotton bud and the thumb with very low risk of injury to the rat (Rivard, Simura et al. 2006).

Although the oro-pharyngeal wedge intubation technique was perfected using euthanized rats, further intubation attempts with a live, anaesthetised rat confirmed that insertion of the oro-pharyngeal wedge took too much time with the rat anaesthetised with isoflurane. The authors of the intubation method state that intubation will take approximately 30 seconds (Jou, Tsai et al. 2000). However in their experiments, the rats were anaesthetised with 50 mg/kg intra-peritoneal pentothal (sodium thiopentone). Unfortunately, intra-peritoneal anaesthesia is in-appropriate for this protocol as it may induce changes in the mesothelium. In the current model, the rat was anaesthetised with isoflurane via a nose cone that needed to be removed to allow intubation and had to be replaced after approximately 15 seconds to maintain adequate anaesthesia. This meant that further anaesthesia was required after

inserting the wedge, before intubation. However, delivery of isoflurane via a nose cone over the wedge is technically difficult. Several different nose cones were designed and trialled and none was able to deliver adequate anaesthesia while still keeping the oro-pharyngeal wedge in place. Therefore either a different anaesthesia or a different technique was required.

#### 4.6.4 Upper airway manipulation with head stabilisation and a cotton bud

Due to the incompatibility of the oro-pharyngeal wedge technique and isoflurane anaesthesia, further published techniques to manipulate the upper airway for direct line of sight to the trachea were sought. An earlier publication reported an intubation technique in which the maxilla was secured, prior to use of a cotton swab to roll the tongue out of the way (Rivard, Simura et al. 2006). The reverse, flat end of the cotton bud is then used to compress the base of the tongue. With a light positioned over the neck, the authors reported unobstructed visualisation of the entrance to the trachea (Rivard, Simura et al. 2006). This technique was successfully trialled and adopted in the current model. The technique has several advantages over the oro-pharyngeal wedge method. The rat is swiftly secured with a rubber band attached to the operating table. Securing the rat has led to a more consistent intubation technique than could be achieved with oro-pharyngeal wedge. Visualisation of the entrance to the trachea was much quicker with the cotton bud method and therefore visualisation, intubation and delivery of anaesthesia could be achieved quickly while anaesthesia was successfully maintained. A rubber band was found to be a simpler method to stabilise the head

than the 'two loop' technique previously described that requires oppositional retraction of the upper and lower incisions with silk loops (Fuentes, Hanly et al. 2004).

#### 4.6.5 Overcoming the glottic reflex

Two animals displayed strong glottic reflexes, causing the rats to move suddenly as the intubation stylet approached the trachea. This made intubation under visual guidance very difficult. After three attempts and one attempt, respectively in the two animals, intubation was aborted in both these animals to seek a solution to the reflex.

Several intubation techniques have not mentioned the need to overcome the glottic reflex and there are mixed reports of whether a muscle relaxant prior to intubation of rats is necessary (Vaghadia, Jenkins et al. 1989; Jou, Tsai et al. 2000; Frank, Gutierrez et al. 2002; Fuentes, Hanly et al. 2004). This may be due to the use of different anaesthetic agents. However, one group did report that intubation while glottic reflexes are present is difficult (Rivard, Simura et al. 2006). They used 5 % enflurane to anaesthetise the rats and routinely also used a cotton bud soaked in 2 % lignocaine to anaesthetise the posterior pharynx and glottis prior to intubation (Rivard, Simura et al. 2006). Enflurane has similar anaesthetic characteristics to isoflurane used in the current model (Davis 2008). In the current model, a cotton bud soaked in 2 % lignocaine was applied to the posterior pharynx and glottis for 10 seconds. Suppression of the glottic reflexes was successfully obtained and intubation was much easier and more predictable.

#### 4.6.6 Resistance on Insertion of the Intubation tube into the trachea

During method development, high resistance to insertion of the intubation tube was encountered in one rat. The rat subsequently developed subcutaneous emphysema and was euthanized. Autopsy revealed that the rat had a perforated trachea. This was likely caused by the excessive force used to overcome the resistance encountered at the entrance to the trachea being exerted on the tracheal wall on entry to the trachea was achieved. This resistance may be overcome by using a lignocaine soaked swab to massage the glottis open (Rivard, Simura et al. 2006). In addition, the 14 G intubation tube used was likely too large, further increasing the resistance at the entrance to the trachea. This reinforced that a 16 G tube was more appropriate for initial intubation.

#### 4.6.7 Depth of Insertion of the Intubation Tube

To ensure that adequate mechanical ventilation is achieved, the endotracheal tube must be inserted far enough into the trachea so that it is secure. However, the tube should not go so far to reach the bifurcation of the trachea at the carina where it could injure the rat or occlude one main bronchus. Previous research has reported both length of the trachea and distance from the base of the incisors to the vocal cords in 18 Sprague Dawley and Wistar rats (weight 300 – 530 g), see Table 4-1 (Jou, Tsai et al. 2000). The authors concluded that neither of these measurements was related to the body weight of the rat. The authors recommend inserting the intubation tube 20-30 mm beyond the entrance to the trachea (Jou, Tsai et al. 2000).

**Table 4-1: Length of the oropharynx and trachea in 18 rats (Jou, Tsai et al. 2000).**

Measurement	Min (mm)	Average $\pm$ SD (mm)	Max (mm)
Upper incisors to vocal cords	28	30.3 $\pm$ 2.5	35
Vocal cords to bifurcation of the trachea	30	35.8 $\pm$ 4.3	45

The 16 G IV catheter used in the current model had a total length of 70 mm, 50 mm past the base of the hub. When the hub of the tube was level with the base of upper incisors, in the shortest and longest reported rats, 22 mm and 15 mm of tube will be within the trachea respectively. This was safely within the 30 mm reported minimum length of the trachea. The observation of the position of the hub relative to the base of the upper incisors allowed visual verification of the insertion depth and that the tube had not moved during manipulation of the animal. Any future change to a tube of different length should also be accounted for.

#### 4.6.8 Size of Intubation Tube

Use of a 16G tube was reported by several authors (Rivard, Simura et al. 2006).

Ineffective anaesthesia delivery has also been reported to be overcome by replacement of the intubation tube with a larger bore tube (Yasaki and Dyck 1991). In one case using the current model with a small rat (244 g), resistance to the insertion of the intubation was high despite the intubation attempt being timed with the rat taking a deep breath, as visualised by wide opening of the vocal cords. Intubation was unsuccessful on three successive attempts as the resistance was too high, and intubation was therefore aborted to avoid iatrogenic injury. The rat recovered without complication. The low weight of this rat and high resistance to insertion of the tube

while the vocal cords were open suggests that this rat may have required an even smaller intubation tube, perhaps 18 G.

In a further case, a larger diameter intubation tube may have been necessary to generate sufficient pressure to inflate the lungs. In that case a 16 G then 14 G intubation tube was inserted into the trachea with very low resistance. On each attempt tracheal intubation was suspected due to visualised placement, because the distinct cartilaginous rings of the trachea were felt as the tubes were advanced and because ventilation did not cause inflation of the gastro-intestinal tract. However, no inflation of the lungs was observed and the rat continued to take deep breaths. Air could be heard gushing from the intubation tube despite there being no leaks present. Further intubations were not carried out so as to avoid iatrogenic injury and the rat recovered with no complications. One published recommendation to prevent the escape of anaesthetic gases is placement of a small piece of silicon tubing over the endotracheal tube, 2 cm from the end to sit behind the vocal cords (Van Dongen, Remie et al. 1990). This was not found to be necessary in the current model. However, it is possible that the silicon may overcome the rare need to use a larger diameter tube when delivery of anaesthetic is insufficient.

In the current model a 16 G intubation tube was used routinely, with 12, 14 and 18 G tubes available for substitution. If resistance to insertion of the tube was too high, the tube was substituted for a smaller 18 G tube. Conversely, if ventilation or anaesthetic delivery was ineffective despite high confidence that the tube was in the trachea and

low resistance to insertion of the tube into the trachea was observed, a larger 14 G and 12 G were available.

#### 4.6.9 Assessment of endotracheal tube location

Following intubation, it is critical to assess that the endotracheal tube has been placed correctly in the trachea. Protocol for the current model required the observation of bilateral chest movement as this is the most reliable method to verify oesophageal intubation (Vaghadia, Jenkins et al. 1989). Both condensation of moisture on the endotracheal tube and abdominal distension are unreliable signs, prone to false negative assessment (Vaghadia, Jenkins et al. 1989).

#### 4.6.10 Oral Secretions

In a pilot of control condition rats, the first rat did not recover from anaesthesia, most likely due to a pulmonary obstruction caused by a large volume of fluid in its lungs. Movement of the fluid was audible with a stethoscope and judged as severe (10/10) from approximately 2 hours of anaesthetic. After several spontaneous breaths and beginning to rouse, the rat stopped breathing. Fluid was visible passively draining from the rat's mouth. Attempts to resuscitate the rat resulted in inflation of the gastro-intestinal tract, suggesting that the lungs were blocked with fluid.

One possible cause of fluid in the lungs is too large a tidal volume causing pulmonary oedema. Previous research has shown that fluid accumulation in the lungs of rats is reduced at smaller tidal volumes, 12 vs. 6 vs. 3 ml/kg (Frank, Gutierrez et al. 2002). Therefore the tidal used in the current rat model was minimised.

A second possible cause of fluid in the lungs is draining of oral secretions. Oral secretions will be increased by both the presence of the intubation tube in the mouth and also by the partially open position of the jaw. In the second pilot case a gauze swab was placed in the rat's mouth to passively collect oral secretions. Movement of fluid in the lungs was audible with a stethoscope, however, was only very mild (1/10). The swab was consistently damp when removed at the end of procedures, suggesting the collection of oral secretions. It is possible that the reaction to the presence of the swab itself may have contributed to oral secretions. However, it is unlikely to have been the only cause of all oral secretions as suction at the mouth is sometimes required during rat anaesthesia (Davis 2008). The swab appeared to be successful at reducing drainage of oral secretions into the lungs and so was adopted into the model protocol.

A third possible cause of fluid in the lung is un-even ventilation of the left and right lung. Despite assumptions about the length of the endotracheal tube relative to the length of the trachea, it is possible that the intubation tube may have been inserted past the carina and into the main bronchus of one of the lungs. If this occurs fluid is then likely to accumulate in the opposite lung. In subsequent cases, a stethoscope was used to compare the right and left breath sounds. If breath sounds on one side were deemed to be louder than the other, the intubation tube was gently retracted until symmetrical breath sounds were achieved. In all cases in which symmetrical ventilation was confirmed, and the gauze swab was placed, no fluid was heard in the lungs and therefore these two techniques became part of the model protocol

If fluid in the lungs becomes a problem in future experiments, anticholinergic agents such as atropine sulphate or glycopyrrolate can be used as a premedication for suppression of salivation and bronchial secretion in rats (Hanusch, Hoeger et al. 2007; National Health and Medical Research Council 2008). Premedication is recommended 15-40 minutes before anaesthetic agents (National Health and Medical Research Council 2008), recommended dosage (0.05 mg/kg s.c.) (Hanusch, Hoeger et al. 2007) or administered as a drop on the tongue (Davis 2008).

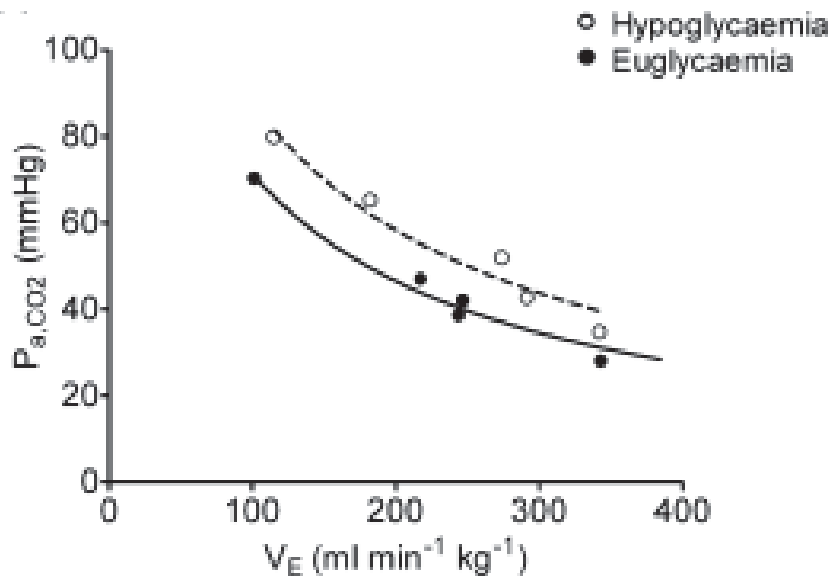
#### 4.6.11 Extubation and recovery of spontaneous breathing

The research planned with the current model required a long period of general anaesthesia, which increased the risk of inadvertent death (Davis 2008). Having the rat mechanically ventilated with an endotracheal tube has the advantage of providing immediate control of the airway if respiratory or cardiac arrest occurred. It was therefore critical to ensure that the endotracheal tube was not taken out before the rat was sufficiently recovered from general anaesthesia so that spontaneous breathing was initiated and continued. It is difficult to re-intubate a rat that is not breathing since the vocal cords are closed. The current model found that the most reliable method for determining the correct time to extubate was to wait for the rat to regain the righting reflex. The presence of hypothermia can prolong the time to recover from anaesthesia (Davis 2008), and so in this case extra time was planned for prior to extubation.

## 4.7 Exploratory trial to determine minute ventilation

### 4.7.1 Background: Exploratory trial to determine minute ventilation

There are no published guidelines stating ventilation stroke rate and volume that will ensure normal blood gas levels are maintained in rats during abdominal surgery under general anaesthesia. The relationship between minute ventilation, normalised to body weight, and  $P_aCO_2$  in an anaesthetised rat is hyperbolic (Bin-Jaliah, Maskell et al. 2005), see Figure 4-8. Minute ventilation needs to be optimised to a specific rat model, as blood gases will also depend on other model specific parameters such as the method of anaesthesia, the concentration of inspired oxygen delivered and also the depth of anaesthesia maintained. However, it is not feasible to take blood gas measurements for all rats because the blood gas disposables are obstructively expensive. Blood gas measurement also increases in the likelihood of unexpected death due to complications of femoral artery cannulation, and the cannulation adds time and equipment to the already crowded operating space. Therefore an exploratory investigation was conducted to establish the best ventilation parameters to maintain normal  $P_aCO_2$  measurements during open abdominal surgery in the current rat model.



**Figure 4-8: Relationship between ventilation and  $P_{a,CO_2}$  in an anaesthetised rat during euglycaemia and hypoglycaemia (Bin-Jaliah, Maskell et al. 2005)**

In addition to establishing ventilation parameters for use during abdominal surgery, different ventilation parameters may be required when rats are exposed to  $CO_2$  gas at atmospheric pressure, as the  $CO_2$  may affect  $P_{a,CO_2}$  independently. Exposure of the abdominal cavity to pressurised  $CO_2$  gas during  $CO_2$  pneumoperitoneum causes hypercapnia in humans (Neudecker, Sauerland et al. 2002). It is common practise to increase ventilation rate during laparoscopic surgery to allow the excess  $CO_2$  to be expelled in order for  $P_{a,CO_2}$  to remain in the normal range (Neudecker, Sauerland et al. 2002). In mice,  $CO_2$  pneumoperitoneum increases  $P_{a,CO_2}$  compared with both laparotomy without  $CO_2$  insufflation and anaesthesia only (Bourdel, Matsuzaki et al. 2007).  $P_{a,O_2}$  and arterial pH did not change (Bourdel, Matsuzaki et al. 2007). To ensure that  $P_{a,CO_2}$  is within the normal range for each group, the authors adopted a protocol in which mice in the laparoscopy group were ventilated at the same stroke volume but at a stroke rate 114% that of the mice in the laparotomy and anaesthesia alone groups (Bourdel, Matsuzaki et al. 2007; Matsuzaki, Jardon et al. 2010). As the  $CO_2$  gas in the

current model was insufflated at atmospheric pressure, it was unclear whether  $P_a\text{CO}_2$  would increase. Therefore, in order to avoid hypercapnia, this exploratory trial was designed to investigate whether insufflation of  $\text{CO}_2$  into the abdominal cavity during laparotomy would increase  $P_a\text{CO}_2$ , in order to determine whether a higher ventilation rate was necessary during  $\text{CO}_2$  insufflation. Furthermore, some research suggests that a muscle relaxant is necessary to avoid acidosis during mechanical ventilation in mice (Bourdel, Matsuzaki et al. 2007). Therefore, it was necessary to establish whether it is possible to achieve normal  $P_a\text{CO}_2$  without the use of a muscle relaxant.

The objections of this exploratory trial were

1. Establish whether it is possible to achieve normal  $P_a\text{CO}_2$  without the use of a muscle relaxant.
2. Establish optimum minute ventilation to maintain normal  $P_a\text{CO}_2$  measurements in the current rat model.
3. Investigate whether insufflation of  $\text{CO}_2$  into the abdominal cavity during laparotomy increases  $P_a\text{CO}_2$  and, if necessary, establish higher minute ventilation during periods of  $\text{CO}_2$  insufflation.

#### 4.7.2 Method: Exploratory trial to determine minute ventilation

##### 4.7.2.1 Establishing a normal $P_a\text{CO}_2$ and pH range

An early attempt to quantify normal  $P_a\text{CO}_2$  in rats reported that the average  $P_a\text{CO}_2$  of 120 adult male rats weighing > 400g was  $41.2 \pm 1.87$  mmHg (all results presented as mean  $\pm$  standard deviation) (Pepelko and Dixon 1975). A later publication documented

what they referred to as 'normal reference' blood gas values in 77 awake, unrestrained male Wistar rats, 250-380 g, as  $P_a\text{CO}_2$   $34.5 \pm 3.0$  mmHg;  $P_a\text{O}_2$   $90.0 \pm 5.5$  mmHg ; pH  $7.47 \pm 0.02$  (Brun-Pascaud, Gaudebout et al. 1982). By combining the results of these two reports, including one standard deviation, the target  $P_a\text{CO}_2$  range for the current investigation was set as 31 – 43 mmHg with a pooled average of 38 mmHg.

Assessment of acidosis and alkalosis will be made when pH is below or above, respectively, the range 7.30 – 7.48 (Vaghadia, Jenkins et al. 1989; Fuentes, Hanly et al. 2004). This range is a combination of pH values reported for awake rats, approximately 7.47 (Pepelko and Dixon 1975; Brun-Pascaud, Gaudebout et al. 1982), and anaesthetised rats, which tend to exhibit lower pH values even when mechanically ventilated (Brun-Pascaud, Gaudebout et al. 1982; Vaghadia, Jenkins et al. 1989).

#### 4.7.2.2 Choice of starting ventilation parameters

Reported and recommended minute ventilation, stroke volume and ventilation rates are extremely variable, see Table 4-2 and Table 4-3. In preliminary investigations stroke volume of 0.15 ml/10 g was used, taken from a published recommendation (Hanusch, Hoeger et al. 2007). However, in the current using this stroke volume rats accumulated fluid in the lungs, which was greatly improved by reducing the stroke volume and increase stroke rate in order to maintain the same minute ventilation. This is consistent with previous research that has shown that low tidal volumes reduce fluid accumulation in the lungs in rats (Frank, Gutierrez et al. 2002). In addition, it was observed that the distension of the lungs with a stroke volume of 0.15 ml/10 g body weight was much more obvious compared with an awake rat. Therefore, ventilation

rate was increased to 100—120 breaths per minute with a tidal volume calculated to achieve a minute ventilation volume of 0.85 mL/min/g (Fuentes, Hanly et al. 2004), for a 250g rat the tidal volume is 2.1 ml. This ventilation rate is in line with previous published protocols (Vaghadia, Jenkins et al. 1989; Kuo, Yuan et al. 2010).

#### 4.7.2.3 Experimental design.

Six rats were used for this exploratory trial. Calibration and quality check of the blood gas analyser was carried out at the start of each day of experimentation following the manufacturer's instructions. Prior to anaesthesia, each rat was weighed and the ventilation rate was calculated (0.85 mL/min/g with a tidal volume of 2.2 ml for the first and adjusted for subsequent rats based on results). General anaesthesia was induced with inhaled isoflurane in air via a nose cone. Endotracheal intubation was performed for mechanical ventilation with room air, and femoral artery cannulation to allow the collection of blood and analysis of arterial blood gases. Blood gas measurement was then taken and the ventilation rate adjusted to achieve  $P_aCO_2$  31 – 43 mmHg, confirmed by additional blood gas measurement. The abdominal incision was performed and another blood gas measurement was taken, again adjusting ventilation rate if necessary. Insufflation of  $CO_2$  was then conducted and blood gases were taken every 15 minutes for an hour. The rats were euthanized at the end of data collection.

**Table 4-2: Previously reported ventilation parameters and blood gas measurements in rats**

Source	Rat strain	Minute ventilation (mL/min/kg)	Tidal volume (mL/kg)	Rate (/min)	Gas	PEEP (cmH <sub>2</sub> O)	Anaesthetic agents	Muscle relaxant	pH	P <sub>a</sub> O <sub>2</sub> (mmHg)	P <sub>a</sub> CO <sub>2</sub> (mmHg)
Peoples et al, 2014 (Peoples and McLennan 2014)	Wistar	600	10	60	Room air	NS	Pentobarbital i.v. 60 mg/kg	N	7.54 ± 0.02	95 ± 3	33 ± 2
Peoples et al, 2013 (Peoples, Hoy et al. 2013)	Wistar	1080	10	60	Room air	NS	Pentobarbital i.v. 60 mg/kg	N	7.42 ± 0.01	100 ± 3	33 ± 1
Peoples et al, 2010 (Peoples and McLennan 2010)	Wistar	600	10	60	Room air	NS	Pentobarbital i.v. 60 mg/kg	N	7.53 ± 0.2	90 ± 4	33 ± 2
Fuentes, 2004 (Fuentes, Hanly et al. 2004)	Sprague–Dawley	850	NS	NS	NS	NS	3 % Isoflurane	N	7.408 ± 0.011	-	36.8 ± 1.5
Frank, 2002 (Frank, Gutierrez et al. 2002)	Sprague-Dawley	780	12	65*	100 % O <sub>2</sub>	10	Pentobarbital i.v. 500 µg/kg/h	Pancuronium i.v. 2 mg/kg/h	7.33 ± 0.08	-	43 ± 7
		780	12			5			7.36 ± 0.08	-	45 ± 9
		390	6			10			7.33 ± 0.08	-	48 ± 4
		195	3			10			7.31 ± 0.06	-	42 ± 8

Source	Rat strain	Minute ventilation (mL/min/kg)	Tidal volume (mL/kg)	Rate (/min)	Gas	PEEP (cmH <sub>2</sub> O)	Anaesthetic agents	Muscle relaxant	pH	P <sub>a</sub> O <sub>2</sub> (mmHg)	P <sub>a</sub> CO <sub>2</sub> (mmHg)
Jou, 2000 (Jou, Tsai et al. 2000)	Sprague-Dawley and Wistar	200	5	40	100 % O <sub>2</sub>	NS	Sodium thiopentone i.p. 50 mg/kg	N	7.4 ± 0.03	318.1 ± 34.9	40.3 ± 3.4
Vaghadia, 1989 (Vaghadia, Jenkins et al. 1989)	Wistar	1751	18	100	50 % O <sub>2</sub> / 50 % NO	NS	1.5 % Halothane	N	7.52 ± 0.04	147.8 ± 54.5	28.2 ± 4.1

\*Respiratory rate adjusted to maintain arterial pH between 7.30 and 7.45.

**Table 4-3: Recommended ventilation parameters for rats**

Source	Minute ventilation (mL/min/kg)	Tidal volume (mL/kg)	Rate (/min)	Blood gas results reported
Van Dongen, 1990 (Van Dongen, Remie et al. 1990)	434-930	6.2	70-150	No
Hanusch, 2007 (Hanusch, Hoeger et al. 2007)	900-1500	15	60-100	No

#### 4.7.3 Results: Exploratory trial to determine minute ventilation

Calibration and quality check of the blood gas analyser (ABL 77, Radiometer, Brønshøj, Denmark) were successful on all occasions. Two rats exhibited hypocapnia. In one,  $P_aCO_2$  remained at approximately 9 mmHg despite dropping minute ventilation from 849 mL/min/kg to 388 mL/min/kg over a 40 minute period. In a second rat  $P_aCO_2$  remained below 10 mmHg despite dropping minute ventilation from 1000 to 532 mL/min/kg over a 15 minute period. Rat two also showed signs of respiratory alkalosis, high pH in the presence of low  $P_aCO_2$ , further suggesting that the rats were over-ventilated. Lower minute ventilation was therefore used for the next rat, which exhibited high  $P_aCO_2$ , 48 and 50 mmHg, at 254 mL/min/kg, suggesting under-ventilation. Pooling of the results following further adjustment of minute ventilation in subsequent rats demonstrated that both  $P_aCO_2$  and  $P_aO_2$  vary with minute ventilation, see Figure 4-9 and Figure 4-10. Regression analysis suggests good correlation, with a hyperbolic relationship between  $P_aCO_2$  and minute ventilation ( $R^2=0.79$  for laparotomy without  $CO_2$  insufflation,  $R^2=0.77$  for laparotomy with  $CO_2$  insufflation). There appeared to be a small increase in  $P_aCO_2$  during  $CO_2$  insufflation, although there is not sufficient statistical power to make this conclusion with confidence. A paired analysis of  $P_aCO_2$  and pH taken from the same rat at the same minute ventilation was

conducted using a Wilcoxon matched pairs signed rank test, and was also underpowered to show a significant difference in either variable ( $P_aCO_2$ : median (25<sup>th</sup> - 75<sup>th</sup> percentile) increase during  $CO_2$  insufflation 8.1 (5.7- 12.8) mmHg,  $p=0.125$ ; pH: median (25<sup>th</sup> -75<sup>th</sup> percentile) decrease during  $CO_2$  insufflation 0.08 (0.04-0.12),  $p=0.125$ ). The regression analysis suggested that a  $P_aCO_2$  of 38 would be best achieved with minute ventilation of 315 ml/kg/min during anaesthesia only, and a minute ventilation of 350 ml/kg/min during anaesthesia with  $CO_2$  insufflation Table 4-4. There was no increase in  $P_aO_2$  during  $CO_2$  insufflation, see Figure 4-10.

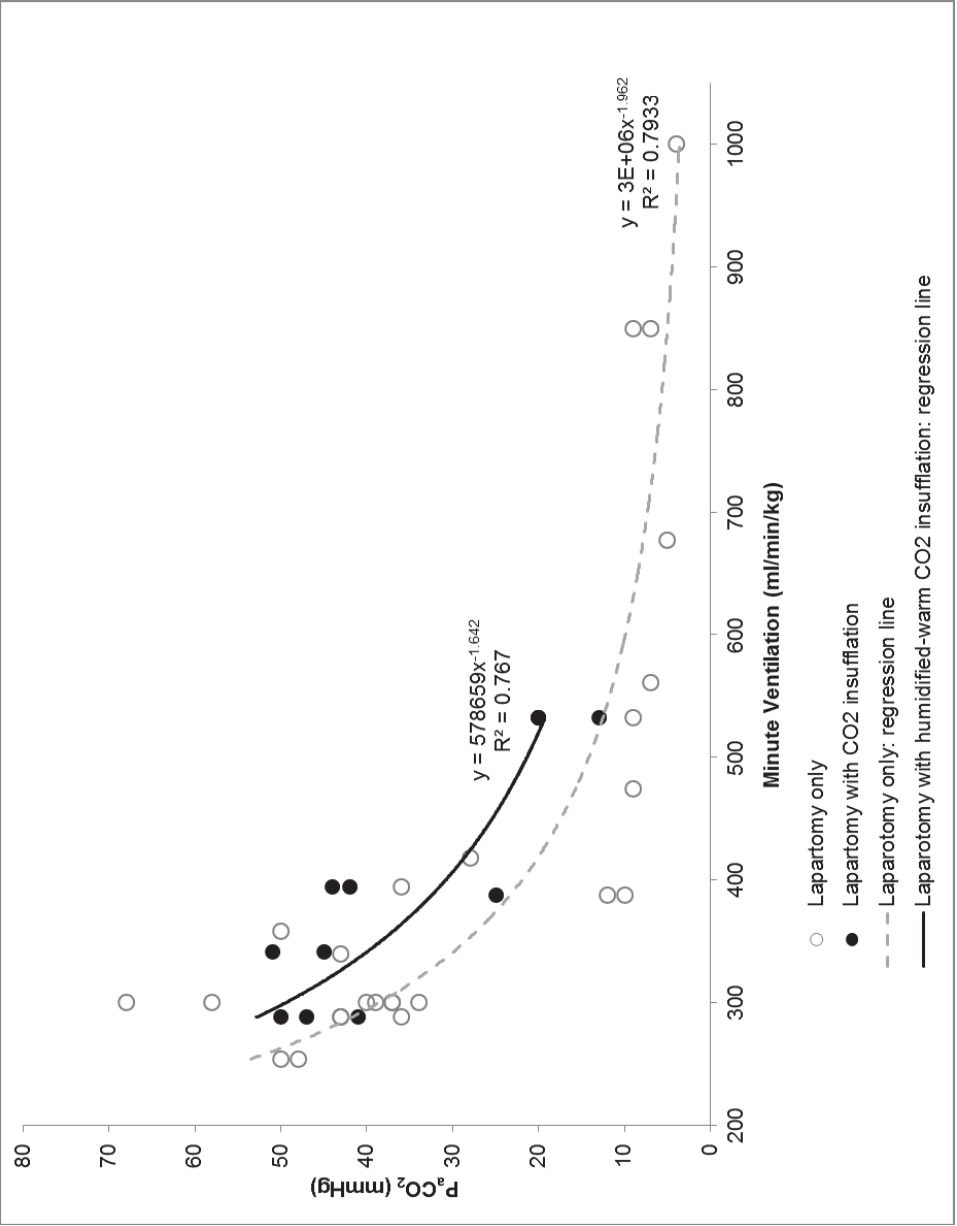


Figure 4-9:  $P_a\text{CO}_2$  vs minute ventilation for all rats

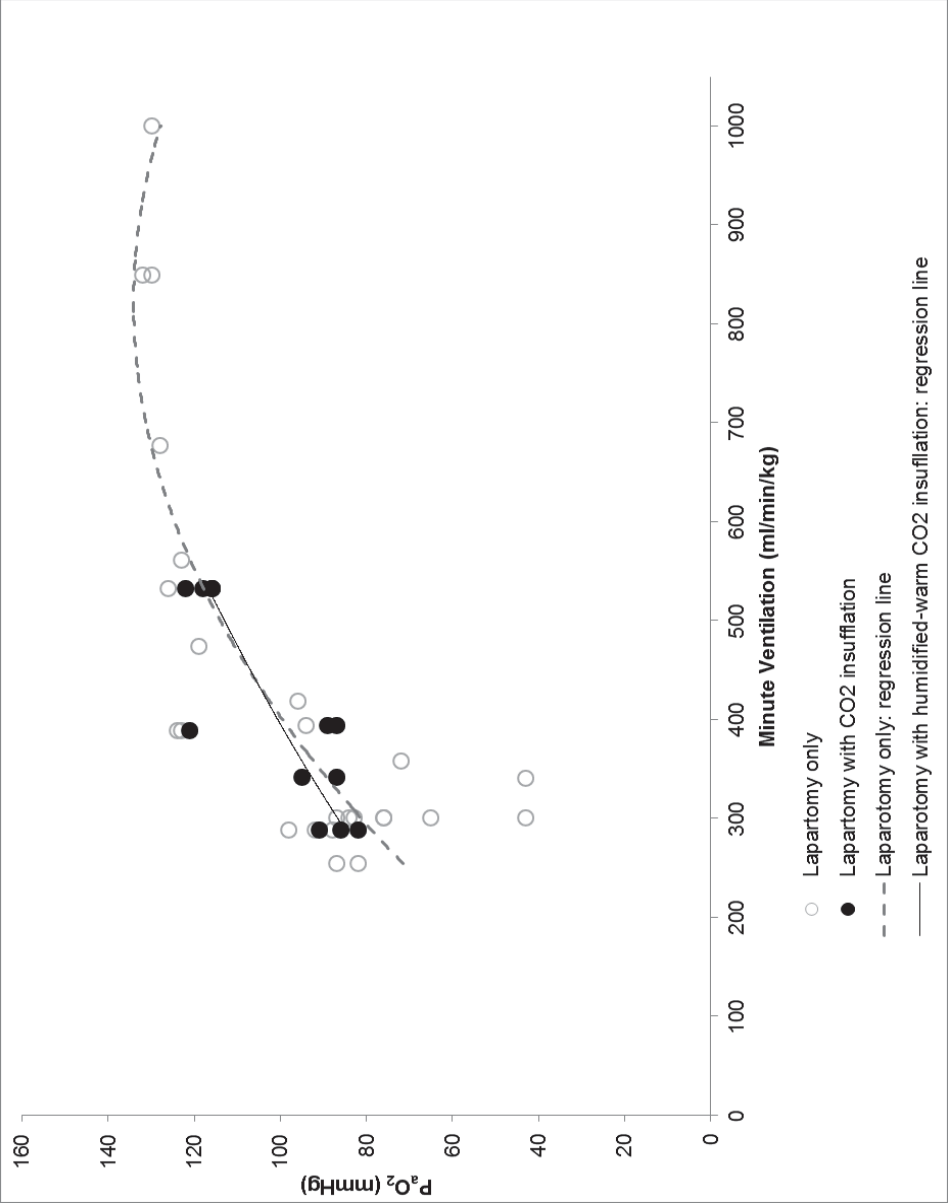


Figure 4-10:  $P_aO_2$  vs minute ventilation for all rats

**Table 4-4: Minute ventilation required to achieve specific  $P_a\text{CO}_2$  values.**

Calculated by the hyperbolic regression curve in Figure 4-9. The target value of 38mm Hg is highlighted in grey. Predicted pH is also presented as calculated by linear regression.

Laparotomy only			Laparotomy with humidified-warm $\text{CO}_2$ insufflation		
Minute ventilation	$P_a\text{CO}_2$	pH	Minute ventilation	$P_a\text{CO}_2$	pH
mL/min/kg	mmHg		mL/min/kg	mmHg	
294	43	7.27	327	43	7.31
305	40	7.28	342	40	7.32
315	38	7.29	350	38	7.32
350	31	7.32	400	31	7.34

#### 4.7.4 Discussion: Exploratory trial to determine minute ventilation

A hyperbolic relationship between minute ventilation and  $P_a\text{CO}_2$  was observed, as expected from previous results (Bin-Jaliah, Maskell et al. 2005). A muscle relaxant was not required to avoid respiratory acidosis, contrary to the suggestion of authors of a surgical mouse model (Bourdel, Matsuzaki et al. 2007). Conversely, without the use of a muscle relaxant one of the rats was alkalotic throughout the entire 2 ½ hour procedure, likely due to hyperventilation. The optimised minute ventilations in the current study are specific to this rat model and do differ from other models, see Table 4-2. Of particular interest, low  $P_a\text{CO}_2$  values recorded from two rats would fall below the horizontal asymptote of the ventilation/ $P_a\text{CO}_2$  curve if plotted onto previously published data shown in Figure 4-8 (Bin-Jaliah, Maskell et al. 2005). The differences are likely explained by dissimilarities in the experimental models, including metabolic rate, depth of anaesthesia, anaesthetic agent and leakage of ventilatory gases. A drop in metabolic rate reduces  $P_a\text{CO}_2$  at a given minute ventilation and changes the shape of the ventilation/ $P_a\text{CO}_2$  curve, including the location of the horizontal and vertical

asymptotes (Bin-Jaliah, Maskell et al. 2005). This is because less CO<sub>2</sub> is produced by cellular respiration and the gain of the chemoreceptors reduced in parallel with metabolic rate (Bin-Jaliah, Maskell et al. 2005). Hypothermia is a possible cause of reduced metabolic rate (reviewed by (Davis 2008)). In the current investigation, body temperature was monitored and both rats that exhibited low P<sub>a</sub>CO<sub>2</sub> remained normothermic (body temperature >36.5 °C) during all blood gas measurements. Another possible explanation for the low P<sub>a</sub>CO<sub>2</sub> values in some rats in the current model is cardiac depression caused by deep anaesthesia. In the current investigation, adequate depth of anaesthesia to conduct surgery was ascertained by loss of pedal reflex. Once the pedal reflex is lost, the depth of anaesthesia is monitored by heart rate and pulse strength using a pulse transducer around the neck of the rat so as to avoid overdose. It is difficult to judge the depth of anaesthesia precisely, due to the inter-animal variation in heart rate. Therefore, it is possible that the depth of anaesthesia contributed to the low P<sub>a</sub>CO<sub>2</sub> values. Deep anaesthesia may also explain the metabolic acidosis, low pH in the presence of low P<sub>a</sub>CO<sub>2</sub>, and final anaesthetic overdose of one of the rats. Overly anaesthetising should be avoided and may require further monitoring than only blood pressure and heart rate changes. Monitoring of arterial oxygen saturation may be a useful adjunct for identifying a drop in cardiac output caused by deep anaesthesia. Finally, another explanation for the low P<sub>a</sub>CO<sub>2</sub> in the current results compared with the findings of Bin-Jaliah, Maskell et al (Bin-Jaliah, Maskell et al. 2005) could be leakage of ventilatory gases in the latter model, resulting in inadequate lung ventilation. This could have been caused by discrepancy between the inner diameter of the trachea and the outer diameter of the endotracheal tube.

The results of this exploratory trial suggest that CO<sub>2</sub> insufflation increases P<sub>a</sub>CO<sub>2</sub>. This is consistent with the effect of intra-abdominal CO<sub>2</sub> in clinical practice (Neudecker, Sauerland et al. 2002) and the results of rodent experimentation (Bourdel, Matsuzaki et al. 2007). However, the data collected from this exploratory trial did not produce enough statistical power to make confident conclusions on optimised minute ventilation for anaesthesia only versus anaesthesia with CO<sub>2</sub> insufflation, and further expense on blood gas measurements to increase the sample size could not be justified. The current model was planned to be used for two different types of measurement. The first investigated desiccation of the peritoneal mesothelium with and without humidified-warm CO<sub>2</sub> insufflation. The second investigated how tissue oxygenation is effected by insufflation of humidified-warm CO<sub>2</sub>. The results of the latter experiment were likely be more affected by high P<sub>a</sub>CO<sub>2</sub> and the use of a higher ventilation rate may be more important. However, given that a firm conclusion of the optimal ventilation rate for each condition cannot be made from these results, it may be prudent to adopt the higher ventilation rate for both experimental conditions.

#### 4.7.5 Conclusions: Exploratory trial to determine minute ventilation

1. Minute ventilation of 315-350 ml/kg/min was the best range for maintenance of normal P<sub>a</sub>CO<sub>2</sub> in the current rat model.
2. Insufflation of CO<sub>2</sub> into the abdominal cavity at atmospheric pressure appears to increase P<sub>a</sub>CO<sub>2</sub>, although the results of this exploratory investigation were not statistically significant. The regression model suggests that the optimum minute ventilation to achieve P<sub>a</sub>CO<sub>2</sub> of 38 mmHg is 315 mL/min/kg during

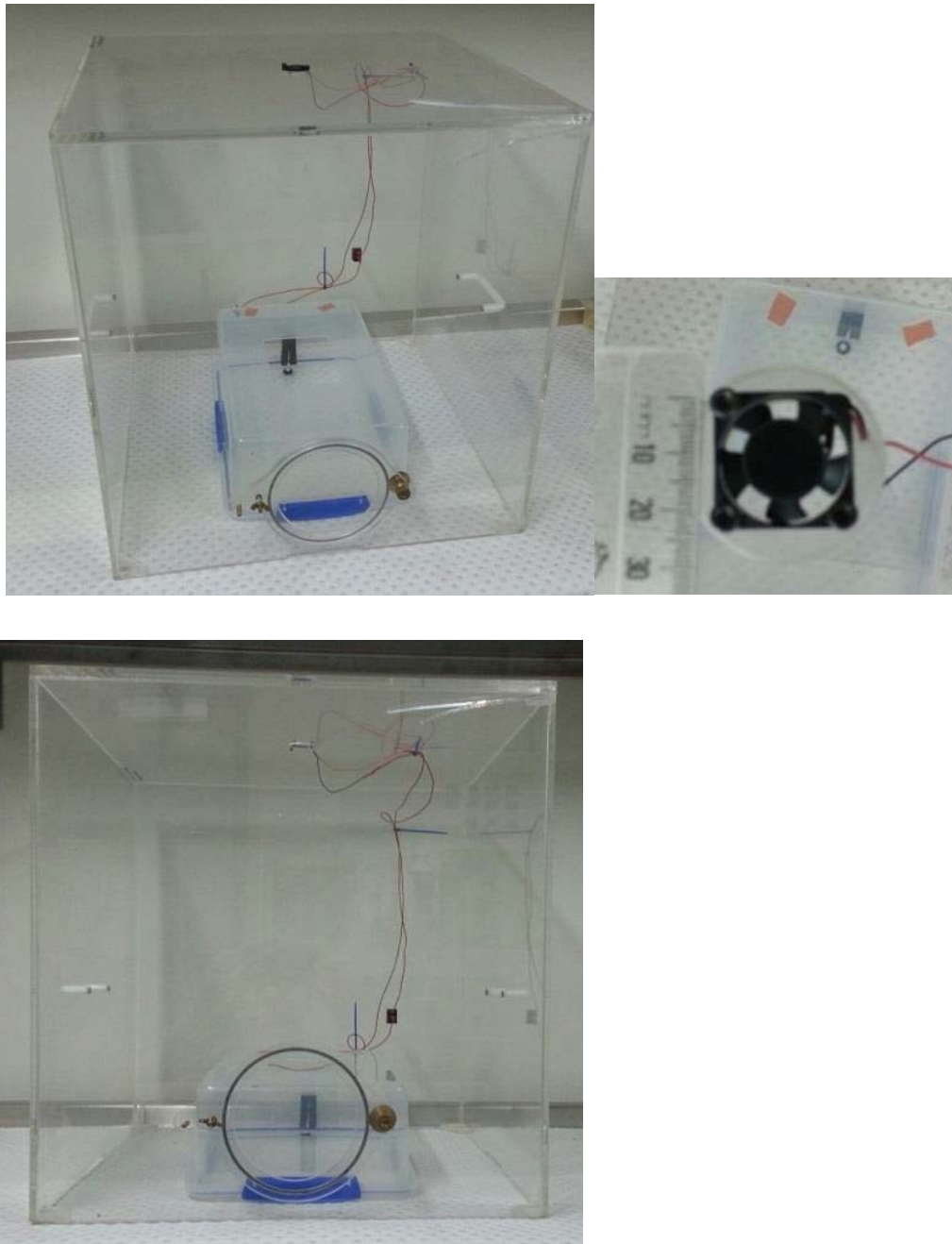
anaesthesia without CO<sub>2</sub> insufflation and 350 mL/min/kg during anaesthesia with CO<sub>2</sub> insufflation.

3. The results were underpowered to justify a mandatory increase in ventilation rate during CO<sub>2</sub> insufflation. When tissue oxygenation is being measured, and therefore avoidance of high P<sub>a</sub>CO<sub>2</sub> is of particular concern, the use of 350 mL/min/kg for both anaesthesia only and anaesthesia with CO<sub>2</sub> insufflation may be prudent.
4. Deep anaesthesia should be avoided to minimise the risk of blood pressure drop, hypoperfusion with resulting metabolic acidosis and the effect of deep anaesthesia on P<sub>a</sub>CO<sub>2</sub>. Monitoring of arterial oxygen saturation may be a useful adjunct for identifying a drop in cardiac output caused by deep anaesthesia.

#### 4.8 Mimicking the air flow rate of an operating room

Air flow around the rat was standardised to simulate normal operating room ventilation. A custom made Perspex chamber (460 x 460 mm square and 480 mm high with a small fan in the ceiling) was placed over the rat, Figure 4-11. The height of the chamber allowed for a camera tripod to be set up within the box if required for intra-operative photography. The chamber had an air gap of 3 mm around its base, so as to direct room air entrained into the fan down over the rat and the exposed peritoneal cavity, before exiting through the base of the chamber. A circular door of diameter 160 mm was placed in one side of the box to allow temporary access to the rat during surgery if necessary. The door was sealed with an O-ring. The speed of the fan was adjusted to create 20 exchanges of the chamber air per hour (volume flow of 0.034 m<sup>3</sup>

x min<sup>-1</sup> for this chamber) as this is the minimum number of operating room air changes per hour recommended for operating theatres (Ninomura and Hermans 2008) and is a conservative ventilation design (Memarzadeh and Manning 2002).



**Figure 4-11: Perspex box with ceiling mounted fan used to simulate conservative airflow normally present in an operating room.**

#### 4.9 Creation of an intra-abdominal surgical CO<sub>2</sub> microclimate in rats

In order to investigate the effect of insufflation of the open abdominal cavity in rats, it was first necessary to ensure that the CO<sub>2</sub> microclimate was successfully created. The efficacy of a CO<sub>2</sub> diffuser (Cardia Innovation AB, Sweden) to successfully de-air the cardiothoracic cavity during open heart surgery has been shown in both bench (Persson and Van der Linden 2003; Svenarud, Persson et al. 2003; Svenarud, Persson et al. 2003; Persson, Svenarud et al. 2004) and human investigations (Persson, Svenarud et al. 2004). In comparison with the abdomen of a rat, a human cardio thoracic cavity, with the heart removed, is large. Measurements of CO<sub>2</sub> concentration needed to be repeated in a rat model to ensure effective creation of the CO<sub>2</sub> microclimate.

##### 4.9.1 Description of the human cardio thoracic model and results

To test the efficacy of the diffuser, researchers in Sweden built two different cardiothoracic wound models based on maximum intra-operative measurements of 5 patients. The first was a cylindrical model (diameter 160 mm, depth 80 mm, volume 2.5 L) (Persson and Van der Linden 2003). The second was a more sophisticated anatomical torso model (midline incision length 200 mm, width 120 mm wide, volume 2.5 L) (Svenarud, Persson et al. 2003). The investigations (Persson and Van der Linden 2003) found that to successfully de-air the cavity it is necessary to:

1. Overcome diffusion of the gas into the surrounding environment by delivering the gas at  $\geq 5$  L/min (10 L/min is recommended to overcome suctioning and movement of tools in the surgical field).

2. Avoid turbulence by delivering the CO<sub>2</sub> at a low velocity of  $\leq 0.1 \text{ m.s}^{-1}$ . This is achieved by using a gas diffuser to reduce gas velocity and explains why open ended tubes are ineffective.
3. Deliver the gas from within the cavity.

#### 4.9.2 Achieving a surgical CO<sub>2</sub> microclimate in rats

In-vitro, the 2.5 litre volume of the cardiothoracic cavity is created by the support of the ribcage. Conversely, when the thin abdominal wall of a rat is opened the depth of the cavity is almost zero, see Figure 4-3. Preliminary testing confirmed that the resulting abdominal cavity of a rat following laparotomy is too shallow to create a local environment of high CO<sub>2</sub> gas concentration. The shallowness of the cavity is due to the relatively thin abdominal wall of the rat compared to humans, and also to the minimal use of retraction in this model in order to protect the peritoneum from mechanical trauma. During optimisation of the laparotomy wound, several methods were trialled that would increase the depth of the wound see Figure 4-1. However, none met the requirements of the model, namely that exposure of the peritoneal sample to the gaseous environment was assured and that trauma to the peritoneum by stretching and mechanical damage was avoided.

Therefore, in order to ensure that the abdominal cavity was exposed to an environment of high concentration of CO<sub>2</sub>, the rat was placed inside a box with a hole in the top from within which CO<sub>2</sub> was insufflated, see Figure 4-12. CO<sub>2</sub> being heavier than air, air was then displaced from the container by the CO<sub>2</sub> and the entire rat, including the open abdominal cavity, was in a stable, high CO<sub>2</sub> concentration

environment. The rats were mechanically ventilated and therefore were not breathing from the CO<sub>2</sub> environment. A 7 L box was chosen (length 355 mm, width 235 mm, height 120 mm), see Figure 4-12, as this was large enough to fit the rat and necessary equipment. The hole in the top was 90 x 120 mm. The CO<sub>2</sub> was insufflated into the container at 9 L/min via a gas diffuser (CarbonVITA™, Cardia Innovation AB, Sweden) so as to ensure the CO<sub>2</sub> environment is maintained despite any movement of tools in the surgical field (Persson and Van der Linden 2003). The gas diffuser ensures the gas enters the container at a low velocity, thereby reducing turbulence and allowing high CO<sub>2</sub> gas concentrations within the container. Validation of the setup showed that a CO<sub>2</sub> concentration >90 % was maintained within the box when CO<sub>2</sub> was insufflated at >5 L/min (CheckMate II gas analyser, PBI Dansensor, Denmark).



**Figure 4-12:** Illustration of the use of a 7 L plastic container (top left) and CarbonVITA™ diffuser (top right) to create a surgical environment within which to insufflate CO<sub>2</sub> (bottom photo).

**Note that the container was used upside down and a hole was cut out of the top to allow insertion of the diffuser to enter the cavity so CO<sub>2</sub> gas was delivered from within the cavity insufflation and for access to the rat when required. The hole is outlined in black for clarity. The diffuser is not included in this photo. A slit was cut in one end of the box to allow the oesophageal intubation tube to enter the box (arrow), and was partially sealed with a piece of foam glued to the slit.**

## 4.10 Measurement of tissue oxygen partial pressure (PtO<sub>2</sub>)

### 4.10.1 Methods for measurement of PtO<sub>2</sub>

Tissue oxygen partial pressure (PtO<sub>2</sub>) can be measured directly and indirectly in several ways, each with advantages and disadvantages, see Table 4-5. The delivery of oxygen to tissue requires oxygen to be available in blood for delivery, either dissolved or bound to haemoglobin, plus adequate perfusion of the tissue of interest. Therefore, indirect measurements of PtO<sub>2</sub> include measuring arterial oxygen partial pressure (P<sub>a</sub>O<sub>2</sub>), arterial haemoglobin oxygen saturation (SpO<sub>2</sub>), tissue oxygen haemoglobin saturation, or tissue perfusion. P<sub>a</sub>O<sub>2</sub> is commonly measured clinically, and SpO<sub>2</sub> is a simple non-invasive measure, making translation of conclusions in rats regarding P<sub>a</sub>O<sub>2</sub>

or  $\text{SpO}_2$  to humans simple and direct. However,  $\text{PtO}_2$  is often not well predicted by either  $\text{P}_a\text{O}_2$  or by  $\text{SpO}_2$ . Tissue oxygen partial pressure tends to increase with  $\text{P}_a\text{O}_2$ , with the relationship curvilinear at  $\text{P}_a\text{O}_2$  below 100-150 mmHg, then tending to become linear at higher  $\text{P}_a\text{O}_2$  levels (Chang, Goodson *et al.* 1983). The slope of  $\text{PtO}_2/\text{P}_a\text{O}_2$  curve is dependent on perfusion (Chang, Goodson *et al.* 1983), and thus the slope can be increased by fluid infusion (Chang, Goodson *et al.* 1983; Jonsson, Jensen *et al.* 1987), and is decreased in presence of hypo-perfusion, such as associated with obesity (Kabon, Nagele *et al.* 2004). Surgical patients experience a downward shift in the slope of the  $\text{PtO}_2/\text{P}_a\text{O}_2$  curve, and the shift appears to be exaggerated in patients undergoing vascular and cardiac operations compared with those undergoing abdominal operations (Chang, Goodson *et al.* 1983). A drop in  $\text{PtO}_2$  has been reported during open abdominal surgery despite maintenance of  $\text{P}_a\text{O}_2$  (Fleischmann, Kugener *et al.* 2007). Furthermore, increasing  $\text{P}_a\text{CO}_2$  increases  $\text{PtO}_2$  without effecting  $\text{P}_a\text{O}_2$  (Akça, Doufas *et al.* 2002). Therefore,  $\text{P}_a\text{O}_2$  can be an unreliable measure of  $\text{PtO}_2$ .

Similar to  $\text{P}_a\text{O}_2$ , measurement of  $\text{SpO}_2$  by spectroscopy is easily obtained but it is a poor predictor of  $\text{PtO}_2$  as it does not account for whether adequate perfusion exists to deliver the oxygen to the tissues. Microbial killing has also been shown to be dependent on tissue oxygen partial pressure, not oxygen saturation (Allen, Maguire *et al.* 1997). The utility of arterial  $\text{SpO}_2$  to measure oxygen in tissue may be improved by measuring average oxygen haemoglobin saturation in an area of tissue below a larger probe than that used for arterial measurements (Govinda, Kasuya *et al.* 2010). There are few reports of use of this measurement and thus far it appears to be an unreliable

predictor of SSI (Govinda, Kasuya et al. 2010), and therefore requires further investigation. Most importantly, however, SpO<sub>2</sub> is an inappropriate measure of oxygenation during CO<sub>2</sub> insufflation in the current model, as CO<sub>2</sub> insufflation is expected to decrease SpO<sub>2</sub>. SpO<sub>2</sub> measurements may lead to an incorrect conclusion that CO<sub>2</sub> insufflation decreases tissue oxygenation.

In addition to P<sub>a</sub>O<sub>2</sub> and SpO<sub>2</sub>, tissue perfusion is also an indirect measure of PtO<sub>2</sub>. Tissue perfusion can be measured non-invasively by fluorescent imaging following the intravascular injection of a fluorescent dye, or by laser Doppler analysis. Fluorescent imaging has recently been shown to assist with real time assessment of intestinal viability (Diana, Noll et al. 2014) and is available integrated with new surgical robots (da Vinci Si, Intuitive Surgical, San Francisco and FireFly, Novadaq, Bonita Springs). However, despite perfusion being of interest to clinicians, it is only an indirect measure of PtO<sub>2</sub>. With regard to the investigation of the effect of CO<sub>2</sub> insufflation in the current model, perfusion is only one of the hypothesised mechanisms of action. CO<sub>2</sub> insufflation is also expected to increase PtO<sub>2</sub> by increasing release of O<sub>2</sub> from haemoglobin. Any increase in PtO<sub>2</sub> attributed to this second mechanism of action would not be measured by changes in perfusion. Should insufflation of CO<sub>2</sub> be shown to increase PtO<sub>2</sub>, measurement of perfusion could then be used to investigate the mechanism of actions of CO<sub>2</sub> insufflation.

Direct measurement of PtO<sub>2</sub> has the advantage of being able to measure the total effect of PtO<sub>2</sub> regardless of the mechanism of action and without additional confounding variables of indirect measurement. Of the methods for direct

measurement of  $PtO_2$ , both a polarographic oxygen sensor and a fluorescent optical sensor are able to be inserted into the tissue of choice to directly measure  $PtO_2$ . Polarographic sensors are more widely used in surgical research, perhaps because the available fluorescent oxygen sensor has been described as difficult to use (Akça, Sessler et al. 2006). A further method of direct measurement of  $PtO_2$  is using a transcutaneous polarographic sensor, which relies on the diffusion of  $O_2$  from the skin into the sensor. This transcutaneous measurement only measures  $PtO_2$  within the underlying skin and is not sterile. It is therefore not appropriate for intra-abdominal measurements. Therefore the current model utilised a tissue-embedded polarographic oxygen sensor to measure  $PtO_2$ .

**Table 4-5: Direct and in-direct methods for measuring tissue oxygen partial pressure**

Method	Physical Principle	Advantages	Disadvantages
Insertion of a polarographic oxygen probe into the tissue of interest.	A voltage is placed across an electrolyte fluid within an O <sub>2</sub> permeable membrane (silicon). Current within the electrolyte relates to PtO <sub>2</sub> .	<ul style="list-style-type: none"> <li>• Direct measure of PtO<sub>2</sub></li> <li>• Temperature compensation</li> <li>• Strongly correlated to the risk of surgical site infection (Hopf, Hunt et al. 1997)</li> </ul>	<ul style="list-style-type: none"> <li>• Invasive</li> <li>• Difficult to move to take measurements in other locations</li> </ul>
Fluorescent dye containing optical sensor inserted into the tissue of interest (Sheffield, Sessler et al. 1996; Hopf, Hunt et al. 1997)	Oxygen quenches fluorescent light emitted from the dye; the change in emission intensity is related to PtO <sub>2</sub> .	<ul style="list-style-type: none"> <li>• Direct measure of PtO<sub>2</sub></li> <li>• Temperature compensation</li> <li>• Comparable accuracy to a polarographic system (<math>r=0.92</math>) (Akça, Sessler et al. 2006)</li> </ul>	<ul style="list-style-type: none"> <li>• Invasive</li> <li>• Reported as difficult to use due to light sensitivity, motion artefact, &amp; large temperature sensor (Akça, Sessler et al. 2006).</li> </ul>
Transcutaneous tissue oxygen partial pressure sensor placed on the skin at the area of interest (Sheffield, Sessler et al. 1996)	O <sub>2</sub> within the underlying skin diffuses into a polarographic sensor.	<ul style="list-style-type: none"> <li>• Direct measure of underlying cutaneous PtO<sub>2</sub></li> <li>• Non-invasive</li> <li>• Temperature compensation</li> </ul>	<ul style="list-style-type: none"> <li>• Only for cutaneous PtO<sub>2</sub></li> <li>• Not related to sub-cutaneous PtO<sub>2</sub> (Sheffield, Sessler et al. 1996).</li> <li>• Does not create a local wound</li> <li>• Requires heating of the area</li> </ul>
Near infra-red spectroscopic (NIRS) imaging positioned over the tissue of interest (Singh, Stansby et al. 2009).	Change in reflectivity of infra-red light relates to the average percentage of haemoglobin saturated by oxygen in vessels within a volume of tissue.	<ul style="list-style-type: none"> <li>• Non-invasive</li> <li>• Easily repositioned to allow measurements at several sites</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect measure of PtO<sub>2</sub>.</li> <li>• CO<sub>2</sub> hypothesised to decrease haemoglobin O<sub>2</sub> saturation.</li> <li>• Weaker predictor of surgical site infection risk than PtO<sub>2</sub> (Ives, Harrison et al. 2007)</li> </ul>
Fluorescent imaging of an intra-vascular fluorescent dye injected in the systemically.	Measures perfusion by detecting presence of intra-vascular fluorescent dye.	<ul style="list-style-type: none"> <li>• Non-invasive</li> <li>• Investigates one of the mechanisms of action of CO<sub>2</sub></li> <li>• Measurement over large area</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect measure of PtO<sub>2</sub></li> <li>• Would not measure increase in haemoglobin O<sub>2</sub> release</li> </ul>

Method	Physical Principle	Advantages	Disadvantages
Laser Doppler flow meter positioned above the area of interest (Singh, Stansby et al. 2009)	Measures perfusion as movement of fluid (blood) by directing lasers and utilising the Doppler effect.	<ul style="list-style-type: none"> <li>• Non-invasive</li> <li>• Investigates one of the mechanisms of action of CO<sub>2</sub></li> <li>• Measurement over large area</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect measure of PtO<sub>2</sub></li> <li>• Would not measure increase in haemoglobin O<sub>2</sub> release</li> </ul>
Direct measurement of arterial oxygen partial pressure (P <sub>a</sub> O <sub>2</sub> )	Measurement of oxygen partial pressure either continuously or in arterial blood samples	<ul style="list-style-type: none"> <li>• Commonly measured in clinical practise and therefore easily translated to humans.</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect measure of PtO<sub>2</sub>.</li> <li>• P<sub>a</sub>O<sub>2</sub> and PtO<sub>2</sub> are not related linearly as PtO<sub>2</sub> also depends on tissue perfusion</li> </ul>
Arterial haemoglobin oxygen saturation (SpO <sub>2</sub> )	Change in transmitted light relates to the arterial percentage of haemoglobin saturated by oxygen.	<ul style="list-style-type: none"> <li>• Routinely used in clinical practise and therefore easily translated to humans.</li> </ul>	<ul style="list-style-type: none"> <li>• Indirect measure of PtO<sub>2</sub>.</li> <li>• CO<sub>2</sub> hypothesised to decrease haemoglobin O<sub>2</sub> saturation.</li> </ul>

#### 4.10.2 Licox™ CC1P1 polarographic oxygen probe

Licox™ polarographic oxygen probes are considered the gold standard for tissue oxygenation measurement (Govinda, Kasuya et al. 2010). Licox™ polarographic oxygen probes have been used in numerous clinical investigations with humans to measure the impact of different interventions on intra-operative and post-operative sub-cutaneous tissue oxygen partial pressure (Greif, Akça et al. 2000; Plattner, Akça et al. 2000; Akça, Doufas et al. 2002; Akca, Liem et al. 2003; Kabon, Nagele et al. 2004; Akça, Sessler et al. 2006; Hager, Reddy et al. 2006; Bakri, Nagem et al. 2008).

The Licox™ CC1P1 Combined Oxygen and Temperature Catheter Micro-Probe (Integra Lifesciences, Plainsboro, USA) (for brevity referred to simply as Licox™ CC1P1 oxygen probe) is a modern polarographic electrode that continuously measures  $PtO_2$  and tissue temperature, which is used to correct the  $PtO_2$  reading for temperature influences. The oxygen electrode within the Licox™ CC1P1 oxygen probe consists of a polarographic sensor in an electrolyte solution contained within an oxygen permeable silicon membrane. Oxygen diffuses through the silicon membrane. A voltage is placed across the electrolyte solution, and the electrical current generated is proportional to the  $PO_2$  of the surrounding tissue. The oxygen permeable silicon membrane allows measurement of average oxygen partial pressure over an area of tissue ( $18 \text{ mm}^2$ ) (GMS Advanced Tissue Monitoring 2010), rather than only at the tip of the sensor as was the case with earlier models. The probe is 1.3 mm in diameter.

#### 4.10.2.1 Justification for not using an additional silicon tonometer

The Licox™ probe is often placed within a custom made additional silicon tonometer (Hopf, Hunt et al. 2004). One of the reasons for the additional silicon tonometer is to contain both the PtO<sub>2</sub> and temperature probes within the same measurement area. However, the modern Licox™ CC1P1 oxygen probe has the advantage of combining oxygen and temperature into one probe. The additional silicon tonometer also allows easy removal and re-insertion of the probe to allow for multiple measurements to be made over several days without having to leave the probe in place (Hopf, Hunt et al. 2004). This reason is not relevant to the current model as the probe will only be in place duration surgery. Finally, the additional silicon tonometer has the potential to increase the sensing area of the probe, and is generally tunnelled within the tissue for 70 mm (Hopf, Hunt et al. 2004). Exposure of a 70 mm length of peritoneum is unrealistic for a rat model. The Licox™ CC1P1 oxygen probe used without an additional silicon tube needs to be inserted into the tissue only 30 mm. Therefore, the Licox™ CC1P1 oxygen probe was used without an additional silicon tonometer.

#### 4.10.3 Location and placement of the PtO<sub>2</sub> probe

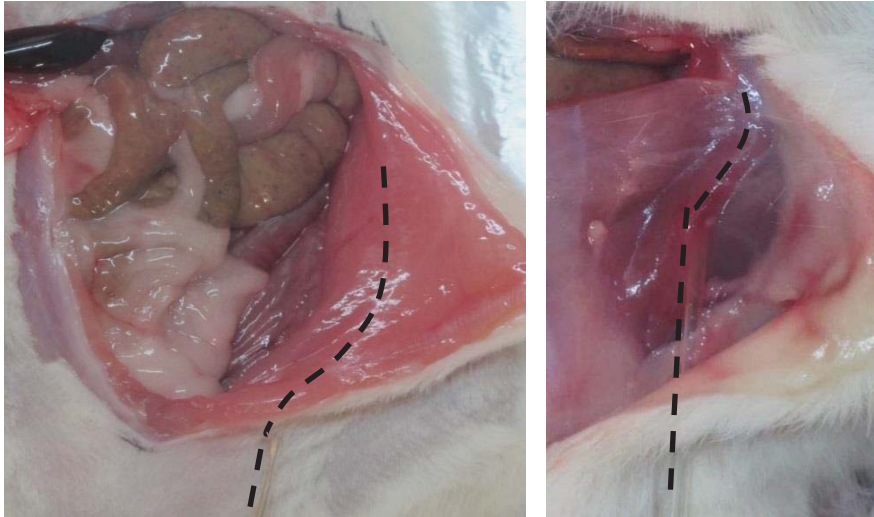
Of note, most human studies place a Licox™ probe in a surrogate wound in the upper arm (Chang, Goodson et al. 1983; Jonsson, Jensen et al. 1987; Hopf, Hunt et al. 1997; Akça, Melischek et al. 1999; Akça, Doufas et al. 2002; Akca, Liem et al. 2003; Akça, Sessler et al. 2006; Hager, Reddy et al. 2006). Three human studies took measurements directly parallel to the surgical incision (Plattner, Akça et al. 2000; Kabon, Nagele et al. 2004; Bakri, Nagem et al. 2008), but only post-operatively. The

current model required intra-operative measurements as the intervention is only administered during surgery. The invasive nature of sub-peritoneal measurement also meant that the measurement was most appropriately taken intra-operatively. In addition, intra-operative measurements allowed the use of a cross-over trial design that greatly reduced the required sample size. The decisive period for the development of surgical site infection is thought to be during surgery and the first few hours thereafter (Gottrup 2000), therefore intra-operative measurements are important. Measurement at a remote site such as the upper arm was not relevant to the current model as the interest in the direct local effect of CO<sub>2</sub>, rather than a systemic effect. Because CO<sub>2</sub> insufflation was hypothesised to increase PtO<sub>2</sub> by causing local vasodilation and shifting of the oxygen haemoglobin dissociation curve, it was necessary to measure local, sub-peritoneal, PtO<sub>2</sub>. Due to the small cross sectional area of the abdominal wall of the rat, it was not possible to place the probe in the wound edge. In the current model, the Licox™ CC1P1 oxygen probe was inserted from the lower left quadrant to the upper left quadrant of the abdominal wall, ensuring at least 30 mm of the distal end of the probe was embedded in the tissue, see Figure 4-13. A catheter with introducer was first inserted. The introducer was then removed and the Licox™ CC1P1 oxygen probe was inserted within the catheter. Finally the catheter was retracted so that only the Licox™ CC1P1 oxygen probe remained within the tissue.

#### 4.10.4 Summary: Measurement of tissue oxygen partial pressure (PtO<sub>2</sub>)

In the current model, PtO<sub>2</sub> was measured with a Licox™ CC1P1 Combined Oxygen and Temperature Catheter Micro-Probe (Integra Lifesciences, Plainsboro, USA). Following

laparotomy, the probe was tunnelled into the sub-peritoneal tissue from the left lower quadrant to the upper left quadrant, ensuring that at least 30 mm of the distal portion of the probe was embedded with the tissue.



**Figure 4-13: Position of the polarographic oxygen probe.**

The dotted line in both images indicates the approximate location of the probe. Left: The probe in place while the abdominal wall and spleen are retracted for surgical exposure. Note that the embedded portion of the probe is within tissue that is directly adjacent to the gaseous environment of the peritoneal cavity. Right: The abdominal wall has been reflected back over the abdominal contents and the skin has been resected to show that the probe was embedded in sub-peritoneal musculature of the abdominal wall.

## **4.11 Tissue fixation**

### **4.11.1 Tissue collection: Perfusion vs. immersion fixation**

Perfusion fixation is achieved by intra-vascular injection of tissue fixative into an anaesthetised animal, in which the heart has been arrested. This results in the delivery of fixative to all tissues by drainage through the vascular system. In some cases, this is followed by immersion of the whole animal in fixative prior to excision of tissue for analysis (Johnson, Lang et al. 1978). The advantage of perfusion fixation is that the tissue is fixed in-situ and therefore the effect of any trauma inflicted during excision is

minimised. Conversely, immersion fixation describes the excision of tissue prior to fixation, followed by placing the excised tissue into a bath of fixative. Immersion fixation is technically simpler than perfusion fixation and was therefore favoured in the current model. It also enables the use of more than one type of fixative, allowing appropriate fixation for multiple modes of analysis.

Early publications reported that perfusion fixation resulted in better preservation of the morphology of various rat tissue viewed under light microscopy, compared to immersion fixation (Johnson, Lang et al. 1978). However, more recently authors have reported that careful handling of peritoneal tissue prior to immersion fixation produces equally well preserved tissue for morphological analysis by scanning electron microscopy (von Ruhland, Newman et al. 2003). This finding is supported by another investigation that showed no difference in the ultrastructure of cardiac tissue viewed by scanning electron microscopy when fixed by either immersion or perfusion (Lenzi, Gesi et al. 1998).

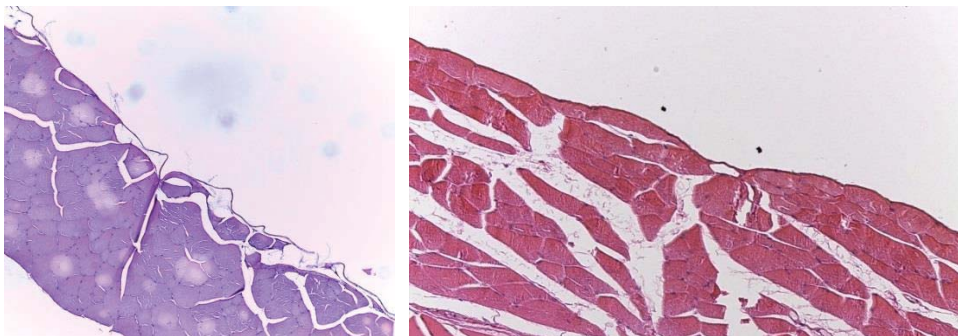
Despite evidence that immersion fixation is an effective fixative method, pilot work in the current model supported previous reports that immersion fixation results in rolling up of peritoneal tissue, distorting the tissue for analysis (von Ruhland, Newman et al. 2003). Further pilot work showed that tissue rolling was prevented by pre-fixing in-situ via an injection of fixative into the peritoneal cavity prior to immersion fixation, a method previously reported for peritoneal tissue (Hirabayashi, Yamaguchi et al. 2002). However in the current model, including a large laparotomy incision, pre-fixing in-situ resulted in fixation of blood contamination to the surface of some tissue samples that

obscured the mesothelium during examination. Another method to avoid tissue rolling is pinning of the tissue onto a piece of cork board (von Ruhland, Newman et al. 2003). This method allows contaminants to be rinsed from the tissue with a physiologic solution prior to immersion fixation. Therefore, in the current model the tissue was excised, pinned onto a piece of cork board, rinsed with a physiologic solution and fixed by immersion fixation.

#### 4.11.2 Choice of fixative

The type of fixative is especially important for fixing of the parietal peritoneum, due to reports of different shrinkage rates of the abdominal muscle fibres compared to the mesothelium, which can cause tissue damage during fixation (Liu, Li et al. 2001; Duman and Şen 2009). Following fixation in 4 % formaldehyde, such shrinking artefact was observed in the abdominal wall mesothelium, but not liver, omentum or intestine (Duman and Şen 2009). An alternative fixative to simple formaldehyde is Bouin's solution, which consists of approximately 10 % formaldehyde within a solution of picric acid and acetic acid to balance tissue shrinkage rates, and is recommended for delicate soft tissues (Ortiz-Hidalgo 1992). In comparison to 10 % formaldehyde, fixation with Bouin's solution results in less mesothelial cell loss, less tissue shrinkage and less loss of sub-mesothelial extracellular matrix (Liu, Li et al. 2001). Preliminary processing of tissue from the current model fixed in paraformaldehyde showed extensive loss of extracellular matrix and peeling of the mesothelium from the underlying connective tissue during sectioning, which was avoided with the use of Bouin's solution, see Figure 4-14.

While Bouin's solution is a suitable fixative for light microscopy, the vacuum employed in scanning electron microscopy requires a higher level of tissue fixation. The use of Osmium Tetroxide, which is a relatively toxic substance, has been superseded by glutaraldehyde with a 0.2 % solution producing good results for scanning electron microscopy examination of peritoneal tissue (von Ruhland, Newman et al. 2003). In order to optimise protection of the mesothelium during tissue fixation for scanning electron microscopy, the current model used Bouin's solution with the addition 0.2 % glutaraldehyde for all processing.



**Figure 4-14: Tissue shrinkage during fixation.**

**Left: Example of uneven shrinkage of muscle and peritoneal tissue in a section fixed in 4 % paraformaldehyde, resulting in the peritoneum bunching and peeling away from the underlying tissue. Right: Example of peritoneum adherent to the underlying tissue in a section of tissue fixed in Bouin's solution that allows for more even shrinkage of tissues during fixation.**

## 4.12 Tissue drying

### 4.12.1 Background: Tissue drying – Critical point drying versus hexamethyldisilazane (HMDS)

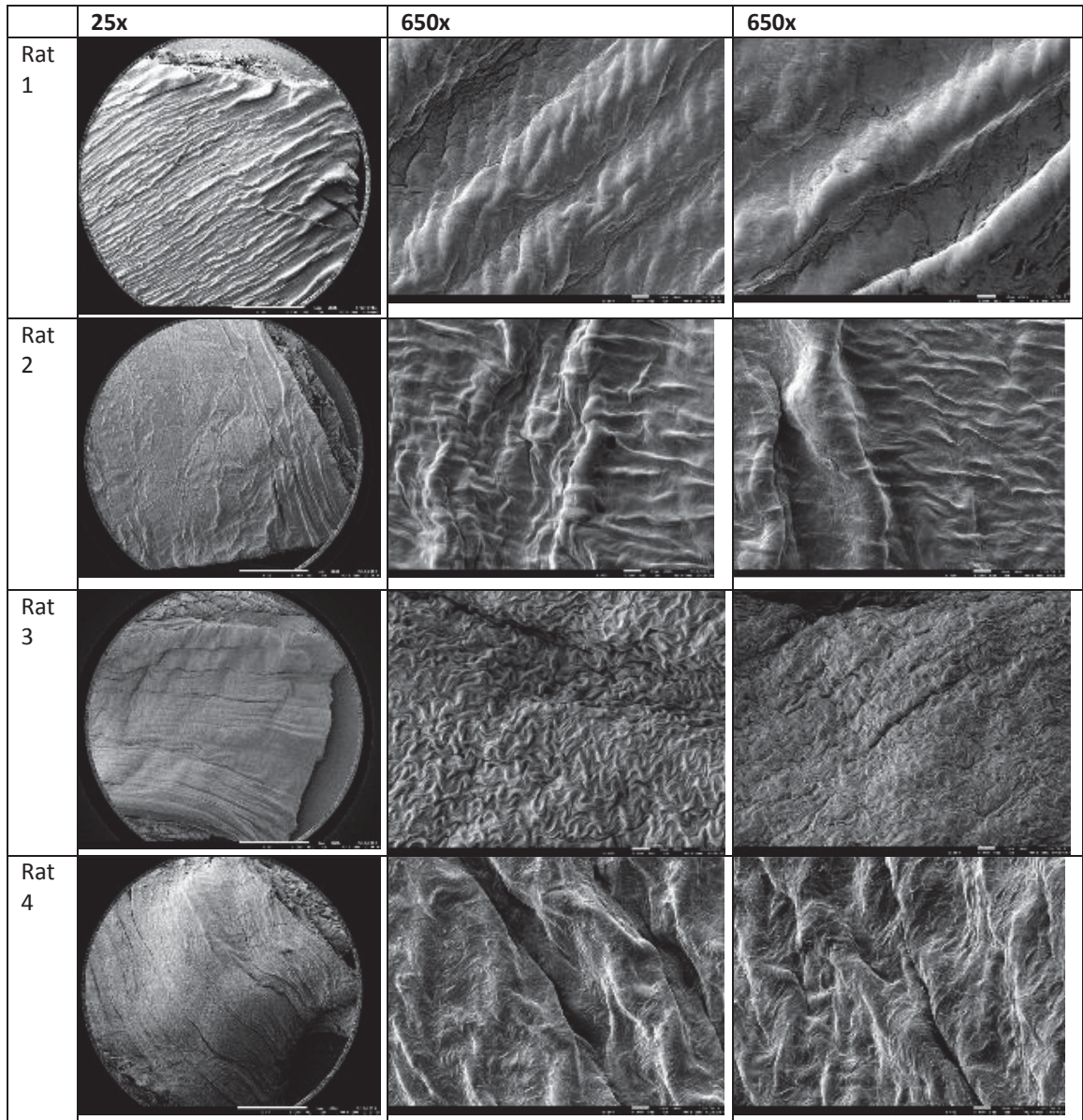
Scanning electron microscopy exposes the specimen to a vacuum in order to focus the electron beam. The vacuum will cause evaporation of water and distortion of wet samples. Therefore, it is essential that the specimen is dry to reduce tissue damage

within the vacuum. Unfortunately, air drying of tissue also causes distortion of the sample and so alternative methods of tissue drying have been developed. Critical point drying is the most common method of drying tissue for scanning electron microscopy. In this method, fluid within the specimen is replaced with liquid CO<sub>2</sub> during several washes. Finally the specimen, now immersed within liquid CO<sub>2</sub>, is passed through the critical point of CO<sub>2</sub>, releasing CO<sub>2</sub> as a gas and leaving a dry tissue specimen with less distortion than air drying. However, critical point drying is a laborious process that would take approximately 4 hours to process a maximum of 12 samples 5 x 5 mm. At the current research site, the critical point dryer was also housed at a different campus, limiting other work that can be done at the same time because the critical point dryer requires constant checking.

Drying the specimen with the solvent hexamethyldisilazane (HMDS) is a less common alternative to critical point drying that is much less labour intensive. As excess HMDS evaporates reduced surface tension and cross-linking of proteins by HMDS are believed to protect the sample compared with air drying without HMDS (Braet, De Zanger et al. 1997). Hexamethyldisilazane has been shown to protect the ultrastructure of biological samples equally as well as critical point drying in reports utilising tissues of comparable origin and dimension, and to higher resolution than required in the current study (Nation 1983; Bray, Bagu et al. 1993; Braet, De Zanger et al. 1997). Furthermore, although no comparison with critical point drying was made, HMDS has been used successfully in several investigations specifically of the

mesothelium using scanning electron microscopy (Hazebroek, Schreve et al. 2002; Ordemann, Jakob et al. 2004; Du, Yu et al. 2011).

In the current model, drying with HMDS was preferential to critical point drying as it is much less labour intensive. Based on promising published results with short duration HMDS treatment, preliminary tissue processing was carried out with a fast, 5 minute, HMDS drying protocol. However, significant tissue shrinkage was observed, as visualised by wrinkling and ridges along the length of the tissue, see Figure 4-15. As no comparison with tissue dried by critical point drying could be made, it was unclear if the shrinkage was also typical to critical point drying, or whether it was due to the use of HMDS. Furthermore it was possible that a slower dehydration protocol may reduce tissue shrinkage. Therefore, further investigation was required to determine whether the shrinking artefact following fast and slow HMDS dehydration was similar to that resulting from drying using a critical point dryer. In addition, labour could be further reduced by using an automated tissue processing machine to conduct the ethanol dehydration steps required prior to final drying, regardless of whether HMDS or critical point drying is used. However, it was possible that the automated tissue processing machine may damage the delicate mesothelial surface compared to delicately processing the tissue by hand. Therefore a further comparison was required to determine whether tissue drying artefact is the same following manual or automated ethanol dehydration.



**Figure 4-15: Scanning electron microscopy images taken following the fast HMDS protocol prior to the optimisation.**

All images show abdominal wall tissue fixed for 36 h in 4% PFA 0.3 % GA for 36 hr. Images show significant tissue shrinkage, visualised by wrinkling and ridges along the length of the tissue.

#### 4.12.2 Aim: Optimisation of tissue drying for scanning electron microscopy

- To investigate whether any shrinkage artefact caused by drying tissue samples by critical point drying is similar to shrinkage and cracking artefact caused by using fast and slow HMDS drying protocols that are much less labour intensive than critical point drying.
- To investigate whether cracking and mesothelial damage following manual ethanol dehydration is similar to cracking and mesothelial damage observed following automated ethanol dehydration in an automated tissue processing machine.

#### 4.12.3 Method: Optimisation of tissue drying for scanning electron microscopy

Abdominal wall tissue was collected from two rats that were euthanized for other purposes and whose peritoneum had not been surgically, or otherwise, manipulated. The tissue was fixed for 36 hours in 4% paraformaldehyde/0.3 % glutaraldehyde (note that this optimisation was carried out prior to the decision to use Bouin's fixative). A total of twelve 5 x 5 mm tissue samples from each of the two rats were dried for imaging. Three samples from each rat were used in the first comparison in which all samples were dried using manual ethanol dehydration. One sample from each rat was dried following a 'Fast' HMDS protocol, 'Slow' HMDS protocol or critical point drying, as described in Table 4-6.

The remaining nine samples from each rat were used in the second set of comparisons. Three samples from each rat were dried using either automated ethanol dehydration followed by the 'Slow' HMDS protocol, manual ethanol dehydration followed by the

‘Slow’ HMDS protocol, or manual ethanol dehydration followed by critical point drying. Following dehydration, samples were sputter coated with gold and observed by scanning electron microscopy. Representative photos were taken of each sample to allow subjective comparison of the degree of sample shrinking, as judged by wrinkling of the tissue, and tissue cracking.

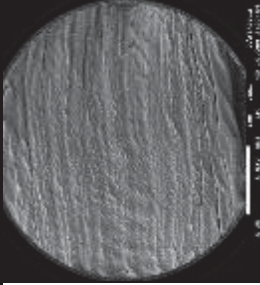
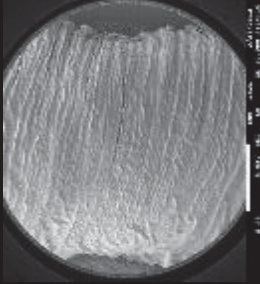
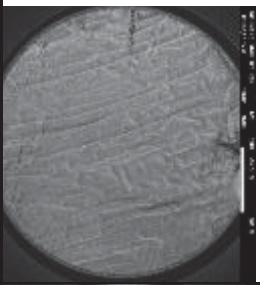
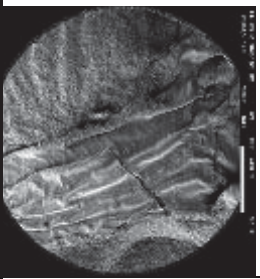
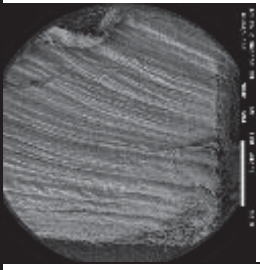
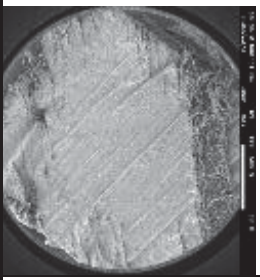
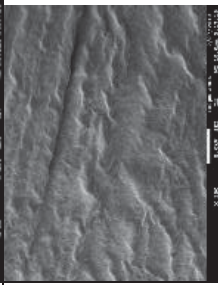
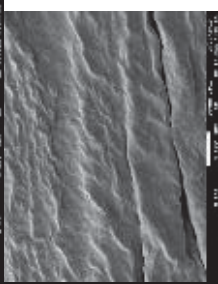
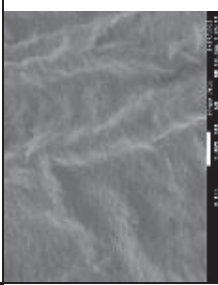
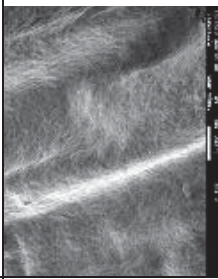
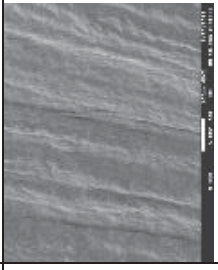
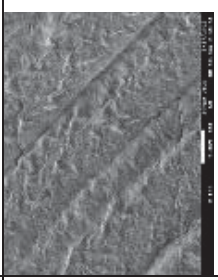
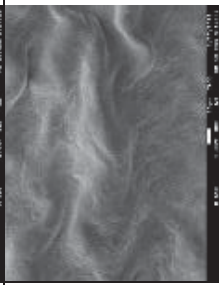
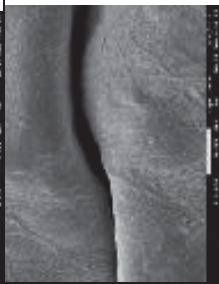
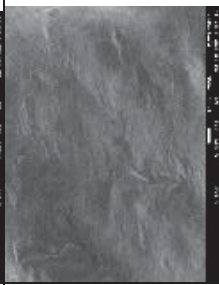
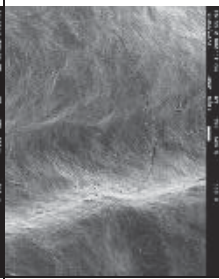
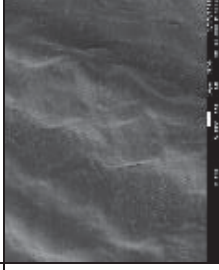
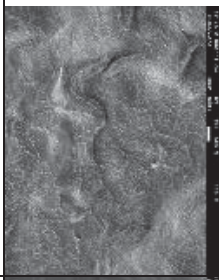
**Table 4-6: Detailed description of the tissue drying protocols**

‘Fast’ HMDS		‘Slow’ HMDS		Critical point drying	
Ethanol concentration (%)	Duration (min)	Ethanol concentration (%)	Duration (min)	Ethanol concentration (%)	Duration (min)
30	10	30	30	30	10
50	10	50	30	50	10
70	10	70	30	70	10
100	10	80	30	100	10
100	10	90	30	100	10
HMDS 100 %	5	100	30	Critical point drying	n/a
Air dry with desiccator overnight		100	30		
		HMDS 100 %	5		
		HMDS 100 %	5		
		HMDS 100 %	60		
		Air dry with desiccator overnight			

#### 4.12.4 Results: Optimisation of tissue drying for scanning electron microscopy

Regardless of drying by HMDS or critical point drying, all manually processed tissue samples showed obvious shrinkage, clearly seen by wrinkling and distortion of the rectangular shape of samples at 25 x magnification, see Figure 4-16. Despite this shrinkage, the mesothelium appeared to be intact in all six samples, supported by the visualisation of microvilli on the cellular surface at 2000x magnification and the presence of a continuous cobblestone appearance of cells. When manual ethanol dehydration was compared to automated ethanol dehydration, cracking of the surface of the tissue was common but occurred more often in tissue processed manually.

Cracking occurred in all the manually processed tissues prior to slow HMDS drying, 2 of the 6 tissue samples processed with the automated machine prior to slow HMDS drying, and 5 of the 6 tissue samples processed with manually ethanol dehydration prior to critical point drying, see Figure 4-17. Further comparison of drying artefact following manual and automated ethanol dehydration at 2000 x magnification showed microvilli on all tissue samples, except sample 1 manual ethanol dehydration and some areas of sample 5 automated ethanol dehydration, see Figure 4-18.

Mag	Fast HMDS		Slow HMDS		Critical Point Dried	
	Rat 1	Rat 2	Rat 1	Rat 2	Rat 1	Rat 2
25x						
150x						
650						

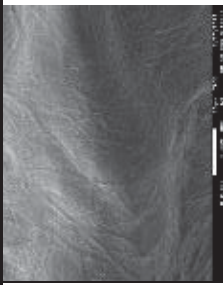
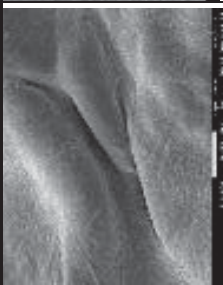
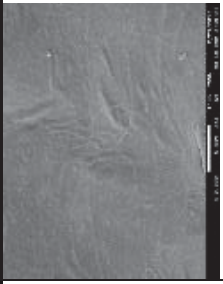
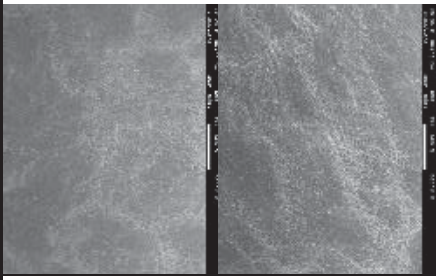
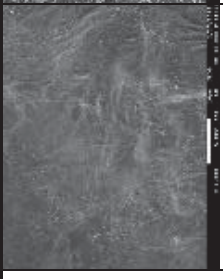
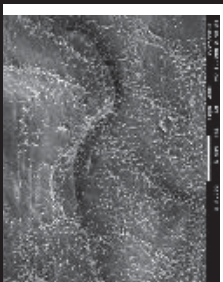
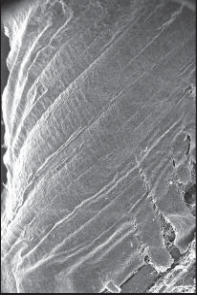
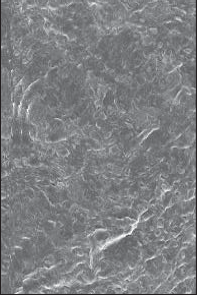
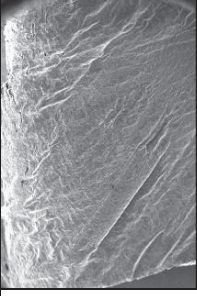

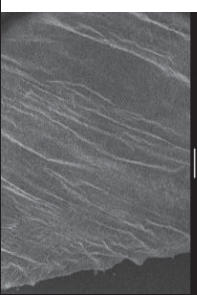
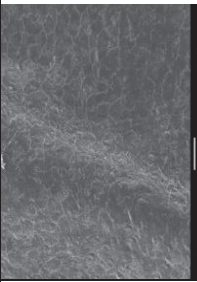
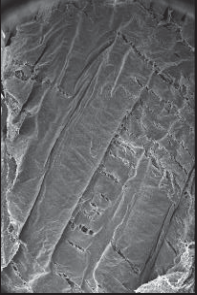
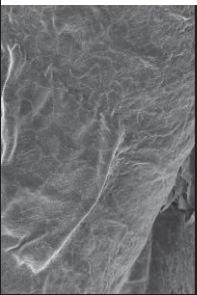
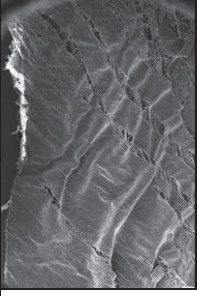
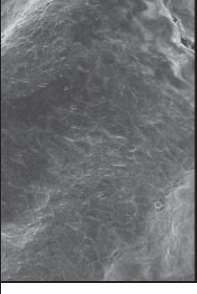
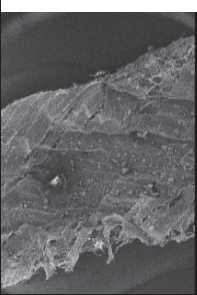
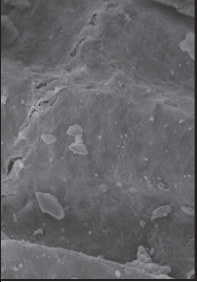
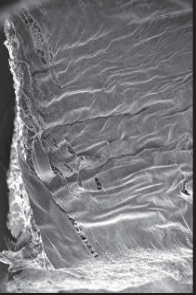
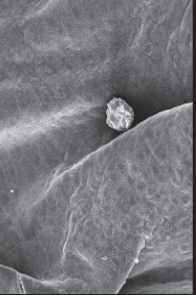
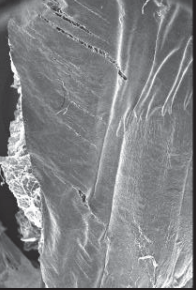
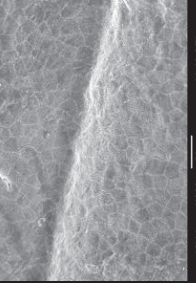
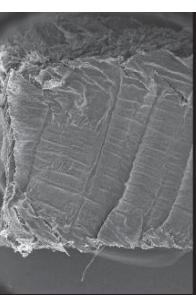
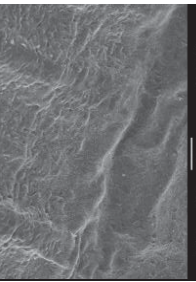

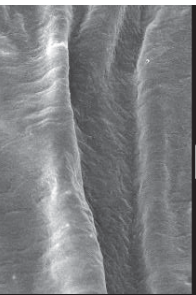
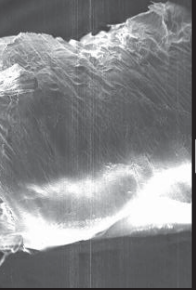

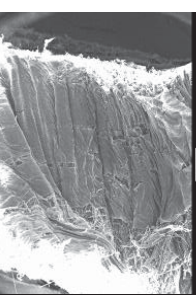

Mag	Fast HMDS		Slow HMDS		Critical Point Dried	
	Rat 1	Rat 2	Rat 1	Rat 2	Rat 1	Rat 2
2000						

Figure 4-16: Scanning electron microscopy images following tissue drying of samples one and two using each of the three drying protocols using manual ethanol dehydration.

#	Slow HMDS				Critical Point Dried			
	Manual Ethanol Dehydration		Automated Ethanol Dehydration		Manual Ethanol Dehydration		Automated Ethanol Dehydration	
	30 x	300 x	30 x	300 x	30 x	300 x	30 x	300 x
1								
2								
3								
4								


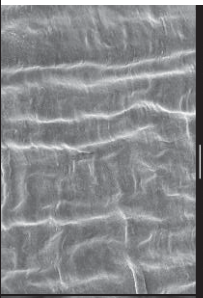
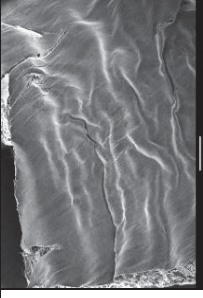
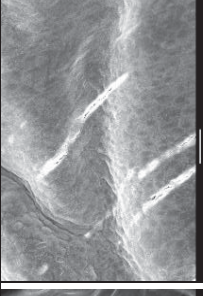
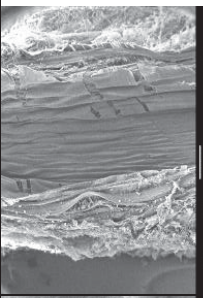
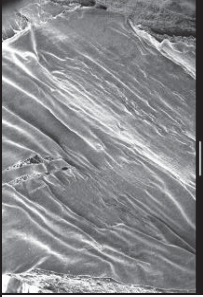
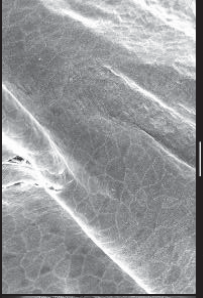
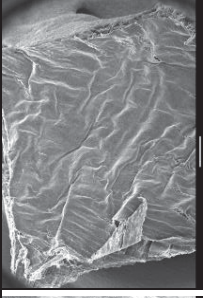
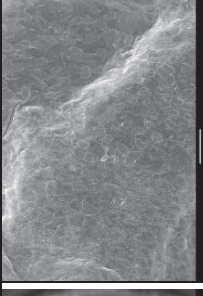
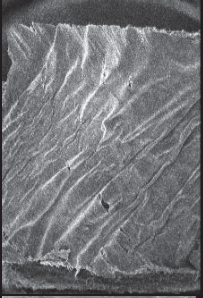
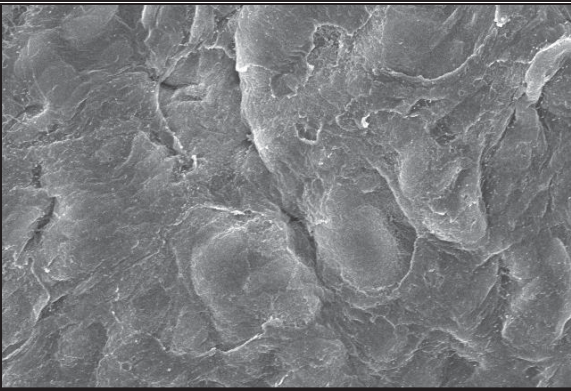
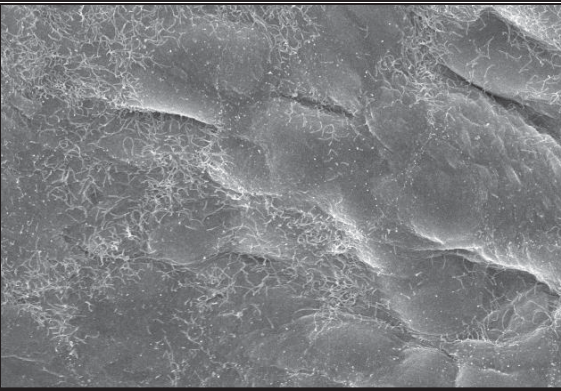
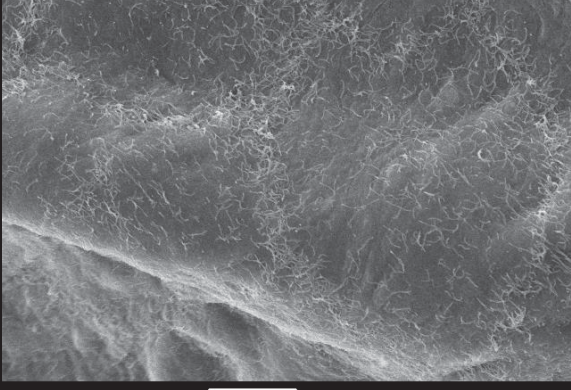
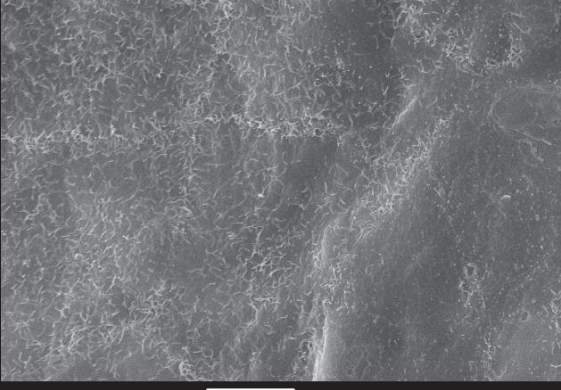
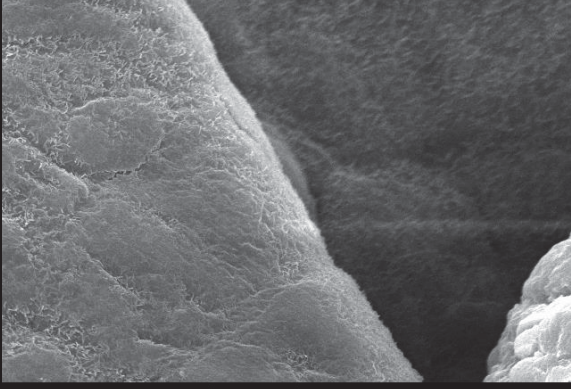

#	Slow HMDS				Critical Point Dried			
	Manual Ethanol Dehydration		Automated Ethanol Dehydration		Manual Ethanol Dehydration			
	30 x	300 x	30 x	300 x	30 x	300 x		
5								
6								

Figure 4-17: Scanning electron microscopy images following tissue drying of samples using a slow HMDS protocol with manual ethanol dehydration or a slow HMDS protocol with automated ethanol dehydration, or manual ethanol dehydration with critical point drying.

#	Slow HMDS	
	Manual Ethanol Dehydration	Automated Ethanol Dehydration
	2000 x	2000 x
1	 <p>15kV X2,000 10µm 11 40 SEI</p>	 <p>15kV X2,000 10µm 11 40 SEI</p>
2	 <p>15kV X2,000 10µm 13 40 SEI</p>	 <p>15kV X2,000 10µm 13 40 SEI</p>
3	 <p>15kV X2,000 10µm 11 40 SEI</p>	 <p>15kV X2,000 10µm 11 40 SEI</p>

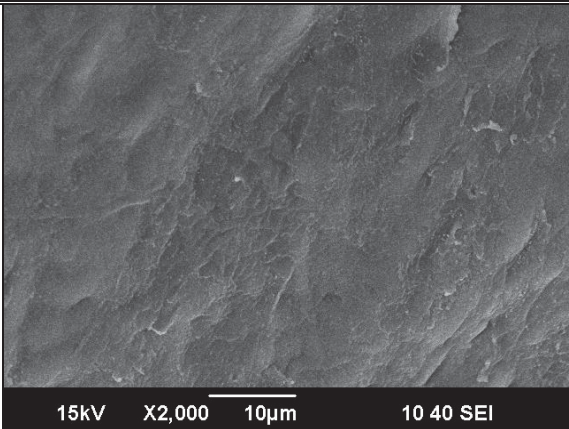
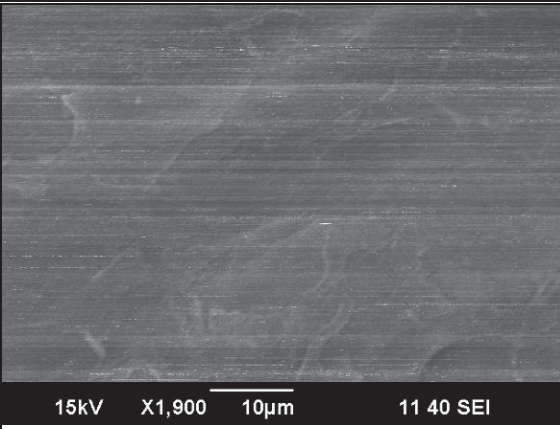
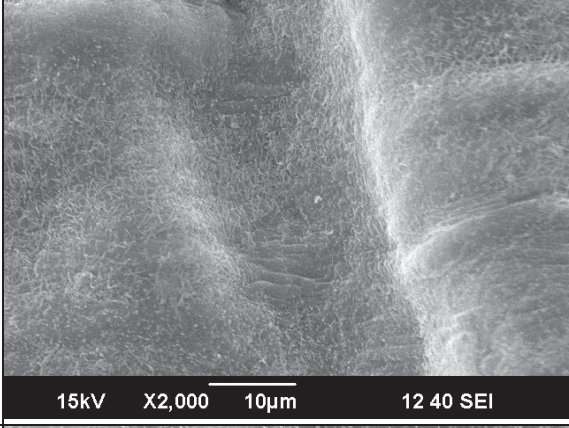
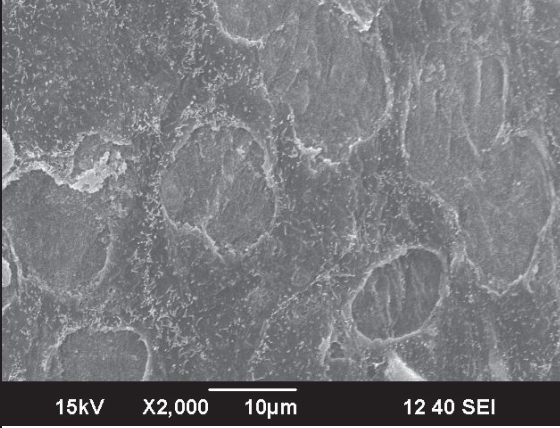
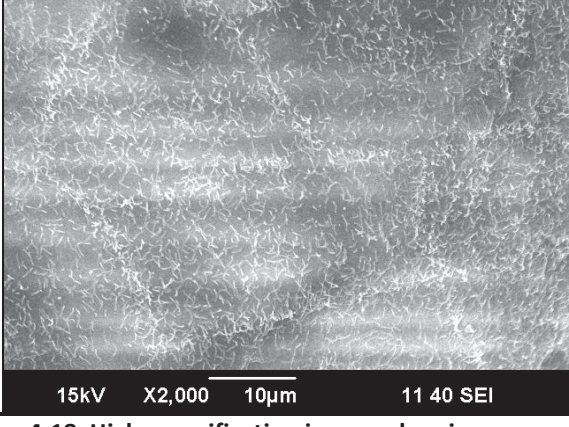
#	Slow HMDS	
	Manual Ethanol Dehydration	Automated Ethanol Dehydration
	2000 x	2000 x
4		
5		
6		

Figure 4-18: High magnification images showing presence of microvilli.

Microvilli are visible on all tissue surfaces except 'sample 1 manual ethanol dehydration' and some areas of 'sample 5 automated ethanol dehydration'. Note that sample 4, automated has electron charging and is therefore hard to analyse.

#### 4.12.5 Discussion: Optimisation of tissue drying for scanning electron microscopy

Tissue shrinkage occurred similarly in tissue processed using the slow or fast HMDS protocols or critical point drying. This supports previous publications that drying with HMDS is as effective as CPD drying (Nation 1983; Bray, Bagu et al. 1993; Braet, De Zanger et al. 1997) and that HMDS drying is suitable for imaging of mesothelium on the surface of peritoneal tissue samples (Hazebroek, Schreve et al. 2002; Ordemann, Jakob et al. 2004; Du, Yu et al. 2011). Despite the similar performance of HMDS and critical point drying, HMDS was considerably less labour intensive. Critical point drying took several additional hours. Some of the tissue shrinkage observed during this investigation of tissue drying may have occurred during tissue fixation and may be reduced by pinning the tissue down during fixation, as has been recommended for peritoneal tissue samples (von Ruhland, Newman et al. 2003).

Tissue cracking was more common when ethanol dehydration was performed manually than by an automated tissue processor. This was the opposite result to what was hypothesised and may reflect that more tissue handling is required when processing the tissue manually. The results suggest that the automated machine performs at least as well as manual processing, and should be adopted as it is labour saving.

#### 4.12.6 Conclusions: Optimisation of tissue drying for scanning electron microscopy

To optimise protection of the mesothelium during tissue processing, in the current model tissue drying by ethanol dehydration was carried out using an automated tissue processor. Tissue samples to be analysed by scanning electron microscopy were

dehydrated with graded ethanol steps of 30 minutes each (30, 50, 70, 80, 90, 100 x 2), followed by 100 % hexamethyldisilazane (5 minutes x 2 then 60 minutes), due to equally good results with less labour compared with critical point drying.

## 5 Effect of Humidified-Warm CO<sub>2</sub> Insufflation during Open Abdominal Surgery on Sub-peritoneal Tissue Oxygenation in Rats

### 5.1 Background

Maintenance of adequate tissue perfusion and oxygenation is a fundamental principle taught to surgeons in training (Taflampas, Christodoulakis et al. 2009; Davis and Rivadeneira 2013). Low tissue oxygenation is highly predictive of surgical site infection (Hopf, Hunt et al. 1997; Greif, Akça et al. 2000; Govinda, Kasuya et al. 2010), as tissue oxygen partial pressure (PtO<sub>2</sub>) drives the production of bactericidal superoxide by phagocytes (Babior 1978; Babior 1978; Allen, Maguire et al. 1997). Superoxide production is necessary for microbial killing via driving the release of protease stores and by direct oxidative killing (Reeves, Lu et al. 2002; Roos and Winterbourn 2002; Roos, Van Bruggen et al. 2003; Segal 2005). Tissue oxygen partial pressure predicts collagen deposition, and therefore wound strength (Jonsson, Jensen et al. 1986; Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992) and integrity or leakage of colonic anastomoses (Sheridan, Lowndes et al. 1987). Angiogenesis also depends on adequate oxygenation and is enhanced by high PtO<sub>2</sub> (Hunt, Gimbel et al. 2008). As such, increasing peri-operative PtO<sub>2</sub> is a common recommendation for the prevention of surgical complications (Sessler 2006; Hopf and Rollins 2007; Yoshida, Nabeshima et al. 2007; Hunt, Gimbel et al. 2008; Davis and Rivadeneira 2013).

Interventions to increase peri-operative  $PtO_2$  are traditionally applied systemically, usually under the control of the anaesthetist rather than the surgeon. These include reduction in sympathetic vasoconstriction by minimising pain (Akça, Melischek et al. 1999), cooling (Sheffield, Sessler et al. 1996), hypovolemia (Chang, Goodson Iii et al. 1983; Hartmann, Jonsson et al. 1992; Arkiliç, Taguchi et al. 2003) and nicotine (Jonsson, Jensen et al. 1987; Jensen, Goodson et al. 1991); and by the use of regional anaesthesia (Buggy, Doherty et al. 2002; Kabon, Fleischmann et al. 2003; Treschan, Taguchi et al. 2003). An increase in  $PtO_2$  can be achieved by systemically increasing arterial oxygen tension ( $P_aO_2$ ), by delivery of a high inspired fraction of oxygen in the inspired gas mix (Chang, Goodson Iii et al. 1983; Jonsson, Jensen et al. 1987; Greif, Akça et al. 2000; Kabon, Nagele et al. 2004). It is also possible to increase  $PtO_2$  by systemic hypercapnia, through a combination of increased cardiac output, vasodilation, and decrease in oxygen haemoglobin affinity (Akça, Doufas et al. 2002; Akca, Liem et al. 2003; Akça, Sessler et al. 2006; Hager, Reddy et al. 2006). However, to achieve increased  $PtO_2$  at the surgical site, where it is required to maximize surgical site healing and to minimize surgical site sepsis, these systemic treatments all critically depend on adequate surgical site tissue perfusion, and, in spite of these techniques, surgical site perfusion is frequently suboptimal (Jonsson, Jensen et al. 1987; Hartmann, Jonsson et al. 1992; Hopf, Hunt et al. 1997). Inadequate local wound perfusion likely explains why manipulation of systemic blood gases does not consistently reduce surgical site infection (Al-Niaimi and Safdar 2009; Hunt and Hopf 2009; Brar, Brar et al. 2011; Meyhoff, Jorgensen et al. 2011; Akça, Kurz et al. 2013; Wadhwa, Kabon et al. 2014). An intervention that can locally increase surgical site perfusion and  $PtO_2$  may be

clinically important, as it may be able to overcome poor perfusion that is often observed in surgical patients.

Insufflation of humidified-warm CO<sub>2</sub> into the abdominal cavity has been proposed as a therapy to locally increase surgical site tissue perfusion and PtO<sub>2</sub> during open abdominal surgery (Persson and van der Linden 2008). The CO<sub>2</sub> is heated to body temperature and humidified to avoid evaporative heat loss, as evaporative cooling contributes up to 50 % of heat loss during open abdominal surgery (Roe 1971). Using an active humidification system and a specially designed gas diffuser, humidified-warm CO<sub>2</sub> can be diffused into the open peritoneal cavity at a low velocity, while at a flow rate high enough to create a local environment with a high concentration of CO<sub>2</sub> (Persson and Van der Linden 2003; Svenarud, Persson et al. 2003; Svenarud, Persson et al. 2003; Persson, Svenarud et al. 2004). The therapy is applied locally to the surgical wound (Persson, Elmqvist et al. 2004), and thus may locally increase tissue perfusion.

Insufflation of CO<sub>2</sub> is hypothesised to have a direct vasodilatory effect on local tissue mimicking metabolic regulation of local blood flow and delivering more oxygenated blood to the tissue (Persson and van der Linden 2008). Furthermore, CO<sub>2</sub> causes a decrease in local pH that will likely increase PtO<sub>2</sub> via the Bohr Effect (Persson and van der Linden 2008). Both CO<sub>2</sub> and H<sup>+</sup> cause a right shift in the oxygen haemoglobin dissociation curve resulting in release of more oxygen from haemoglobin. Previous research shows that topical CO<sub>2</sub> increases skin blood flow (Diji 1959; Hartmann, Bassenge et al. 1997; Minamiyama and Yamamoto 2010) and PtO<sub>2</sub> (Hartmann, Bassenge et al. 1997). Intra-abdominal CO<sub>2</sub> in laparoscopy has been shown to increase

sub-peritoneal PtO<sub>2</sub> compared with open abdominal surgery in humans (Gianotti, Nespoli et al. 2011) and in a murine model (Bourdel, Matsuzaki et al. 2007). However, during laparoscopy, the increase in local PtO<sub>2</sub> by exposure to CO<sub>2</sub> is balanced by a relative decrease in local PtO<sub>2</sub> as intra-abdominal pressure increases (Bongard, Pianim et al. 1995; Bourdel, Matsuzaki et al. 2007; Matsuzaki, Jardon et al. 2010). During open abdominal surgery CO<sub>2</sub> is insufflated into the abdominal cavity at atmospheric pressure, and therefore may increase PtO<sub>2</sub> to a greater degree than laparoscopy. However, the effect of insufflation of CO<sub>2</sub> into the abdominal cavity during open abdominal surgery on PtO<sub>2</sub> has not yet been measured.

In addition to increased PtO<sub>2</sub> by exposure to CO<sub>2</sub>, delivery of the CO<sub>2</sub> humidified and warm is hypothesised to further elevate PtO<sub>2</sub> by reducing evaporative heat loss and thereby improving maintenance of tissue temperature. This may cause additional vasodilation and Bohr shift of the haemoglobin dissociation curve (Persson and van der Linden 2008). Previous research has shown that local warming of the skin significantly increases sub-cutaneous PtO<sub>2</sub> in healthy volunteers (Sheffield, Sessler et al. 1996; Ikeda, Tayefeh et al. 1998). Systemic warming can double peripheral capillary blood flow measured by laser Doppler (Sheffield, Sessler et al. 1996). Local heating can increase capillary blood flow by as much as 17-fold in the presence of peripheral vasoconstriction (Sheffield, Sessler et al. 1996). Intra-abdominal insufflation of humidified-warm CO<sub>2</sub> provides a mechanism by which the surgical site can be locally heated. Research has shown that intra-abdominal insufflation of humidified-warm CO<sub>2</sub> increases abdominal wound temperature during open colorectal surgery compared

with exposure to ambient air (Frey, Janson et al. 2012; Frey, Janson et al. 2012). Intra-abdominal insufflation of humidified-warm CO<sub>2</sub> increases local temperature; however, whether this increase in temperature leads to increased PtO<sub>2</sub> has not yet been measured.

Insufflation of humidified-warm CO<sub>2</sub> is a simple therapy with a strong theoretical basis by which it may achieve increased local PtO<sub>2</sub> at the surgical site. High PtO<sub>2</sub> is an outcome that is related to reduction in important surgical complications, however, increases in local PtO<sub>2</sub> are difficult to achieve with systemic treatments in poorly perfused surgical patients. This research was designed to test the hypothesis that insufflation of humidified-warm CO<sub>2</sub> into the open abdominal cavity during surgery will increase local PtO<sub>2</sub> due to a combination of exposure to CO<sub>2</sub>, as well as by reducing evaporative cooling in the abdominal cavity. It was hypothesised that insufflation of either humidified-warm CO<sub>2</sub>, or dry-cold CO<sub>2</sub>, into the abdominal cavity during open abdominal surgery would increase sub-peritoneal PtO<sub>2</sub> compared with laparotomy without gas insufflation. Furthermore, it was hypothesised that insufflation of humidified-warm CO<sub>2</sub> would increase sub-peritoneal PtO<sub>2</sub> compared with insufflation of dry-cold CO<sub>2</sub>.

## 5.2 Methods

### 5.2.1 Ethics and animal care:

Approval for this study was granted by the University of Wollongong Animal Ethics Committee (AE 10-24). Female Wistar rats were used and maintained in accordance

with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council 2013). Prior to surgery, the rats were housed two rats to a cage with ad-libitum access to food and water. The rats were maintained in a temperature controlled environment with diurnal variation of light. Immediately following the experiments, the rats were euthanized by carbon dioxide asphyxiation, while still anaesthetised.

#### 5.2.2 Induction and monitoring of general anaesthesia:

All rats were weighed and then pre-warmed in the animal handler's hands for 10 minutes immediately prior to induction of general anaesthesia, using a blanket and heating lamp. General anaesthesia was induced with inhalant isoflurane in air via a nose cone. Throughout anaesthesia the rats rested on a Small Animal Far Infra-Red Warming Pad (Kent Scientific Corporation, Connecticut, USA) to maintain body temperature. Following induction, the rats underwent endotracheal intubation with a 16 G blunted intravenous catheter during direct vision of the entrance to trachea, illuminated by a red LED shone through the skin of the throat. The endotracheal catheter was connected to a ventilator (Rodent Ventilator 7025, Ugo Basile, Varese, Italy) and ventilated with air at 350 ml/kg/min with a tidal volume of 1 ml, as pilot measurements showed that this minute ventilation maintained  $P_a\text{CO}_2$  in a normal range for all treatment groups (see section 4.7). The endotracheal catheter was secured around the snout of the rat and a small piece of gauze was placed in the mouth to collect oral secretions. Depth of anaesthesia was monitored via continuous measurement of heart rate and pulse pressure using a pressure transducer placed over

the skin of the neck in the area of the carotid artery, and also by oxygen haemoglobin saturation monitoring using a pulse oximeter (Powerlab, AD Instruments, Dunedin, New Zealand). Core body temperature was monitored every 5-10 minutes with a rectal thermometer (Surgipak Flexible Digital Thermometer, Vega Technologies Inc., Taipei, Taiwan). An insulating “sock” was placed over the rat’s tail to reduce radiated heat loss. A heat lamp was positioned approximately 25cm above the rat, and was only used if the rat’s temperature was falling to below 36 °C. Any use of the heat lamp was recorded. Ambient temperature and humidity of the operating room was recorded (HygroPalm22 Portable Humidity & Temperature Meter, Rotronic, Switzerland). Insensible fluid replacement was delivered hourly at 10 ml/kg/hr with sub-cutaneous warmed 0.9 % sodium chloride, according to Australian guidelines for the promotion of wellbeing of animals used for scientific purposes (National Health and Medical Research Council 2008).

#### 5.2.3 Surgical procedure:

Following endotracheal intubation, the abdomen was clipped with an electric hair clipper (Oster Golden A5 two speed, Model 5-50A, Sunbeam Products, Florida, USA). An inverted “L” shaped laparotomy incision was then made to create adequate exposure of the parietal peritoneum for insertion of the tissue oxygen partial pressure (PtO<sub>2</sub>) probe, see Figure 5-1. This consisted of a 60mm long midline laparotomy incision, starting approximately 10 mm caudal to the xiphoid process. A further 40 mm long perpendicular extension of that incision was then made across the left side of the abdominal wall, from the rostral end of the first incision. A small clamp (5mm wide)

was attached to the skin of the abdominal wall flap at the intersection point of the two incisions. The abdominal wall was then gently reflected towards the lower left quadrant so as to expose the parietal peritoneum. The skin was clamped so as to minimise tension on the peritoneum, see Figure 5-1. To further expose the parietal peritoneum, by way of relaxation of the left abdominal wall, the left hind leg was flexed and secured using tape across the foot.

#### 5.2.4 Measurement of tissue oxygen partial pressure:

PtO<sub>2</sub> was measured using a combined temperature and polarographic oxygen tension probe (Licox™ CC1P1, Gesellschaft für Medizinische Sondensysteme, GmbH, Kiel, Germany). Each probe is calibrated by the manufacturer and is supplied with an individual calibration card that is inserted into the monitor prior to use. The accuracy of the probe reported on the manufacturer's user instructions is  $\pm 10\%$  for PtO<sub>2</sub> and  $\pm 0.2$  for temperature. To position the PtO<sub>2</sub> probe a 16G intra venous catheter was tunnelled beneath the peritoneal membrane from the lower left quadrant to the upper left quadrant under direct vision. The needle was then removed and the PtO<sub>2</sub> probe was inserted into the catheter. Finally, the catheter was gently retracted so that a minimum of 30 mm length of probe remained within the tissue, ensuring that the measurement portion of the probe is fully embedded, see Figure 5-1. Dissection conducted during pilot investigations showed that the probe is embedded in the muscle of the abdominal wall. The PtO<sub>2</sub> probe was then connected to a Licox™ CMP Oxygen and Temperature Monitor (Gesellschaft für Medizinische Sondensysteme,

GmbH, Kiel, Germany) to allow continuous recording of  $PtO_2$  and tissue temperature throughout the experiment.

#### 5.2.5 Experimental design:

Three sets of experiments were conducted, all in a randomised cross-over design. The first treatment was randomised and then treatment was alternated so that each rat received both treatments at least twice.

- Experiment 1: 5 rats alternatively exposed to humidified-warm  $CO_2$  insufflation or ambient air
- Experiment 2: 7 rats alternatively exposed to humidified-warm  $CO_2$  insufflation or dry-cold  $CO_2$  insufflation
- Experiment 3: 3 rats alternatively exposed to dry-cold  $CO_2$  insufflation or ambient air

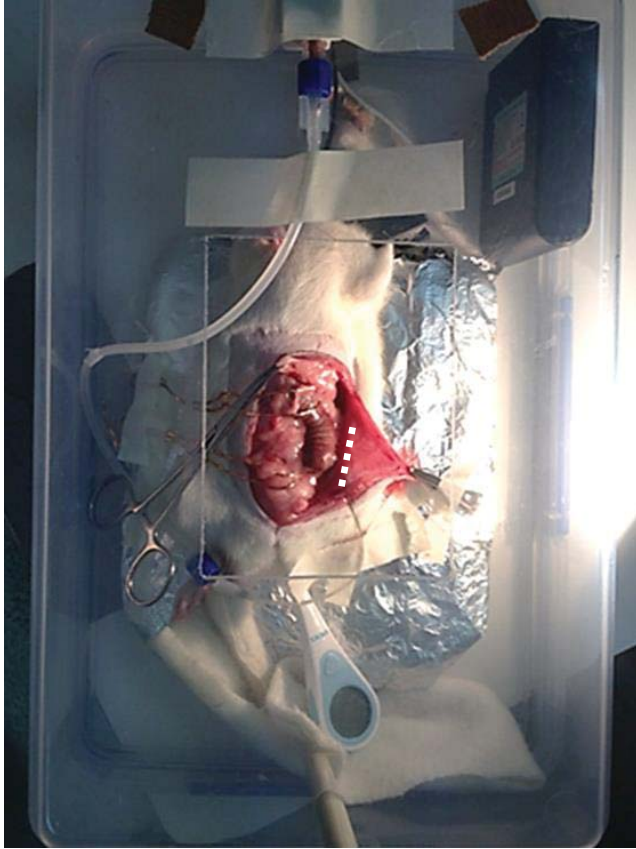
#### 5.2.6 Treatment conditions:

Preliminary testing showed that the abdominal cavity of a rat is too shallow to create a local environment of high  $CO_2$  gas concentration. This is due to the relatively thin abdominal wall of the rat compared with humans, and also to the minimal use of retraction in this model in order to protect the peritoneum from mechanical trauma. To ensure the abdomen was exposed to a high concentration of  $CO_2$ , the rat was placed in a plastic container with an 8 x 10 cm hole in the top through which the  $CO_2$  is insufflated, see Figure 5-1.  $CO_2$  being heavier than air, air was then displaced from the container by the  $CO_2$  and the abdomen was in a stable, high  $CO_2$  concentration

environment. The CO<sub>2</sub> was insufflated into the container at 9 L/min via a gas diffuser (VitaDiffuser, Cardia Innovation AB, Sweden) that ensures the gas enters the container at a low velocity, thereby reducing turbulence and allowing high CO<sub>2</sub> gas concentrations within the container. Pilot measurements of CO<sub>2</sub> concentration using a CheckMate II gas analyser (PBI Dansensor, Denmark) showed that the environment within the box is maintained at > 90 % CO<sub>2</sub>. In the humidified-warm CO<sub>2</sub> conditions, the CO<sub>2</sub> was humidified and warmed using a humidifier controller and heated delivery tube (HumiGard, Fisher and Paykel Healthcare, New Zealand). Independent testing has shown that the humidifier delivers > 98.0% relative humidity at 37 °C (Sammour, Kahokehr et al. 2010).

#### 5.2.7 Data analysis:

PtO<sub>2</sub> and tissue temperature were averaged over the last minute for each treatment condition. Each treatment was paired with the alternate treatment (the first trial with the second, third with the fourth etc.) to give a change in PtO<sub>2</sub> and tissue temperature for each cross-over trial. The Shapiro-Wilk test was used to check the normality of the data set for each experiment. When the assumption of normality was satisfied, a paired student t-test was conducted to test whether the mean change between paired treatment conditions differed from zero. When the assumption of normality was not satisfied, a Hodges-Lehman median difference and Wilcoxon Signed Rank test was used. P-value <0.05 was considered statistically significant for all tests. No adjustments were made for multiple comparisons.



**Figure 5-1: Surgical set-up.**

To ensure adequate exposure of the parietal peritoneum for placement of the PtO<sub>2</sub> probe, an inverted "L" shaped laparotomy incision was performed. The dashed line indicates the position of the PtO<sub>2</sub> probe. To facilitate insufflation of CO<sub>2</sub>, the rat is placed in a plastic container. The container has a hole at the top to allow gas insufflation. This set-up ensures a high CO<sub>2</sub> concentration.

## 5.3 Results

### 5.3.1 Animals and arterial oxygen saturation

The average weight of the rats was 294 g (SD 27), and did not differ between experiments ( $p = 0.34$ ). There was no difference in arterial haemoglobin oxygen saturation during  $PtO_2$  data collection between conditions in any of the experiments (Table 5-1).

### 5.3.2 Sub-peritoneal tissue oxygenation partial pressure ( $PtO_2$ ) and tissue temperature

Upon insufflation of humidified-warm  $CO_2$ , both  $PtO_2$  and tissue temperature increased almost immediately following the start of each period of gas insufflation, and was reversed each time gas insufflation was stopped (Table 5-2). Mean sub-peritoneal  $PtO_2$  increased by 29.8 mmHg (SD 13.3,  $p < 0.001$ ), or 96.6 % (SD 51.9), and tissue temperature by 3.0 °C (SD 1.7,  $p < 0.001$ ) compared with exposure to ambient air experiment 1) (Table 5-2 and Figure 5-3).

Experiment 2 showed an increase in  $PtO_2$  of 10.3 mmHg (SD 5.1,  $p < 0.001$ ), or 32.7 % (SD 16.6), upon the delivery of humidity and warmth, in the constant presence of  $CO_2$  insufflation. Experiment 3 showed an increase in  $PtO_2$  of 14.1 (SD 7.2,  $p = 0.005$ ), or 50.7 % (SD 37.9), upon insufflation of dry-cold  $CO_2$  compared with exposure to ambient air.

With a similar pattern to  $PtO_2$ , tissue temperature increased upon the addition of humidity and warmth by 4.7 °C (SD 1.9,  $p < 0.001$ ) in the constant presence of  $CO_2$

insufflation (experiment 2). However, in contrast to  $PtO_2$ , tissue temperature decreased by 1.4 °C (SD 0.5,  $p = 0.001$ ) during insufflation of dry-cold  $CO_2$  compared with exposure to ambient air (experiment 3).

### 5.3.3 Body temperature maintenance

There was no significant difference in the average heat pad setting between conditions in any of the experiments (Table 5-2). The heat lamp was normally not required to maintain normothermia, but was used half as often in the conditions in which humidity and warmth were delivered. There were small increases in rectal temperature following insufflation of humidified-warm  $CO_2$  of 0.7 °C (SD 0.7,  $p = 0.001$ ) when compared with exposure to ambient air (experiment 1), and 0.9 °C (SD 0.5,  $p < 0.001$ ) when compared with exposure to dry-cold  $CO_2$  (experiment 2) (Table 5-2 and Figure 5-3). There was no significant change in rectal temperature during insufflation of dry-cold  $CO_2$  compared with exposure to ambient air (experiment 3).

**Table 5-1: Summary of animal weights, arterial oxygen saturation, and room and rectal temperatures**

Variable	Experiment 1			Experiment 2			Experiment 3		
	Humidified - warm CO <sub>2</sub>	Ambient air	p	Humidified - warm CO <sub>2</sub>	Dry - cold CO <sub>2</sub>	p	Dry - cold CO <sub>2</sub>	Ambient air	p
Arterial O <sub>2</sub> saturation (%)	90 (6)	92 (7)	0.48	89 (5)	90 (5)	0.510	85 (2)	88 (2)	0.46
Room temp (°C)	22.6 #	-	-	23.5 (0.4)	-	0.16 <sup>^</sup>	24.3 (1)	-	0.16 <sup>^</sup>
Room humidity (%)	21.1 #	-	-	57.0 (3.8)	-	0.73 <sup>^</sup>	55.9 (5)	-	0.73 <sup>^</sup>
Average heat pad setting (maximum 5)	4.5 (1)	4.8 (0.6)	0.16	4.2 (1.2)	4.6 (0.8)	0.05	4.8 (0.5)	4.7 (0.4)	0.80
Number of trials with heat lamp on *	2/16	4/16	-	1/20	3/20	-	2/8	2/8	-
Body temperature at end of intervention (°C)	38.0 (0.7)	37.3 (0.5)	0.001	37.8 (0.5)	37.0 (0.5)	<0.001	37.1 (0.6)	37.0 (0.6)	0.60
Change in rectal temperature (°C)	0.7 (0.7)	-	0.001	0.9 (0.5)	-	0.6	0.1 (0.6)	-	0.60

All results show mean (standard deviation). All p-values are the result of the intervention vs. control within that experiment, unless otherwise stated.

# No standard deviation as temperature and humidity was only collected on one experimental day.

<sup>^</sup> Experiment 2 vs experiment 3 (unpaired t-test)

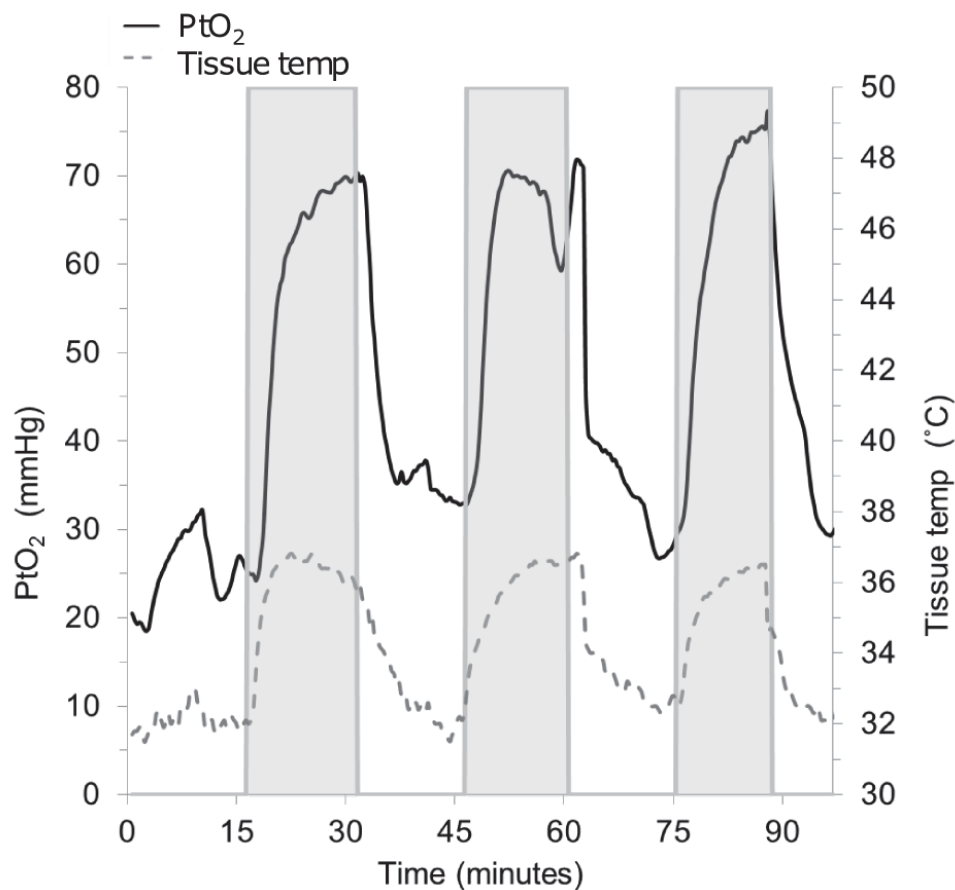
\* Number of trials in which the heat lamp was on in the last 10 minutes of the trial, the period of time immediately prior to collection of the tissue oxygenation data that was used for analysis. Expressed as a fraction of the total number of trials of that treatment condition in the experiment.

Table 5-2: Tissue oxygen tension (PtO<sub>2</sub>) and tissue temperature results for each experiment.

Experiment	Intervention	Control	Number of rats	Number of trials	PtO <sub>2</sub>			Tissue Temperature		
					Mean Control (mmHg)	Mean change with intervention (mmHg)	p	Mean Control (°C)	Mean change with intervention (°C)	p
1	Humidified - warm CO <sub>2</sub>	Ambient air	5	13	33.2 (8.2)	29.8 (13.3)	<0.001	34.0 (1.6)	3.0 (1.7)	<0.001
2	Humidified - warm CO <sub>2</sub>	Dry - cold CO <sub>2</sub>	7	20	33.3 (7.2)	10.3 (5.1)	<0.001*	31.4 (1.6)	4.7 (1.9)	<0.001
3	Dry - cold CO <sub>2</sub>	Ambient air	3	7	28.0 (7.2)	14.1 (7.2)	0.005	32.1 (1.4)	-1.4 (0.5)	0.001

All results show 'mean (standard deviation)' unless stated

\* Related-samples Hodges-Lehman median difference shown, and Wilcoxon matched pair signed rank test used as assumption of normality was not satisfied.



**Figure 5-2: Representative data from one rat in experiment 1 (Insufflation of humidified-warm CO<sub>2</sub> vs exposure to ambient air).**

The shaded areas show each period of gas insufflation. A rapid increase in both PtO<sub>2</sub> and tissue temperature is seen each time gas insufflation is started and is reversed when insufflation is stopped.

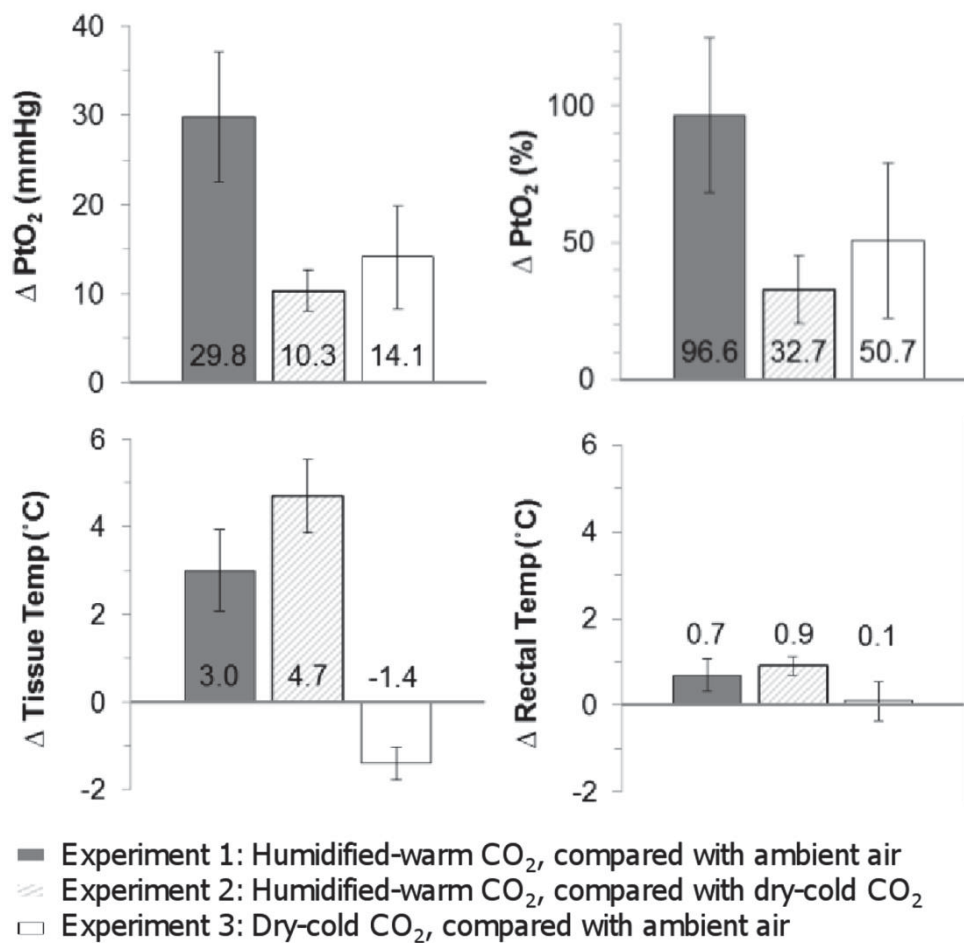


Figure 5-3: Change in  $\text{PtO}_2$ , tissue temperature and rectal temperature affected by the intervention condition of each experiment.

Error bars show 95 % confidence intervals. Where error bars do not cross zero, the intervention had a statistically significant effect compared with the control condition.

## 5.4 Discussion

This research was designed to test the hypothesis that insufflation of humidified-warm  $\text{CO}_2$  into the open abdominal cavity during surgery will increase local  $\text{PtO}_2$  by a combination of the delivery of  $\text{CO}_2$  and reducing evaporative cooling in the abdominal cavity. The first set of randomised cross-over trials showed that insufflation of humidified-warm  $\text{CO}_2$  caused an immediate and significant increase in  $\text{PtO}_2$  compared with exposure to ambient air with an average increase of 29.8 mmHg or 96.6 %. Two

subsequent sets of randomised cross-over trials showed that exposure to CO<sub>2</sub> and exposure to humidity/warmth, individually increased PtO<sub>2</sub> and these effects were additive. Furthermore, the increase in PtO<sub>2</sub> upon the delivery of humidity/warmth may be explained by a concomitant increase in tissue temperature, in contrast to the drop in tissue temperature observed when dry-cold CO<sub>2</sub> was insufflated in comparison with ambient air. This is the first known report of the effect on PtO<sub>2</sub> of CO<sub>2</sub> insufflation during open abdominal surgery.

The observed increase in PtO<sub>2</sub> upon exposure to CO<sub>2</sub> is consistent with previous reports of topical CO<sub>2</sub> exposure; however, there have been disparate reports of the magnitude of the effect. In a report of PtO<sub>2</sub> in patients randomised to either open or laparoscopic colectomy, dry-cold CO<sub>2</sub> pneumoperitoneum resulted in 10 mmHg higher PtO<sub>2</sub> measured in the colon wall (Gianotti, Nespoli et al. 2011). This increase was observed both in comparison with within subject control measurements, taken immediately following cessation of CO<sub>2</sub> pneumoperitoneum, and compared with patients randomised to open colectomy (Gianotti, Nespoli et al. 2011). A similar increase in foot sub-cutaneous PtO<sub>2</sub> was observed when patients with intermittent claudication immersed their foot in CO<sub>2</sub> enriched warm water (Hartmann, Bassenge et al. 1997). Compared with immersion in fresh, warm water alone, PtO<sub>2</sub> increased by 14 mmHg (Hartmann, Bassenge et al. 1997). A much larger increase was observed in a mouse study that showed dry-cold CO<sub>2</sub> pneumoperitoneum increased retro-peritoneal PtO<sub>2</sub> by 54 mmHg compared with laparotomy (Bourdel, Matsuzaki et al. 2007). The differing magnitude of increase in PtO<sub>2</sub> upon exposure to CO<sub>2</sub> may be attributed to

varied placement of the tissue oxygenation probe relative to local blood supply. Importantly, each of these studies utilised within subject comparison that allows control over differences in vascularity between subjects. Furthermore, variation may be due to differences in the relative permeability of different tissues to CO<sub>2</sub>, differences in the pressure at which the CO<sub>2</sub> was insufflated, and the magnitude of concomitant humidity/warmth delivered.

The enhanced maintenance of tissue and core body temperature observed upon exposure to humidified-warm CO<sub>2</sub> (3 °C and 0.7 °C, respectively, compared with ambient air) is consistent with previous reports in human trials (Frey, Janson et al. 2012; Frey, Janson et al. 2012). In a report of 79 patients randomised to CO<sub>2</sub> insufflation or no insufflation during open colo-rectal surgery, insufflation of CO<sub>2</sub> warmed to 37 °C and fully saturated with water vapour increased average abdominal wound temperature by approximately 1.7 °C and core temperature by 0.6 °C (Frey, Janson et al. 2012). Equally, the decrease in local tissue temperature of 1.4 °C observed upon exposure to dry-cold CO<sub>2</sub> is also consistent with published human results. In a randomised cross-over trial in open cardiac surgery, insufflation of dry-cold CO<sub>2</sub> caused a decrease in local tissue temperature of 1.8 °C (Frey, Sveiby et al. 2010).

The 30 mmHg increase in PtO<sub>2</sub> observed upon insufflation of humidified-warm CO<sub>2</sub> is higher than the threshold of 15-25 mmHg that is widely considered as clinically significant (Hopf, Hunt et al. 1997; Bakri, Nagem et al. 2008; Akça, Kurz et al. 2013). A 25 mmHg increase in PtO<sub>2</sub> has been shown to predict a 30 % drop in surgical site infection rate (Hopf, Hunt et al. 1997). Furthermore, a 40-50 % fall in PtO<sub>2</sub> is highly

predictive of anastomotic leakage (Sheridan, Lowndes et al. 1987), suggesting that the 97 % increase in  $PtO_2$  measured upon insufflation of humidified-warm  $CO_2$  may have a clinically important positive impact of anastomotic healing. Importantly, the results suggest that this local (surgical field) therapy may be at least as effective at increasing  $PtO_2$  during the operative period as systemically applied intraoperative therapies. An increase in intra-operative  $P_aO_2$  from 150 to 300 mmHg increases  $PtO_2$  by approximately 19-20 mmHg in non-obese patients (Kabon, Nagele et al. 2004). The increase in  $PtO_2$  drops to 11 mmHg in obese patients, likely due to reduced perfusion related to adiposity (Kabon, Nagele et al. 2004). Manipulation of end tidal  $CO_2$  from 30 to 45 mmHg increases  $PtO_2$  by 12-16 mmHg (Akça, Doufas et al. 2002; Akca, Liem et al. 2003). The observed effect of humidified-warm  $CO_2$  on  $PtO_2$  is twice that of both obesity (Fleischmann, Kurz et al. 2005) and aggressive fluid management (Arkiliç, Taguchi et al. 2003). Furthermore, the effect of humidified-warm  $CO_2$  insufflation is more than twice the increase achieved with the use of thoracic epidural anaesthesia (Buggy, Doherty et al. 2002; Kabon, Fleischmann et al. 2003; Treschan, Taguchi et al. 2003), which has been reported to halve the risk of surgical site infection (Chang, Lin et al. 2010). Similar to  $CO_2$  insufflation, thoracic epidural increases  $PtO_2$  by reducing sympathetic vasoconstriction, however, the increase in  $PtO_2$  with thoracic epidural only became significant after 60 minutes (Kabon, Fleischmann et al. 2003), whereas insufflation of humidified-warm  $CO_2$  had an immediate effect.

While intra-abdominal insufflation of humidified-warm  $CO_2$  during open abdominal surgery may cause an increase in  $PtO_2$  of a similar magnitude to systemic therapies,

the innovation of intra-abdominal insufflation of humidified-warm CO<sub>2</sub> is that it achieves a local increase in PtO<sub>2</sub> that may be able to overcome limitations of systemic therapies. The results of the current study suggest that a local effect was achieved, as the change in tissue temperature in the surgical site (sub-peritoneal in the abdominal wall) was at least 4-fold greater than the observed change in rectal temperature.

It is likely that the insufflation of humidified-warm CO<sub>2</sub> locally increases tissue oxygenation by mimicking normal metabolic regulation of oxygen delivery. This is most likely a combination of both a local increase in micro-perfusion through vasodilation, and a decrease in oxygen haemoglobin affinity. Increase in perfusion has previously been measured in response to topical CO<sub>2</sub> (Diji 1959; Hartmann, Bassenge et al. 1997; Minamiyama and Yamamoto 2010) and local heating (Sheffield, Sessler et al. 1996), and has been reported with concomitant increase in PtO<sub>2</sub> (Sheffield, Sessler et al. 1996; Hartmann, Bassenge et al. 1997). Delivery of oxygen to wounds is critically dependent on micro-perfusion to the surgical site. Oxygen dissipates radially from the vasculature to the tissue and PtO<sub>2</sub> can drop rapidly just 20 µm from the vessel wall (Tsai, Johnson et al. 2003). Micro-perfusion is dramatically altered by vasoconstriction and by arteriovenous shunts, which can allow oxygenated blood to bypass capillary beds resulting in higher venous PO<sub>2</sub> than capillary PO<sub>2</sub> (Tsai, Johnson et al. 2003). The potential for inadequate perfusion to the surgical site is an obstructive hurdle for the effectiveness of systemic interventions to increase PtO<sub>2</sub> (Qadan and Cheadle 2009). Maintenance of a warm, humid, high CO<sub>2</sub> surgical site environment during open

surgery in the abdomen proposes a solution that appears to increase perfusion directly at the target site and thereby increase vital tissue oxygenation.

The implementation of intra-abdominal CO<sub>2</sub> in open abdominal surgery into clinical practice is simplified by the fact that intra-abdominal CO<sub>2</sub> is already established as the recommended gas for the creation of pneumoperitoneum for laparoscopic surgery (Neudecker, Sauerland et al. 2002). Due to early concerns, the effect of intra-abdominal CO<sub>2</sub> exposure has been broadly investigated, especially in respect to post-operative adhesion formation and tumour implantation. Recent mechanistic animal research concludes that exposure to dry CO<sub>2</sub> pneumoperitoneum does not increase post-operative adhesion formation compared with exposure to dry air, when pneumoperitoneum is established at an appropriately low pressure and with adequate ventilatory support so as to avoid tissue hypoxia (Bourdel, Matsuzaki et al. 2007; Matsuzaki, Canis et al. 2007; Matsuzaki, Jardon et al. 2010). Furthermore, human trials have found that the rates of adhesive bowel obstruction (Reshef, Hull et al. 2013; Saklani, Naguib et al. 2013) and cancer survival (Fleshman, Sargent et al. 2007) are at least on-par with open abdominal surgery. In light of this evidence, a recent study that suggested a CO<sub>2</sub> operating environment increases adhesion formation (De Vries, Mårvik et al. 2013) has been criticized as not using an adequately standardized adhesion model (Mynbaev, Eliseeva et al. 2014). Another point of clinical interest is the fate of the excess CO<sub>2</sub> that overflows from an open abdominal cavity. The Coanda effect causes the CO<sub>2</sub> to flow out of the wound attached to the adjacent surface, similar to gas flow in avionics. A combination of the negative buoyancy of CO<sub>2</sub> and the

Coanda effect causes the CO<sub>2</sub> to drop to the floor of the operating room and therefore operating staff are not exposed to high inspired CO<sub>2</sub> (Persson and van der Linden 2003; Cater and van der Linden 2014).

The rat model used for this research was designed to mirror clinical operating conditions, including endotracheal intubation, replacement of insensible fluid loss, and continuous monitoring of depth of anaesthesia by a dedicated anaesthetist. Although applicability to the human clinical setting is inferred, there is some evidence that the PtO<sub>2</sub> response is similar between species, as similar increases in PtO<sub>2</sub> have been reported in rodent (Bourdel, Matsuzaki et al. 2007) and human surgical models in response to increased inspired oxygen (Greif, Akça et al. 2000). The use of a rat model had several advantages over measurement in humans. Most notably, the model allowed for the expensive PtO<sub>2</sub> probe to be re-used for several rats, avoiding the expense of single use probes that would be required in the human clinical setting. This enabled several different experiments to be conducted, which would not have been economically feasible in a human model. The model also allowed complete assurance that the PtO<sub>2</sub> probe remained in place and that the overlaying tissue remained free of surgical fluids, blood or bowel.

Continuous monitoring of arterial CO<sub>2</sub> partial pressure (P<sub>a</sub>CO<sub>2</sub>) was not conducted in this model, as the extra complication of femoral artery cannulation in an already long procedure was not justified. It is possible that a systemic increase in P<sub>a</sub>CO<sub>2</sub> contributed to the measured increase in PtO<sub>2</sub>. During the development of the current rat model, blood gas measurements were taken at different minute ventilation rates in 6 rats with

and without humidified-warm CO<sub>2</sub> insufflation. The results are not shown here, but do suggest that insufflation of humidified-warm CO<sub>2</sub> increases P<sub>a</sub>CO<sub>2</sub> by 7 mmHg at the minute ventilation used in the current study (note that the results were not statistically significant and therefore did not justify increasing minute ventilation during CO<sub>2</sub> insufflation in this protocol). Human data suggests that a 7 mmHg increase in P<sub>a</sub>CO<sub>2</sub> would increase PtO<sub>2</sub> by just 5 – 7 mmHg (Akça, Doufas et al. 2002; Akca, Liem et al. 2003). It is, therefore, unlikely that an increase in P<sub>a</sub>CO<sub>2</sub> alone is sufficient to explain the large increase in PtO<sub>2</sub> observed in the current study.

A further limitation, relevant to the interpretation of the current results, is that differences in inspired fraction of oxygen between studies may have had an impact on PtO<sub>2</sub> responses to interventions. A final limitation to this research is that post-operative measurement of PtO<sub>2</sub> could not be taken due to the cross-over design of this study. The decisive period for the development of surgical site infection is thought to be during surgery and the first few hours thereafter (Gottrup 2000), therefore intra-operative measurements are important. However, it would be interesting to determine whether the increase in PtO<sub>2</sub> observed during surgery is maintained after surgery, as this would be predicted to increase the benefit of the therapy, towards reduction in post-operative complications. The current results suggest that a post-operative increase in PtO<sub>2</sub> is unlikely, due to the rapid drop in PtO<sub>2</sub> following acute exposure to intra-abdominal humidified-warm CO<sub>2</sub>. However, it may be possible that exposure to CO<sub>2</sub> for the duration of surgery, which may be several hours, will result in increased angiogenesis and perfusion (Irie, Tatsumi et al. 2005), and therefore increased PtO<sub>2</sub>. If

PtO<sub>2</sub> is not maintained post-operatively, it may be beneficial to maintain topical CO<sub>2</sub> treatment following surgery.

In conclusion, the current study has shown that insufflation of humidified-warm CO<sub>2</sub> into the abdominal cavity during open abdominal surgery causes an immediate and clinically significant increase in PtO<sub>2</sub>. Furthermore, the effect is an additive result of the delivery of CO<sub>2</sub> and avoidance of evaporative cooling via the delivery of the gas humidified at body temperature. This finding may have important clinical implications, as the local delivery of the therapy may be able to overcome inadequate local micro-perfusion, which limits delivery of supplemental oxygen to the surgical site in many surgical patients.

## **6 Effect of Operating Room Ventilation and Humidified-Warm CO<sub>2</sub> Insufflation during Open Abdominal Surgery on Integrity of the Peritoneal Mesothelium in Rats**

### **6.1 Background**

The peritoneal mesothelium plays an essential role in the prevention of post-operative adhesion formation and peritoneal tumour implantation (Jayne 2007; Mutsaers and Wilkosz 2007). Following damage to the peritoneal mesothelium, as occurs during surgical incision, the presence of neighbouring mesothelial cells is essential to control the delicate balance between the deposition and breakdown of fibrin, to allow the mesothelium to heal adhesion free (Gillett, James et al. 1994; Mutsaers and Wilkosz 2007; Hellebrekers and Kooistra 2011). In the event of intra-peritoneal tumour spillage, mesothelial cells are required to secrete free hyaluronan to bind to intra-peritoneal tumour cells, inhibiting them from adhering and thereby metastasising to the peritoneum (Mutsaers and Wilkosz 2007). Furthermore, it has been shown that tumour cells adhere preferentially to areas where the mesothelium is disrupted in acute *in-vitro* human (Kiyasu, Kaneshima et al. 1981) and animal studies (Buck 1973; Koster, Volz et al. 1998; Van Den Tol, Van Rossen et al. 1998; Aoki, Shimura et al. 1999; Volz, Koster et al. 1999), and in tissue culture investigations (Yu, Kuebler et al. 2010). Therefore, avoidance of inadvertent damage to the peritoneal mesothelium during abdominal surgery is important to avoid surgical complications.

In addition to damage by surgical incisions, inadvertent damage to the peritoneal mesothelium can occur during abdominal surgery by exposing the peritoneal surface to rubbing (Gillett, James et al. 1994; Burns, Skinner et al. 1995), handling (Phillips and Dudley 1984), foreign bodies (Torre, Favre et al. 2002), ischemia (Matsuzaki, Jardon et al. 2010), lavage solutions (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973; Lopes, De Oliveira et al. 2007), and, of particular interest to the current research, desiccation (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973; Verger, Luger et al. 1983; Burns, Skinner et al. 1995; von Ruhland, Newman et al. 2003; Binda, Molinas et al. 2006; Binda, Corona et al. 2014; Carpinteri, Sampurno et al. 2015). However, despite these results that experimentally induced desiccation causes mesothelial damage, there is a lack of evidence as to whether simply exposing the mesothelium to the ambient operating room ventilation during open abdominal surgery causes sufficient desiccation to result in mesothelial cell loss.

Despite this lack of evidence, to reduce inadvertent damage to the mesothelium during abdominal surgery, guidelines recommend the use of microsurgical techniques including avoiding desiccation (DeWilde, Trew et al. 2007; Schnuriger, Barmparas et al. 2010; Koninckx, Ussia et al. 2012; The Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society of Reproductive Surgeons 2013). Desiccation is traditionally reduced using irrigating lavage and by placing wet packs into the abdominal cavity. However, criticism is growing against the unnecessary use of intra-peritoneal lavage, as it may increase the risk of post-operative complications by disrupting the peritoneal mesothelium and it is not effective in

reducing the risk of surgical site infection (Mutsaers and Wilkosz 2007; National Collaborating Centre for Women's and Children's Health and commissioned by the National Institute for Health and Clinical Excellence 2008; Hellebrekers and Kooistra 2011). Furthermore, it is likely that rubbing the peritoneum with a wet pack can also cause mesothelial damage (Van Den Tol, Van Stun et al. 1997; von Ruhland, Newman et al. 2003). Therefore a problem exists as to how to avoid tissue desiccation during open abdominal surgery, in which the peritoneum is exposed to the dry operating room, without using intra-peritoneal lavage or rubbing the peritoneum with wet packs.

Insufflation of humidified-warm CO<sub>2</sub> into the abdominal cavity has been proposed as a therapy to reduce inadvertent damage to the peritoneal morphology caused by desiccation during open abdominal surgery (Persson and van der Linden 2008). The CO<sub>2</sub> is heated to body temperature and humidified to avoid desiccation. Insufflation of humidified-warm CO<sub>2</sub> is significantly more successful at reducing evaporation than insufflation of humidified-warm air {Persson, 2004 #58}. Using an active humidification system and a specially designed gas diffuser, humidified-warm CO<sub>2</sub> can be diffused into the open peritoneal cavity at a low velocity while at a flow rate high enough to create a local environment with a high concentration of CO<sub>2</sub> (Persson and Van der Linden 2003; Svenarud, Persson et al. 2003; Svenarud, Persson et al. 2003; Persson, Svenarud et al. 2004). Laboratory studies show that air is displaced by the heavier CO<sub>2</sub> and an invisible humidified greenhouse is created within the abdominal cavity that reduces desiccation (Persson, Elmqvist et al. 2004; Persson and Van Der Linden 2005). While not yet in widespread use in open abdominal surgery, CO<sub>2</sub> is established as the recommended

gas for the creation of pneumoperitoneum for laparoscopic surgery (Neudecker, Sauerland et al. 2002). For endoscopy reduction of desiccation by humidification of CO<sub>2</sub> has been shown to reduce mesothelial cell loss and inflammatory changes (Mouton, Bessell et al. 1999; Erikoglu, Yol et al. 2005; Binda, Molinas et al. 2006; Peng, Zheng et al. 2009; Davey, Hayward et al. 2013). Therefore, it has been hypothesised that the use of humidified-warm CO<sub>2</sub> will also reduce loss of peritoneal mesothelium in open abdominal surgery (Persson and van der Linden 2009). However, the effect of insufflation of either dry or humidified-warm CO<sub>2</sub> on mesothelial damage in open abdominal surgery has not been investigated.

Other signs of peritoneal inflammation may also be present in addition to loss of peritoneal mesothelium. Investigations conducted on the effect of desiccation during laparoscopy suggest that loss of parietal mesothelium will be proceeding by a change in parietal cellular morphology from a flat to relatively bulged cell (Volz, Koster et al. 1999; Erikoglu, Yol et al. 2005; Peng, Zheng et al. 2009), an increase in presence of apoptotic cells {Tosun, 2007 #272}, and increased expression of the inflammatory marker COX-2 that is an important predictor of cancer progression (Carpinteri, Sampurno et al. 2015). COX-2 is the inducible form of the cyclo-oxygenase enzyme and is up-regulated in inflammation. Increased expression of COX-2 can be used as a marker of inflammation. It is also likely that inflammation will extend to portions of the peritoneum that are not exposed to the desiccating environment, and that sub-mesothelial oedema will occur (Mutsaers, Whitaker et al. 2000). By preventing damage to the mesothelium caused by desiccation, humidified-warm CO<sub>2</sub> may reduce these

signs of inflammation including reducing the presence of bulged mesothelial cells, apoptotic cells, expression of the inflammatory marker COX-2, and sub-mesothelial oedema.

This research was designed to test two primary hypotheses. Firstly, that exposure of the peritoneal mesothelial simply to normal operating room ventilation during open abdominal surgery will cause inadvertent loss of peritoneal mesothelial cells compared with anaesthesia only controls. Secondly, that insufflation of humidified-warm CO<sub>2</sub> into the open abdominal cavity will reduce loss of peritoneal mesothelial cells compared with laparotomy without gas insufflation. Data was also collected to explore the secondary hypotheses that laparotomy without gas insufflation, compared with both surgery with insufflation of humidified-warm CO<sub>2</sub> and anaesthesia only controls, will cause in exposed and un-exposed peritoneal mesothelium: bulging of parietal mesothelial cells; increased expression of the inflammatory marker COX-2; increased sub-mesothelial cell thickness; and apoptosis.

## 6.2 Methods

### 6.2.1 Ethics and animal care

This study was approved by the University of Wollongong Animal Ethics Committee (AE 10-24). Female Wistar rats were used and maintained in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council 2013). Prior to surgery, the rats were housed two rats to a cage with ad-libitum access to food and water. The rats were maintained in a

temperature controlled environment with diurnal variation of light. 12 hours after surgery, the rats were euthanized by slow fill carbon dioxide asphyxiation.

#### 6.2.2 Induction and monitoring of general anaesthesia

All rats were weighed and then pre-warmed in the animal handler's hands for 10 minutes immediately prior to induction of anaesthesia, using a blanket and heating lamp. Anaesthesia was induced with inhalant isoflurane in air via a nose cone.

Throughout anaesthesia the rats rested on a Small Animal Far Infra-Red Warming Pad (Kent Scientific Corporation, Connecticut, USA) to maintain body temperature.

Following induction, the rats underwent endotracheal intubation with a 16 G blunted intravenous catheter during direct vision of the entrance to trachea, illuminated by a red LED shone through the skin of the throat, see model development chapter 4. The endotracheal catheter was connected to a ventilator (Rodent Ventilator 7025, Ugo Basile, Varese, Italy) and ventilated at 315 ml/kg/min in rats not exposed to CO<sub>2</sub> insufflation and 350 ml/kg/min in rats exposed to CO<sub>2</sub> insufflation, with a tidal volume of 1 ml. Pilot measurements showed that this minute ventilation maintained P<sub>a</sub>CO<sub>2</sub> in a normal range (see chapter 4.7). Prophylactic pain relief was administered (subcutaneous meloxicam 1 mg/kg (Roughan and Flecknell 2003)). The endotracheal catheter was secured around the snout of the rat and a small piece of gauze was placed in the mouth to collect oral secretions. Depth of anaesthesia was monitored via continuous measurement of heart rate and pulse pressure using a pressure transducer placed over the neck in the area of the carotid artery. Core body temperature was monitored every 5-10 minutes with a rectal thermometer (Surgipak Flexible Digital

Thermometer, Vega Technologies Inc., Taipei, Taiwan). An insulating “sock” was placed over the rat’s tail to reduce radiated heat loss. A heat lamp was positioned approximately 25 cm above the rat, and was only used if the rat’s temperature was falling below 36 °C. Any use of the heat lamp was recorded. Ambient temperature and humidity of the operating room was recorded (HygroPalm22 Portable Humidity & Temperature Meter, Rotronic, Switzerland). Insensible fluid replacement was delivered hourly at 10 ml/kg/hr s.c. with warmed 0.9 % sodium chloride, according to Australian guidelines for the promotion of wellbeing of animals used for scientific purposes (National Health and Medical Research Council 2008).

#### 6.2.3 Experimental design

Rats were assigned to one of three groups.

1. Group C: Anaesthesia only control (2 rats)
2. Group LO: Laparotomy with controlled air flow (4 rats)
3. Group LI: Laparotomy with insufflation of humidified-warm CO<sub>2</sub> (3 rats)

#### 6.2.4 Surgical procedure

Following endotracheal intubation, the abdomen was clipped with an electric hair clipper (Oster Golden A5 two speed, Model 5-50A, Sunbeam Products, Florida, USA) and the skin was cleaned with Betadine. In groups LO and LI, an inverted “L” shaped laparotomy incision was then made to create adequate exposure of the parietal peritoneum for tissue collection, see Figure 6-1. This consisted of a 60 mm long midline laparotomy incision, starting approximately 10 mm caudal to the xiphoid process. A

further 40 mm long perpendicular extension of that incision was then made across the left side of the abdominal wall, from the rostral end of the first incision. A small clamp (5 mm wide) was attached to the skin of the abdominal wall flap at the intersection point of the two incisions. The abdominal wall was then gently reflected towards the lower left quadrant so as to expose the parietal peritoneum. The skin was clamped so as to minimise tension on the peritoneum, see Figure 6-1. To further expose the parietal peritoneum, by way of relaxation of the left abdominal wall, the left hind leg was flexed and secured using tape across the foot. The relevant treatment condition was then applied for 1 hour.

Following the treatment the peritoneum was sutured closed, the skin was stapled and a bandage was applied to protect the wound. The rat was then returned to an individual cage until CO<sub>2</sub> euthanasia at 12 hours after surgery. 12 hours has previously been shown to be the time of maximum mesothelial inflammation (Volz, Koster et al. 1999; Volz, Koster et al. 1999). The rat was monitored for signs of pain, and an extra dose of pain relief was administered if necessary (subcutaneous meloxicam 1 mg/kg).

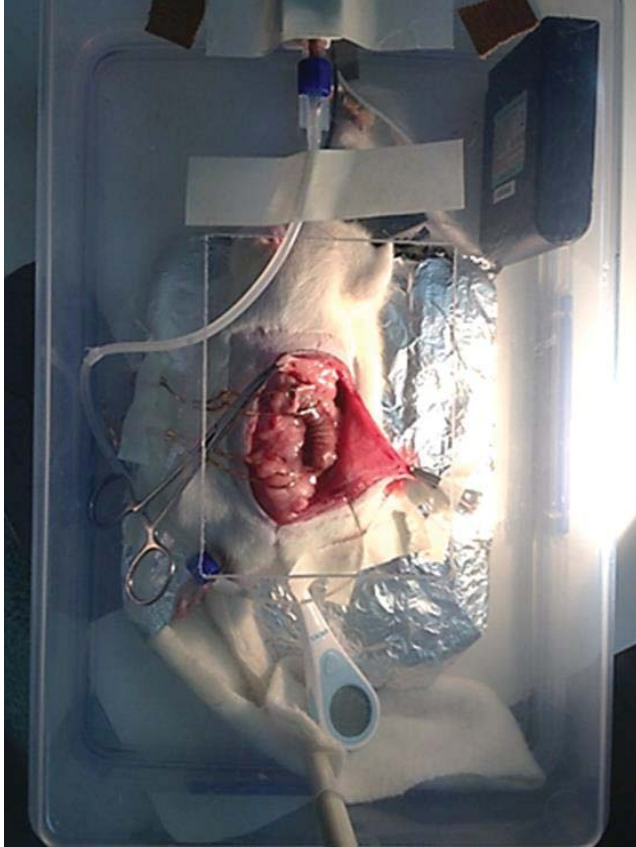
#### 6.2.5 Treatment conditions

##### 6.2.5.1 Controlled air flow

Air flow around the rat was standardised to simulate conservative operating room ventilation of 20 exchanges per hour, as described in section 4.8.

#### 6.2.5.2 Insufflation of humidified-warm CO<sub>2</sub>

To ensure the abdomen was exposed to a high concentration of CO<sub>2</sub>, the rat was placed in a plastic box as describe in section 4.9. Within the box CO<sub>2</sub> was insufflated via a gas diffuser (VitaDiffuser, Cardia Innovation, Sweden) at 9 L/min to ensure that high CO<sub>2</sub> concentration was maintained during any disruption to the CO<sub>2</sub> environment by the hands of the research team (Persson and Van der Linden 2003). In the humidified-warm CO<sub>2</sub> conditions, the CO<sub>2</sub> was humidified and warmed using a humidifier controller and heated delivery tube (HumiGard, Fisher and Paykel Healthcare, New Zealand). Independent testing has shown that the humidifier delivers > 98.0% relative humidity at 37 °C (Sammour, Kahokehr et al. 2010).



**Figure 6-1: Surgical set-up.**

To ensure adequate exposure of the parietal peritoneum for tissue harvesting, an inverted "L" shaped laparotomy incision was performed. To facilitate insufflation of CO<sub>2</sub>, the rat was placed in a plastic container. The container had a hole at the top to allow gas insufflation. This set-up ensured a high CO<sub>2</sub> concentration.

#### 6.2.6 Tissue sample collection and processing

Twelve hours after treatment, rats were euthanized and tissues of the left abdominal wall only (control group) or both abdominal walls (LO and LI groups) and spleen (all groups) were excised and pinned out in 100 mmol/l sodium phosphate buffer with 2 % sucrose, pH 7.3 (sample buffer) (von Ruhland, Newman et al. 2003). Following rinsing with sample buffer, the buffer was replaced with Bouin's fixative containing an additional 0.2 % glutaraldehyde, and left for 36 hours. Bouin's fixative has been shown to preserve peritoneal tissue well (Liu, Li et al. 2001), see discussion in section 4.11.

Following fixation, tissue was rinsed in sample buffer and divided into 5 mm<sup>2</sup> as illustrated in Figure 6-2, and stored in sample buffer at 4 °C. Tissue samples for paraffin embedding were dehydrated in graded alcohol solutions and embedded in paraffin, taking care to mount the tissue parallel to the face of the wax block. Embedded tissues were sectioned to 5 µm (Erikoglu, Yol et al. 2005; Peng, Zheng et al. 2009). One section from each tissue sample was stained with haematoxylin and eosin, another section from each sample for TUNEL analysis and a further section for analysis of COX-2 expression.

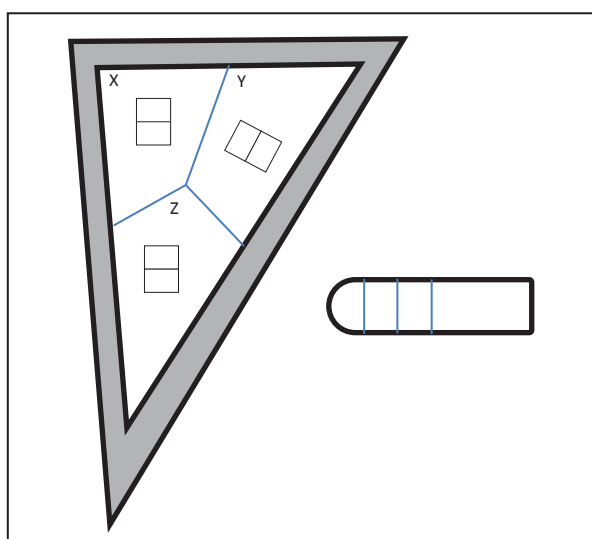


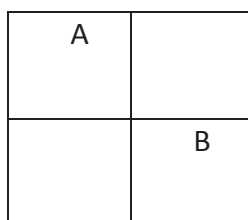
Figure 6-2: Illustration of the division of tissue for microscopic analysis.

The left illustration shows an excised abdominal wall and the right illustration the spleen. The shaded area along the cut edges was discarded. Two pieces of tissue from each of the three areas marked X-Z and the spleen were collected. One piece of tissue from each region was used for paraffin embedding and the other for preparation for scanning electron microscopy analysis. Thus a total of three pieces of parietal peritoneum from different areas were collected for paraffin embedding and three pieces for preparation for scanning electron microscopy from each abdominal wall sample. (Note that the illustrations are drawn not to scale)

#### 6.2.7 Scanning electron microscopy preparation and analysis

Prior to SEM analysis tissue samples were dehydrated in graded ethanol solutions, (30 minutes each at 30 %, 50 %, 70 %, 80 %, 90 %, and two 30 minutes washes in 100 %

ethanol), immersed in 100 % hexamethyldisilazane (HMDS) (two 5 minute washes followed by one 60 minute wash) and air dried in a sealed container with a desiccant for at least two days before mounted onto stubs and sputter coated with gold. Two images were taken from each tissue samples, at 2000x, 650x and 300x magnification in the centre of each of the quadrants A-B, illustrated in Figure 6-3, using a template placed over the low magnification image so as to avoid bias. SEM was conducted on a JEOL JSM-6490LV (JEOL Ltd., Tokyo, Japan). The microscopist was blinded to the group allocation of the tissue sample. All images were evaluated for area percentage of mesothelial cell loss and mesothelial cell bulging, as described below, by two independent evaluators blinded to the group allocation of the images.



**Figure 6-3: Illustration of quadrants for imaging of tissue samples by SEM.**

**Two images were taken in the centre of quadrants A and B, one each of 2000 x, 650x and 300x magnification.**

#### 6.2.7.1 Area percentage of mesothelial cell loss

The area percentage of mesothelial cell loss was quantified using a previously published stereology method (Du, Yu et al. 2011). A transparent grid was placed at random over each SEM photograph and the number of grid points overlying exposed basement membrane was counted. The area percentage of exposed basement membrane was calculated using the following formula:

$$[ A(\text{exposed}) / A(\text{total}) ] * 100 = [ \sum P(\text{exposed}) / \sum P(\text{total}) ] * 100$$

Where:

A(exposed) = Area of mesothelial cell loss

A(total) = Total area of the photograph being analysed

P(exposed) = Number of point hitting the basement membrane exposed,

P(total) = Total number of points covering the photograph

Mesothelial cells were identified by the presence of microvilli and cell borders.

Detailed definitions and SEM analysis instructions given to the evaluators is included in appendix 1.

#### 6.2.7.2 Bulging of mesothelial cells

Mesothelial cell bulging was evaluated on a semi-quantitative three level scale, in which 0 = none or slight; 1 = moderate (20-39% of surface affected); 2 = marked (40-100 % of surface affected). Detailed descriptions are included in appendix 1.

#### 6.2.8 Analysis of haematoxylin and eosin staining sections by light microscopy

Light microscopy imaging was conducted using a Leica™ DM6000 optical microscope (Leica microsystems, Wetzlar, Germany) at 20 x magnitude and mosaic was created to give one image for each section, allowing analysis of the entire width of the tissue section. All images were evaluated by two independent evaluators blinded to the group allocation of the images. Mesothelial cell loss, and mesothelial cell bulging were then evaluated on a semi-quantitative three level scale, as described above for SEM. Detailed description is included in appendix 2. The scores of each of the two evaluators were averaged to give a score for each image. In addition, sub-mesothelial thickness

was measured by one blinded evaluator as the average thickness of the sub-mesothelial connective tissue measured perpendicular to the peritoneal surface, as defined and illustrated by Williams *et al.* (2002) (Williams, Craig *et al.* 2002). The average thickness was calculated by first measuring the sub-mesothelial loose zone area ( $\mu\text{m}^2$ ), and dividing by the average of the apical and basal lengths using the software package LAS v 4.3 (Leica Microsystems, Wetzlar, Germany). Outlining was done at 322 x magnification. Two measurements were made. Firstly, the sub-mesothelial thickness was measured across the entire section. Secondly, sub-mesothelial thickness was measured in the widest portion of the tissue in which the section was parallel to the underlying muscle fibres, as it has been suggest that the measurement is more consistent in this orientation (Duman, Sen *et al.* 2001; Duman and Şen 2009).

#### 6.2.9 Detection of apoptosis by TUNEL assay

Detection of apoptotic mesothelial cells on exposed peritoneal was carried out by labelling cleaved DNA using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (In Situ Cell Death Detection Kit TMR red, Roche Applied Science, Basel, Switzerland). Paraffin embedded sections were de-paraffinised, rehydrated in water, and permeabilised using Proteinase K as per the kit instructions. DNA cleavage was induced in positive controls using DNase I (Roche Diagnostics, Basel, Switzerland).

Sections were imaged with a scanning fluorescent microscope (Aperio FL Multiplexing Immunofluorescence Slide Scanner, Leica Biosystems, Nussloch, Germany), allowing

the whole section to be imaged and analysed (excitation wavelength 590, detection wavelength 617 nm). The microscopist was blinded to the group allocation of the tissue samples. Average fluorescence along the peritoneal surface was quantified by an evaluator blinded to the group allocation of the images blinded evaluator using Image J (free software available at <http://fiji.sc/Fiji>) without any image processing. Background subtraction was carried out using average fluorescence in the non-fluorescing adjacent muscle fibres for abdominal wall tissue or white pulp for spleen tissue. Following background subtraction, the average fluorescence along the peritoneal surface for each section was expressed as a percentage of the average background fluorescence for that section.

#### 6.2.10 Analysis of COX-2 expression by immunohistochemistry

Paraffin embedded sections of peritoneal tissue were de-paraffinised and rehydrated in water. For groups LO and LI, only tissue exposed to the gaseous environment was used for analysis. In addition, tissue from a previous investigation was used as a positive control (Carpinteri, Sampurno et al. 2015). Heat-induced antigen retrieval was conducted in EDTA (pH 8 diluted to 1 mM) within a pressure cooker (13-17 psi at 125 °C) for three mins, followed by blocking of endogenous peroxidase or pseudoperoxidase activity with 3% H<sub>2</sub>O<sub>2</sub> for ten minutes. Each section was then washed in Tris Buffer 20 buffer (0.1 % pH 7.60) for five minutes and incubated with primary COX-2 antibody (sc-1745, Santa Cruz Biotechnology Inc., Texas, USA) 1:1000 at 4 °C overnight in a humid chamber. Sections then underwent three x five minutes washes in Tris Tween 20 buffer, were incubated with the secondary antibody

ImmPRESS™ Anti-Goat-Ig, peroxidase, (Vector laboratories, CA, USA) at room temperature for 30 minutes, and underwent a further three x five minutes washes in with Tris Tween 20 buffer. All sections except the negative controls were then developed with 3,3' diaminobenzidine tetrahydrochloride (DAB) and chromogen (DAB+ substrate-chromogen system, Dako Australia Pty, Sydney, Australia) for three minutes and immediately washed in tap water. Finally slides were counterstained with haematoxylin, dehydrated, mounted with Entellan mounting medium and cover-slipped.

Imaging was conducted using a Leica™ DM6000 optical microscope (Leica microsystems, Wetzlar, Germany) at 50 x magnification. Four representative images were taken from each sample, in areas where mesothelial cells could be identified, if possible. The microscopist was blinded to the group allocation of the tissue samples. All images were evaluated by two independent evaluators blinded to the group allocation of the images. Each image was scored for intensity of staining (0=none; 1=weak; 2=medium; 3=strong). The scores given to each of the four images from each section were averaged to give one score per tissue section.

#### 6.2.11 Statistical analysis

For each duplicated measure, the scores of the two evaluators were averaged to give a score for each image. A Shapiro-Wilk normality test was used to test for normality. An independent samples ANOVA or Kruskal-Wallis test was used to determine whether there was a difference in scores between any groups. Where a difference between groups was detected, an independent samples t-test or Mann-Whitney test was used

to determine whether differences between individual groups LO vs C and LO vs LI were present. P-value <0.05 was considered statistically significant for all tests. For area percentage of mesothelial cell loss and mesothelial cell bulging, the analysis was repeated for tissue exposed to the gaseous environment and for non-exposed tissue. Scores for parietal and visceral peritoneum were pooled for analysis for area percentage of mesothelial cell loss, TUNEL assay, and COX-2 expression. Scores for mesothelial cell bulging were analysed separately for parietal and visceral peritoneum, as the parietal peritoneum is expected to normally consist of predominately flat mesothelial cells, while the mesothelium of the spleen is normally characterised by cuboidal mesothelial cells (Michailova and Usunoff 2006). When non-parametric test were used, the outcomes were reported as (median, 25<sup>th</sup> – 75<sup>th</sup> percentile).

#### 6.2.12 Detection of intra-operative mesothelial damage by fluorescein

As a pilot of the utility of fluorescein to detect mesothelial cell damage, fluorescein was applied to the mesothelial surface of three rats that were to be euthanized for experimental investigations separate to the current research. Animals were anaesthetised and had undergone laparotomy, as described above. Fluorescein 1 % (Bausch and Lomb, Chatswood, Australia) was dripped onto virgin peritoneum and peritoneum that had been exposed to the operating environment for 2-11 minutes, without insufflation of CO<sub>2</sub>. Immediately following application of the fluorescein, the area was thoroughly rinsed with 0.9 % sodium chloride. A cobalt blue pen torch was applied to the tissue and photographic images were taken.

## 6.3 Results

### 6.3.1 Animals

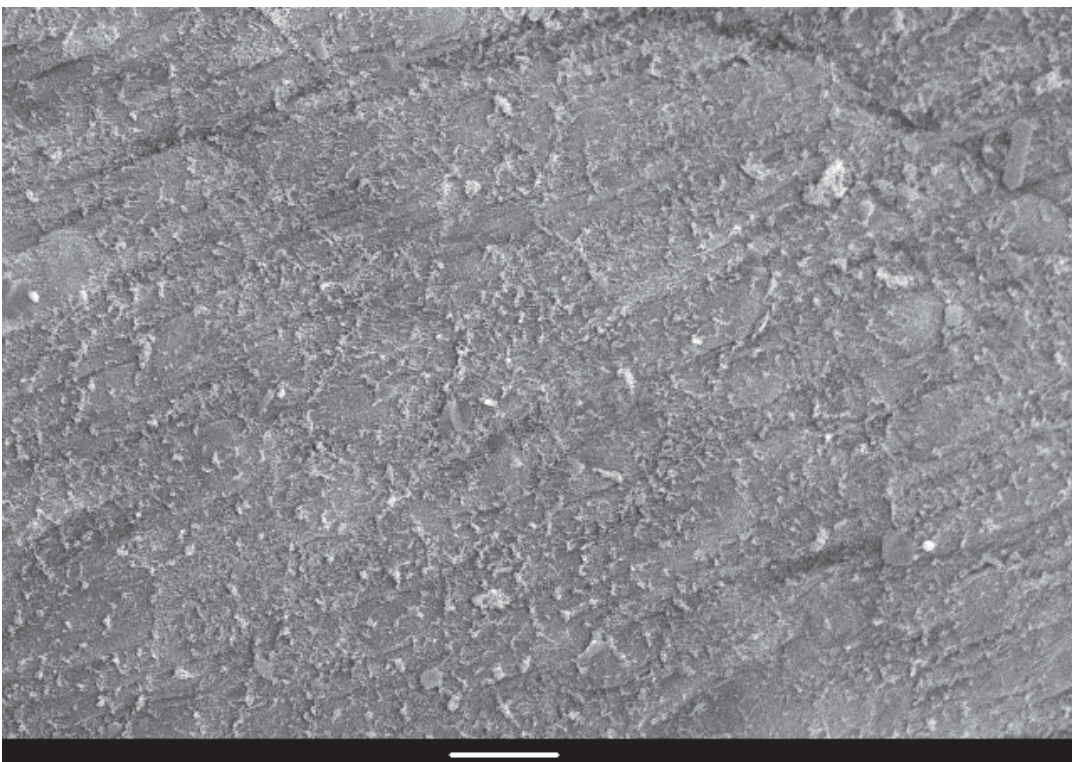
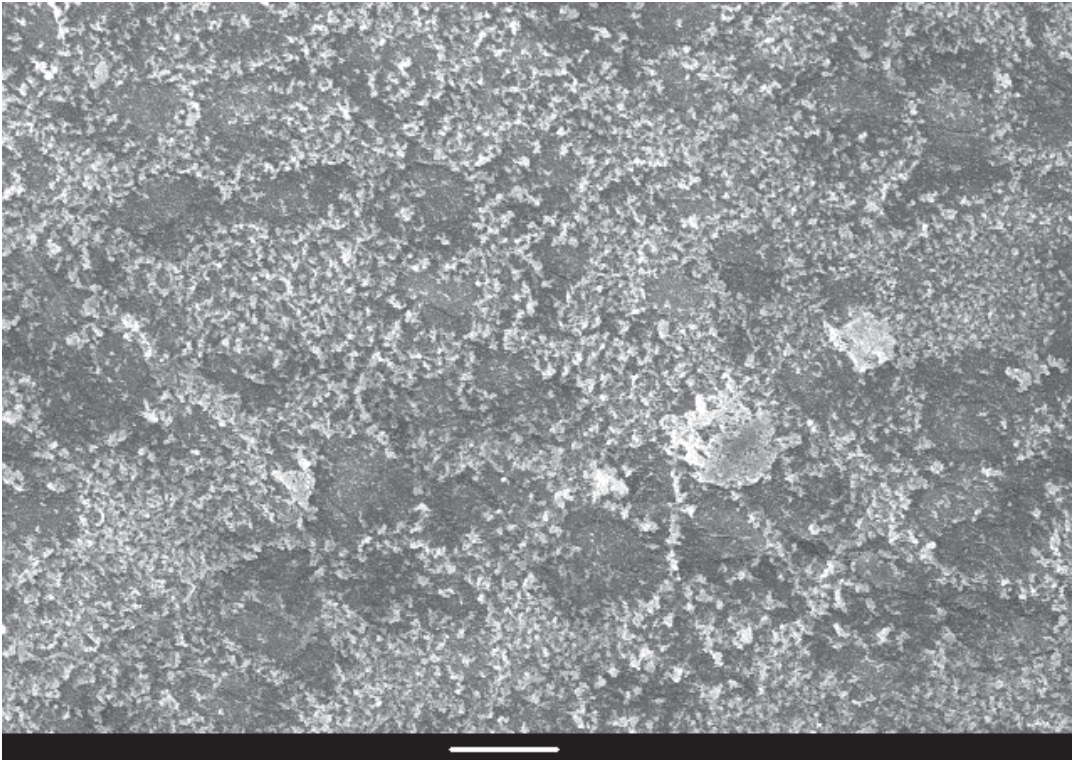
The average weight of the rats was 314 g (SD 58), and did not differ between groups ( $p < 0.05$  for all comparisons).

### 6.3.2 Mesothelial cell loss

Example images analysed by scanning electron microscopy are shown in Figure 6-4, Figure 6-5, and Figure 6-6. The area percentage of mesothelial cell loss from peritoneum exposed to the gaseous environment in the LO group (5.8, 0-50.8 %), was larger than both group C (0, 0-0 %),  $p=0.0005$ , and LI (0, 0-1.7 %),  $p=0.0040$ , see Figure 6-8. Similar loss of mesothelium was seen in haematoxylin and eosin stained peritoneal tissue sections, measured on a scale of 0-2, see example images in Figure 6-7.

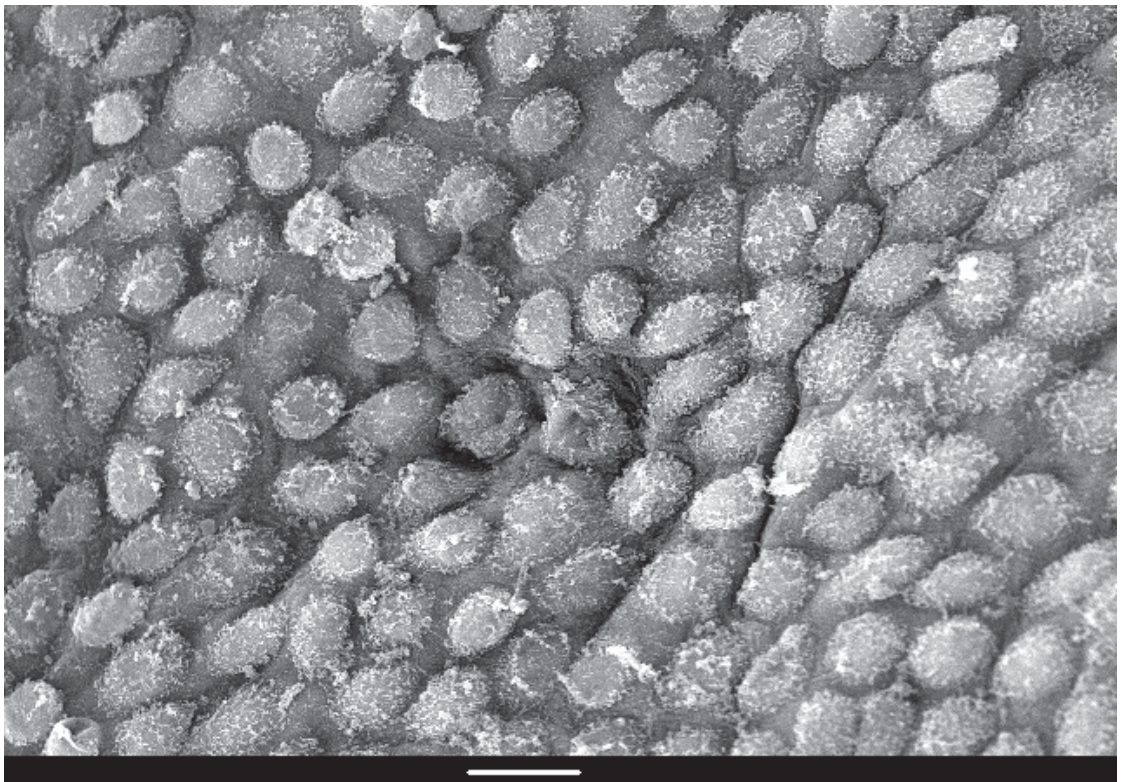
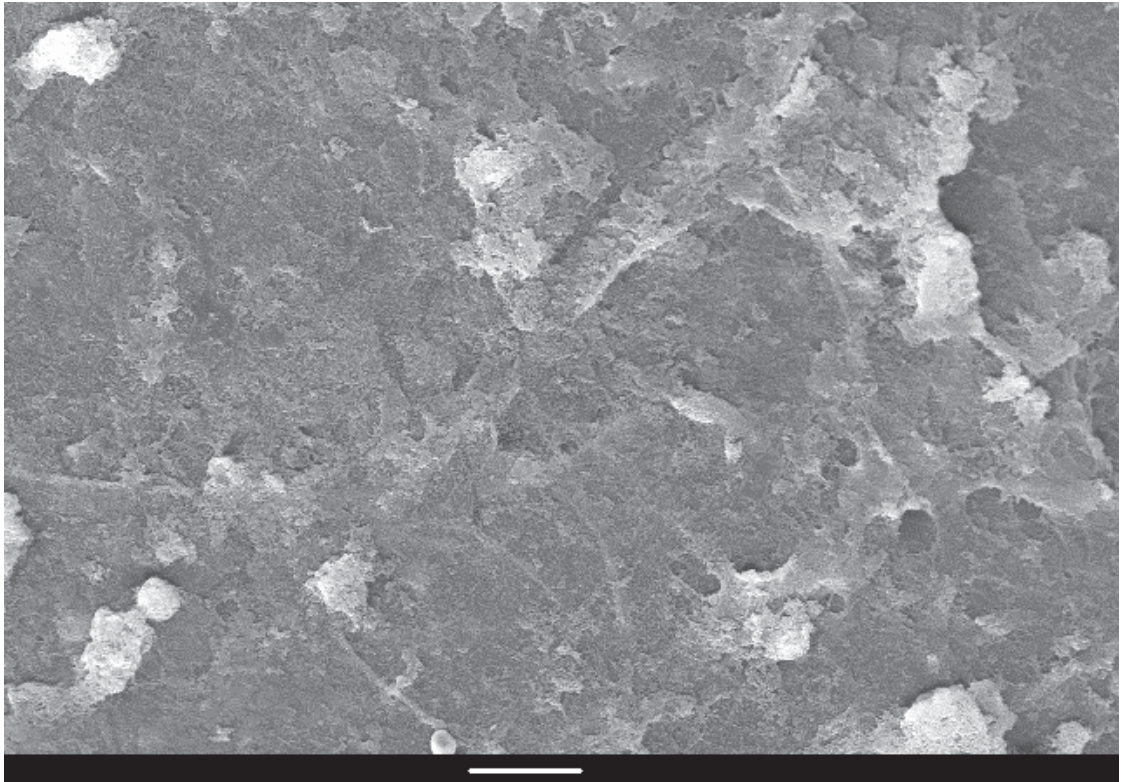
Mesothelial cell loss from peritoneum exposed to the gaseous environment in the LO group (1.25, 0.625-2) was larger than both group C (0, 0-0),  $p=0.0004$ , and group LI (0, 0-0),  $p=0.0003$ , see Figure 6-8.

There was very little mesothelial cell loss seen in non-exposed peritoneum. In non-exposed tissue samples analysed using scanning electron microscopy, the area percentage of mesothelial loss in the LO group (1.2, 0-5.2 %), was slightly larger than the group C,  $p=0.0007$ , and LI groups (0, 0-1.35 %),  $p=0.032$ , see Figure 6-9. No significant differences in mesothelial cell loss was seen by light microscopy analysis,  $p = 0.49$ , see Figure 6-9.



**Figure 6-4: Scanning electron microscopy images of from Group C (anaesthesia only control).**

**Images show continuous coverage with flat mesothelial cells, identified by the presence of microvilli and cell borders. Parietal peritoneum, white bar indicate 20  $\mu$ m.**



**Figure 6-5: Scanning electron microscopy images from Group LO (Laparotomy with controlled air flow).**

Images show massive loss of mesothelium in the upper image (visceral peritoneum) and marked bulging of mesothelial cells in the lower image. Parietal peritoneum, white bar indicates 20  $\mu\text{m}$ .

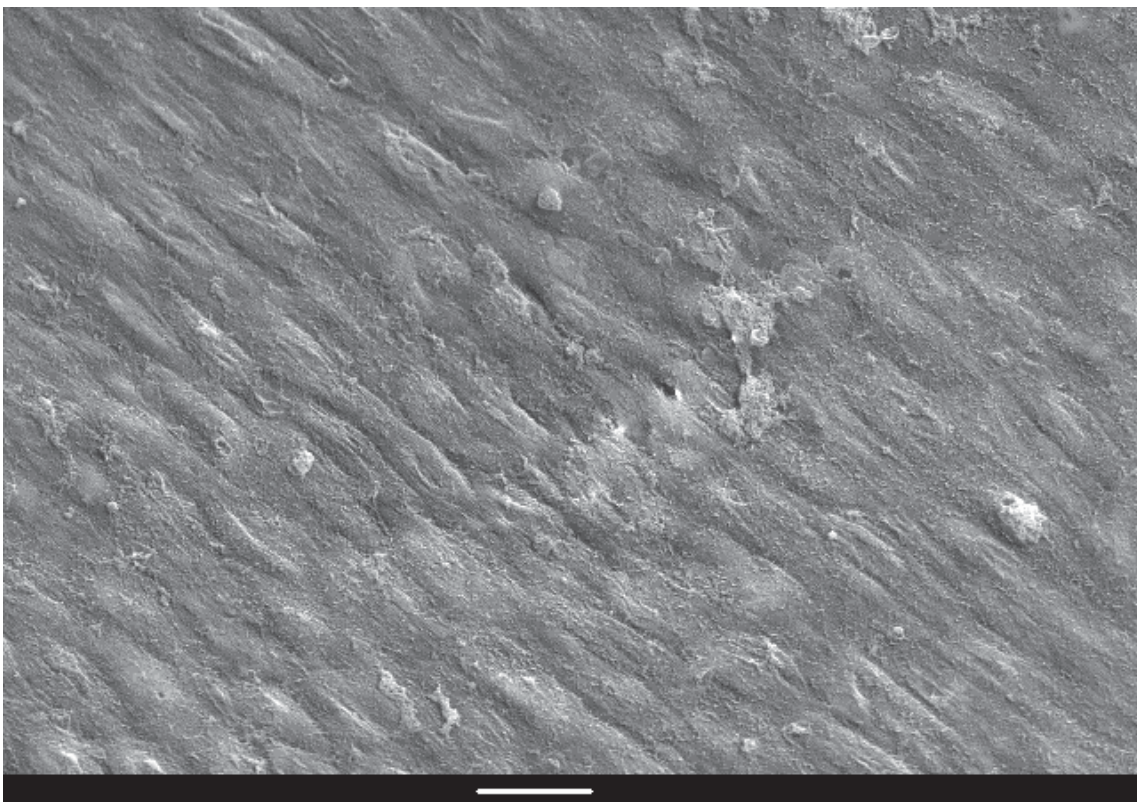
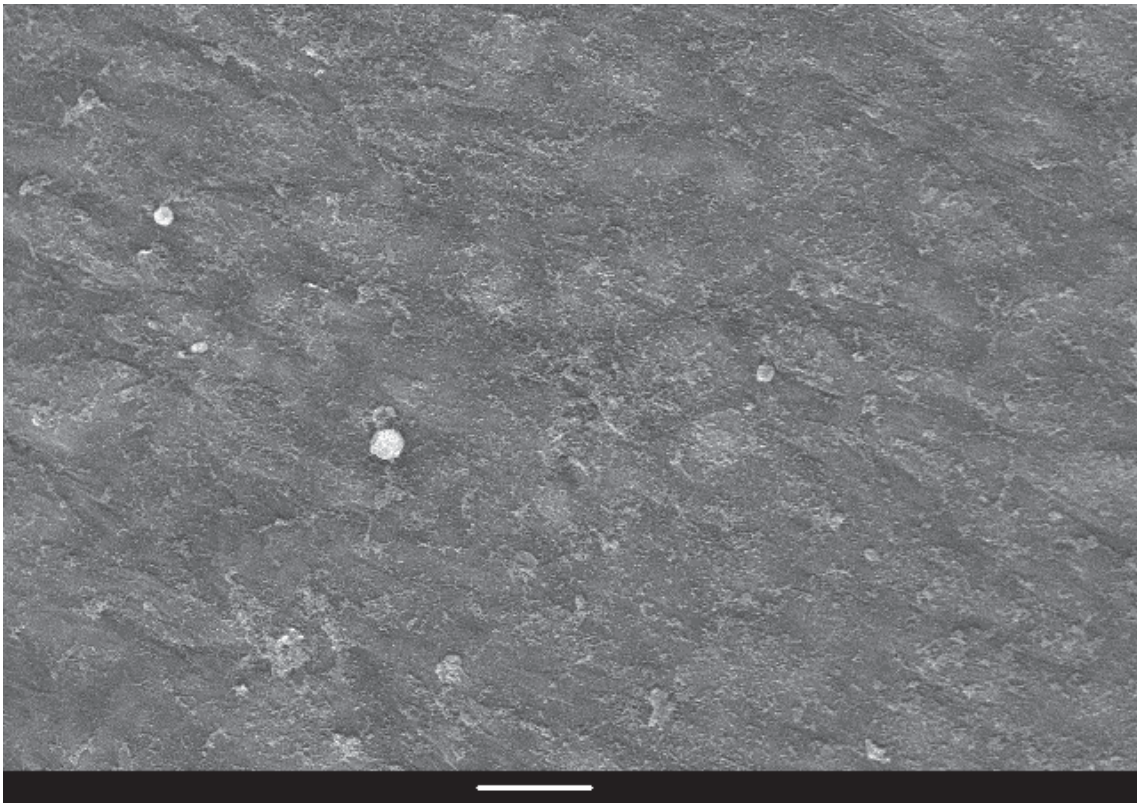


Figure 6-6: Scanning electron microscopy images from Group LI (Laparotomy with CO<sub>2</sub> insufflation)

Images show continuous coverage with mesothelial cells with some slight cellular bulging in the lower image. Parietal peritoneum, white bar indicate 20  $\mu$ m.

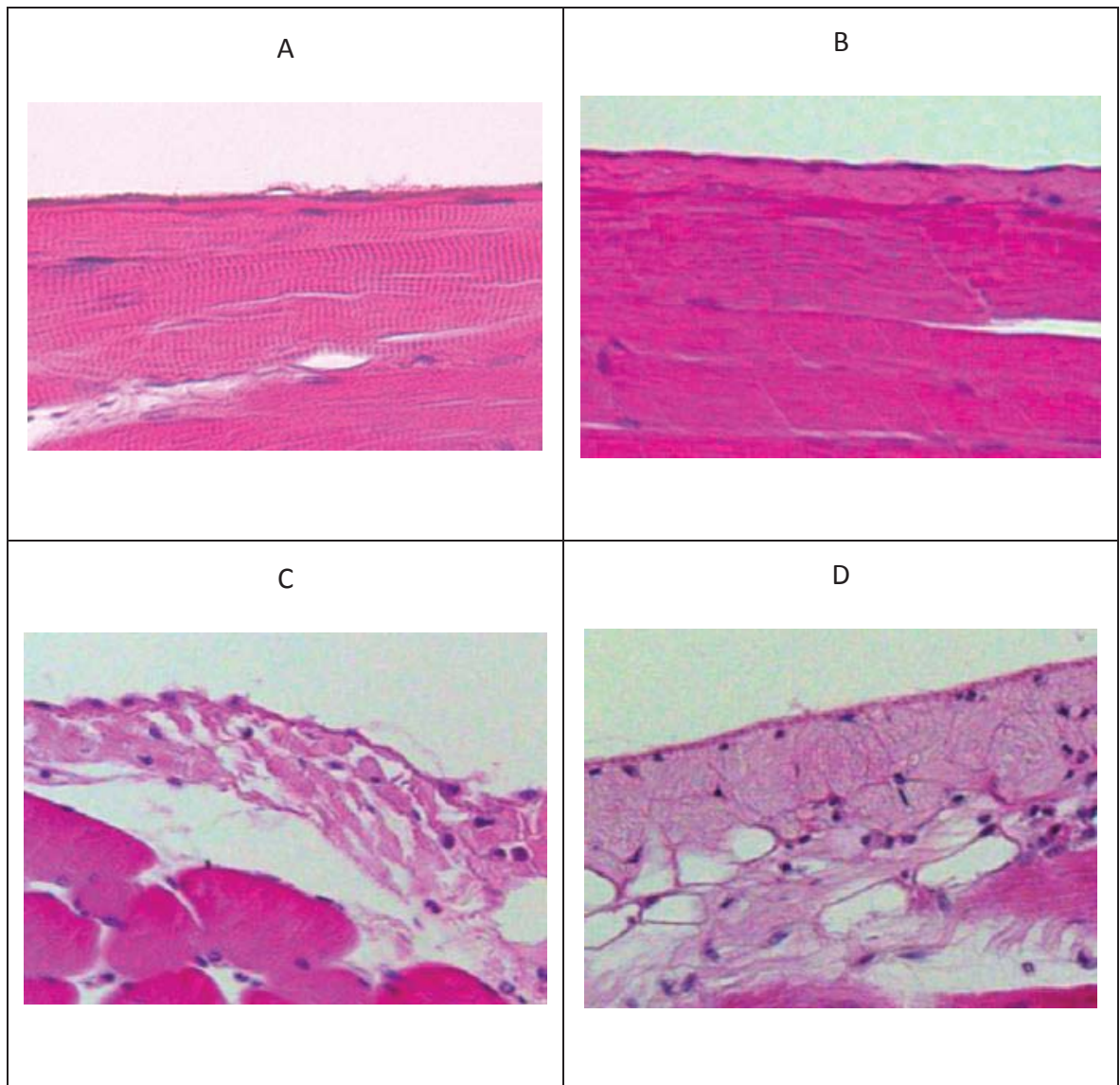


Figure 6-7: Examples of changes seen in light microscopy images (imaged at 20 x magnification).

**A: Anaesthesia only - a continuous layer of flat mesothelial cells. B: LI exposed - a continuous layer of flat mesothelial cells. C: LO exposed - bulging of mesothelial cells and increase in sub-mesothelial thickness. D: LO exposed – total loss of mesothelium and increase in sub-mesothelial thickness.**

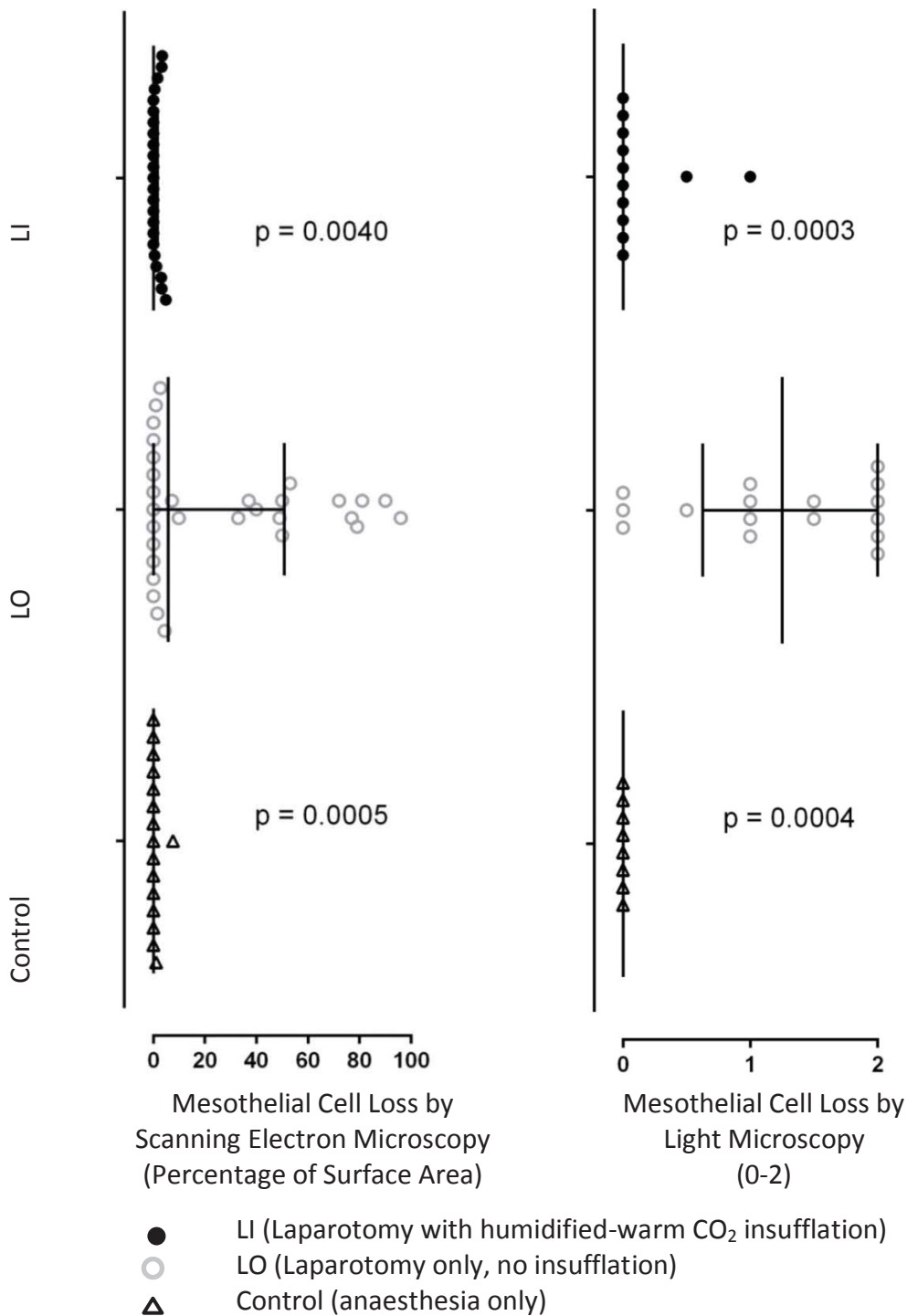


Figure 6-8: Results of analysis of mesothelial cell loss of the exposed peritoneum and anaesthesia only controls.

Tissue was analysed independently by scanning electron (left) and light (right) microscopy. Each symbol represents the average score of one image. Data from both parietal and visceral peritoneum are included together. P values show the results of Mann Whitney U test of the adjacent group compared with the LO group. Error bars show median and interquartile range. Control: 2 rats; LO: 4 rats; LI: 3 rats. Four tissue samples were analysed per animal for light microscopy and four for scanning electron. The whole length of the section was analysed by light microscopy, two areas of interest were analysed by scanning electron microscopy.

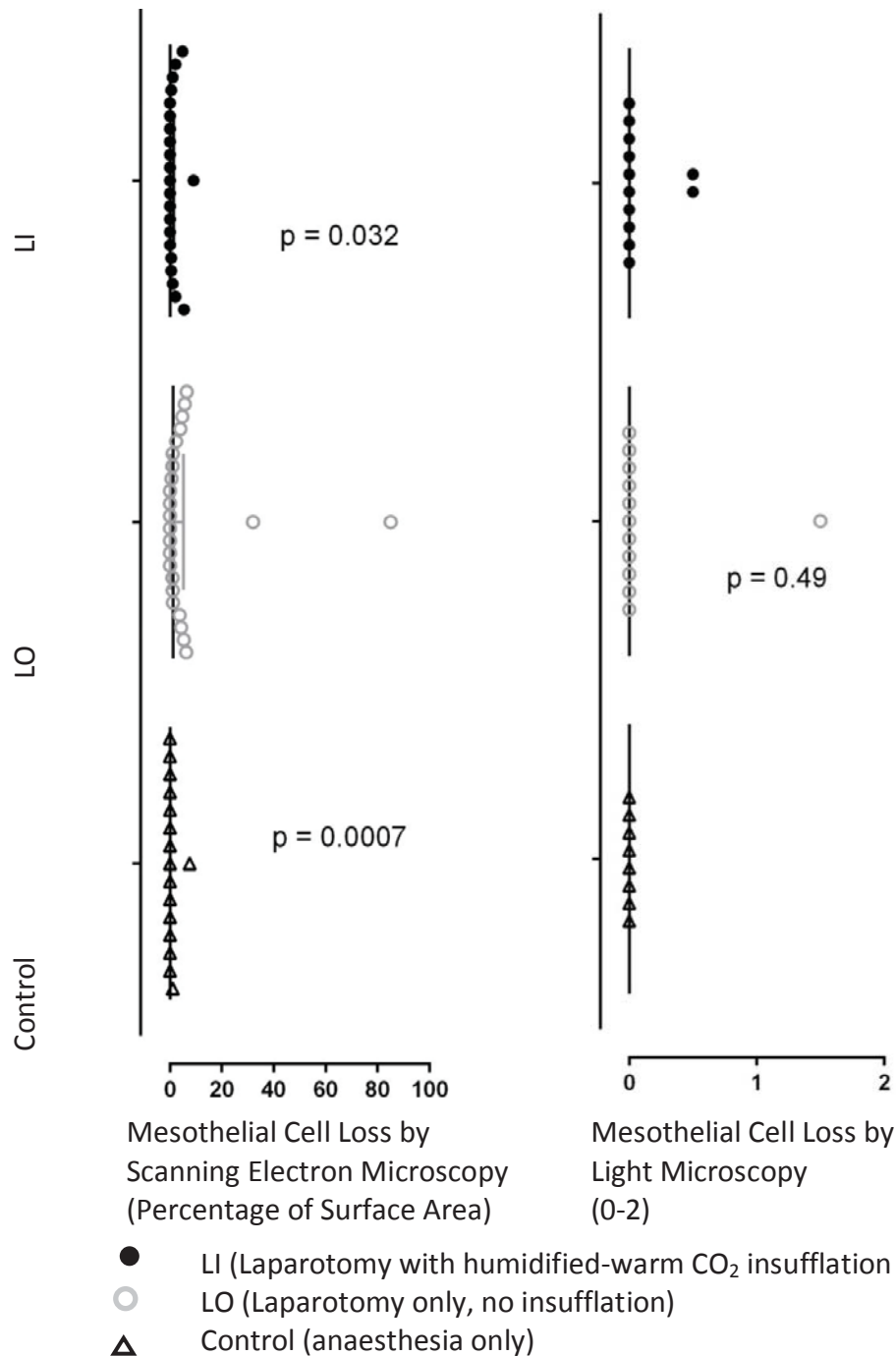


Figure 6-9: Results of analysis of mesothelial cell loss of the non-exposed peritoneum and anaesthesia only controls.

Tissue was analysed independently by scanning electron microscopy (left) and light microscopy (right). Each symbol represents the average score of one image. Data from both parietal and visceral peritoneum are included together. For scanning electron microscopy, p values show the results of Mann Whitney U test of the adjacent group compared with the LO group. For light microscopy the p value shows the result of the Kruskal Wallis test. As the test was not significant, pair wise comparisons were not conducted. Error bars show median and interquartile range. (Control: 2 rats; LO: 4 rats; LI: 3 rats. Four tissue samples were prepared per animal for light microscopy and four for scanning electron microscopy. The whole length of the section was analysed by light microscopy, two areas were analysed by scanning electron microscopy.)

### 6.3.3 Mesothelial cell bulging

Adherent mesothelial cells of the parietal peritoneum scored from scanning electron microscopy images showed significantly greater cellular bulging in the LO group (0.5, 0-1.5) compared with both group C (0, 0-0.38),  $p=0.026$ , and LI (0, 0-0.5),  $p=0.028$  (Figure 6-10). Bulging of the parietal mesothelium was not detected in tissue sections analysed by light microscopy analysis in either exposed or non-exposed samples (C 0, 0-0; LI exposed 0, 0-0; LO exposed 0, 0-0.5; LI non-exposed 0, 0-0.5; LO non-exposed 0, 0-0.25; all  $p$  values  $>0.05$ ).

Bulging scores of spleen tissue was analysed separately due to the expected greater presence of cuboidal shape of cells from this region of the peritoneum (Michailova and Usunoff 2006). Spleen tissue from group C showed variability in normal mesothelial cell morphology (1, 0.125-1.875). In peritoneal samples that were exposed to the gaseous environment, there was less cell bulging in the LO group (0, 0-0.5) compared with the LI group (1.75, 1-2), (Figure 6-10). In peritoneal samples that were not exposed to the gaseous environment, there was no significant difference between the LO group (1.75, 0.75-2) and the LI group (1.5, 1.5-2) (Figure 6-11). There were no significant differences in bulging seen in light microscopy analysis, Kruskal Wallis test  $p=0.39$  for exposed peritoneum and  $p=0.40$  for non-exposed peritoneum (C 1.25, 1-1.5; LO exposed 0.5, 0-1.375; LI exposed 0, 0-1; LO non-exposed 1, 0-1.5; LI non-exposed 0.5, 0-1).

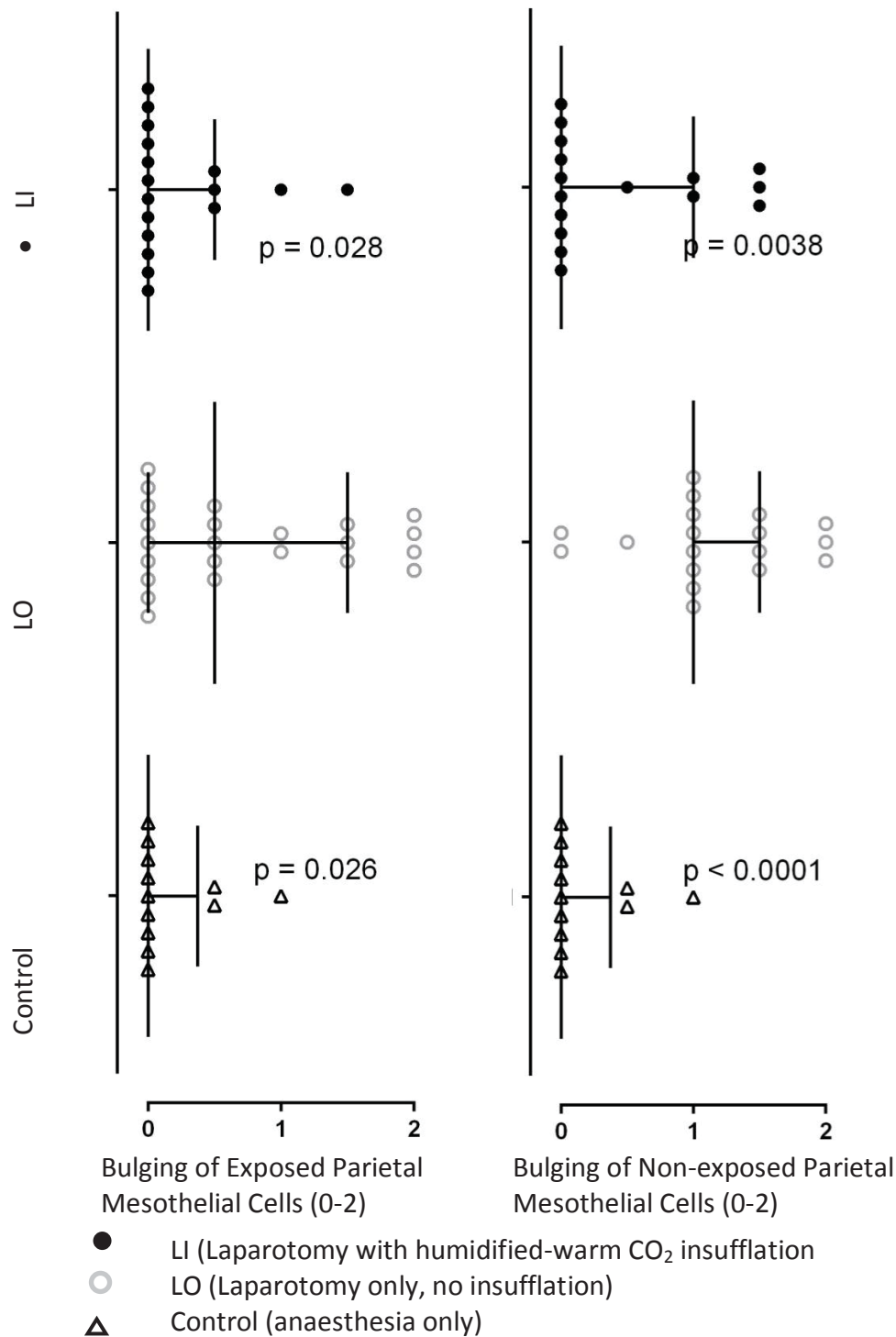


Figure 6-10: Results of SEM analysis of mesothelial bugling of parietal peritoneal tissue.

Tissue exposed to the operating environment is shown on the left and non-exposed on the right. Each symbol represents the average score of one image. P values show the results of Mann-Whitney U test of the adjacent group compared with the LO group. Error bars show median and interquartile range. (Control: 2 rats; LO: 4 rats exposed, 3 rats non-exposed; LI: 3 rats. Three exposed and three non-exposed parietal tissue samples were prepared per animal, and two areas per sample were analysed by scanning electron microscopy.)

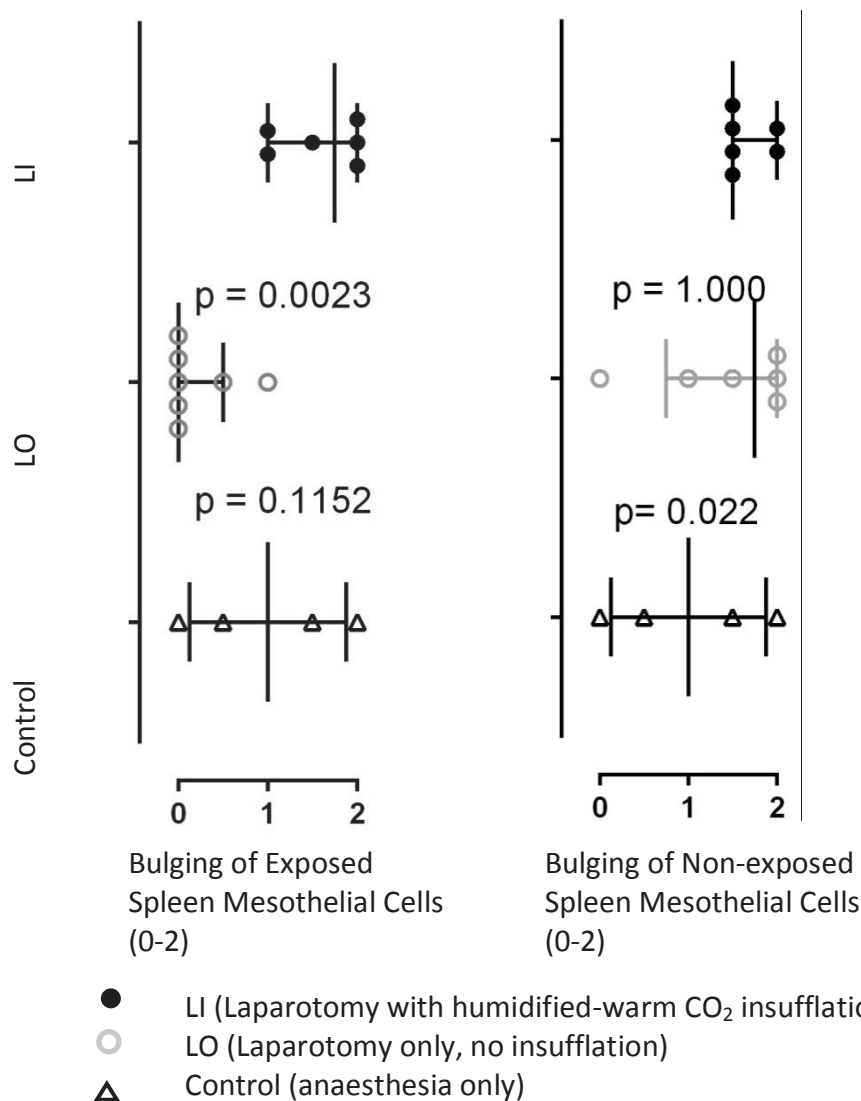


Figure 6-11: Results of SEM analysis of mesothelial bugling of visceral peritoneal tissue.

Tissue exposed to the operating environment is shown on the left and non-exposed on the right. Each symbol represents the average score of one image. P values show the results of Mann Whitney U test of the adjacent group compared with the LO group. Error bars show median and interquartile range. (Control: 2 rats; LO: 4 rats exposed, 3 rats non-exposed; LI: 3 rats. One exposed and one non-exposed visceral peritoneal tissue sample was prepared per animal, two areas from each sample were analysed by scanning electron microscopy. One exposed sample from LO was damaged and could not be analysed.)

#### 6.3.4 Sub-mesothelial thickness

There was no significant difference in sub-mesothelial thickness between any groups when measured across the entire section, Kruskal Wallis  $p = 0.1175$  for exposed peritoneum and  $p = 0.8484$  for non-exposed peritoneum (C 25.99, 13.55-47.23  $\mu\text{m}$ ; LO exposed 36.13, 30.26-62.09  $\mu\text{m}$ ; LI exposed 26.48, 16.96-35.72  $\mu\text{m}$ ; LO non-exposed 34.19, 20.57-44.37  $\mu\text{m}$ ; LI non-exposed 24.63, 15.43-60.98  $\mu\text{m}$ ) (Figure 6-12). However, when measurements were taken only in tissue that was parallel to underlying muscle fibres sub-mesothelial thickness of the LO group (34.19, 28.07-58.06  $\mu\text{m}$ ) was higher than both the C (16.46, 4.825-20.49  $\mu\text{m}$ ),  $p=0.0182$ , and LI (17.83, 13.42-25.26  $\mu\text{m}$ ),  $p=0.0012$ ) groups. There was no significant difference between exposed and non-exposed tissue measurements, so those groups were combined (LO exposed vs non-exposed  $p=0.4290$ ; LI exposed vs non-exposed  $p=0.4103$ ).

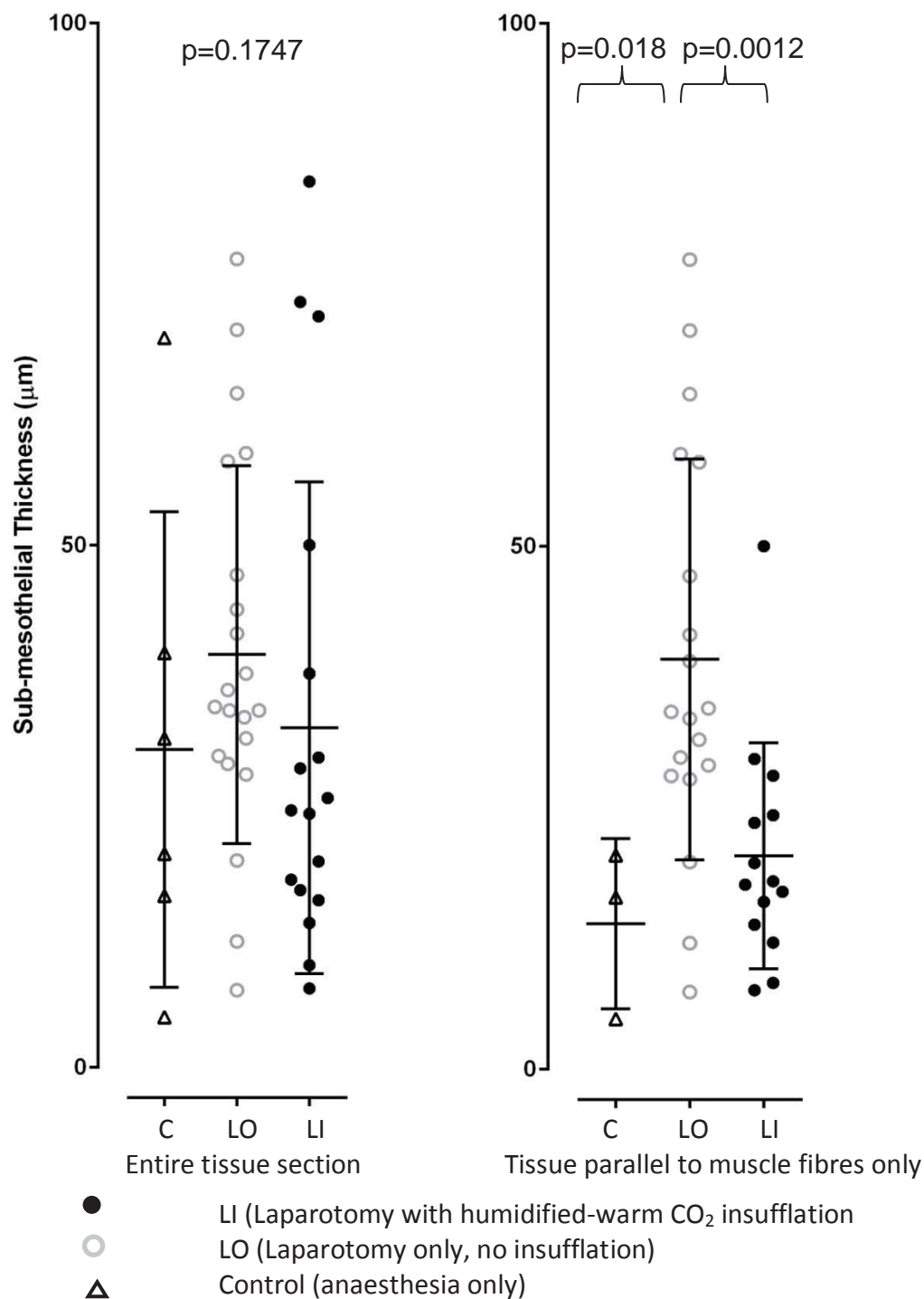
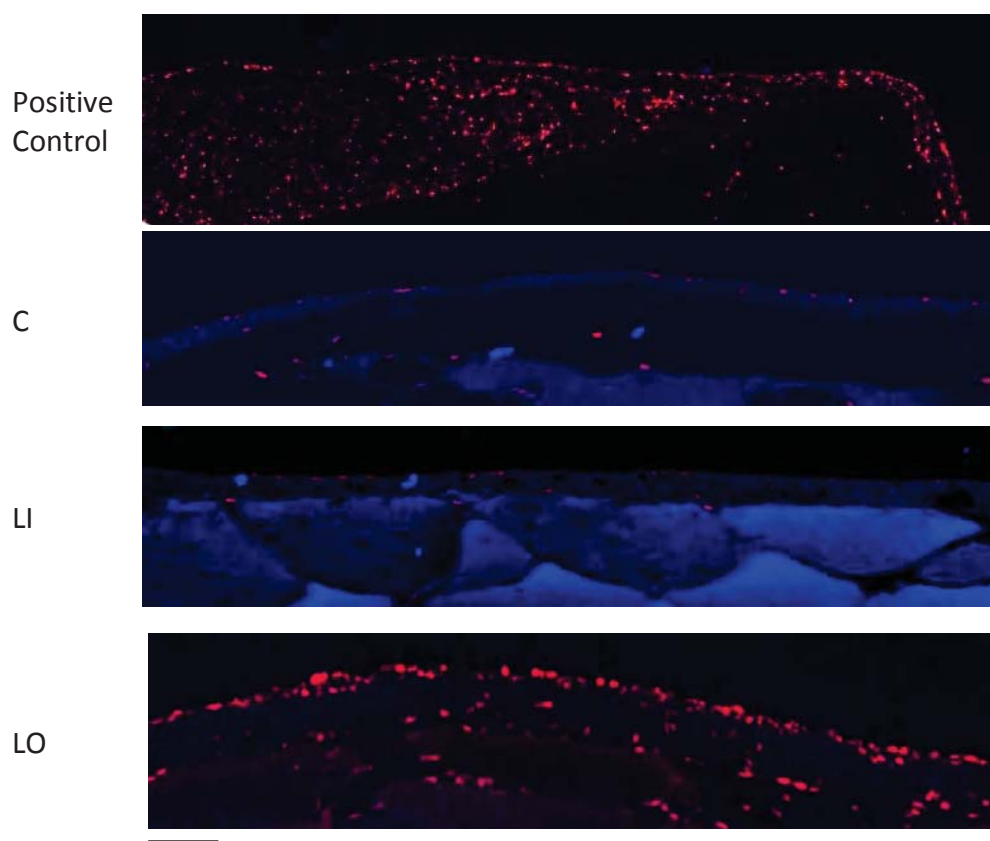


Figure 6-12: Sub-mesothelial thickness of parietal peritoneal tissue.

Thickness was measured in the entire tissue section (left) and in tissue cut parallel to the muscle fibres only (right). Measurements of exposed and non-exposed tissue were combined as there was no significant difference between those groups. Error bars show median and interquartile range. P value on the left shows the result of a Kruskal Wallis test. P values on the right show the results of Mann Whitney U tests of the adjacent group compared with the LO group. (Control: 2 rats; LO: 4 rats exposed, 3 rats non-exposed; LI: 3 rats. Three exposed and non-exposed parietal tissue samples were prepared per animal. Some samples did not have areas in which the tissue was parallel to the muscle fibre, and therefore the sample size is smaller in that analysis.)

#### 6.3.5 Detection of apoptosis by TUNEL assay

There was significantly higher average fluorescence on the surface of exposed peritoneum in the LO group (69.0, 10.5-151.5 %) compared with both group C (1.5, -9.75-8.0%),  $p=0.0055$ , and LI (-20.0, -41.0-6.0 %),  $p=0.0003$ , see Figure 6-13 and Figure 6-14. One spleen tissue sample was discarded from analysis due to massive macroscopic damage during processing. In the spleen tissue samples comparison with adjacent, non-exposed peritoneum was possible. Red fluorescence was observed on the exposed surface that was visible at low magnification. The fluorescence extended beneath the mesothelium and was not seen on the non-exposed surfaces, see Figure 6-15.



**Figure 6-13:** Example fluorescent microscopy images of parietal peritoneal tissue stained with a TUNEL assay for the detection of apoptosis.

C and LI: Little fluorescent red TUNEL staining along the peritoneal edge. LO: Significantly increased fluorescent red TUNEL staining along the peritoneal edge. Bar = 50  $\mu$ m. Blue colouring is included to allow visualisation of adjacent tissue structures and is due to technical difficulties with DAPI staining, likely due to the use of glutaraldehyde fixation.

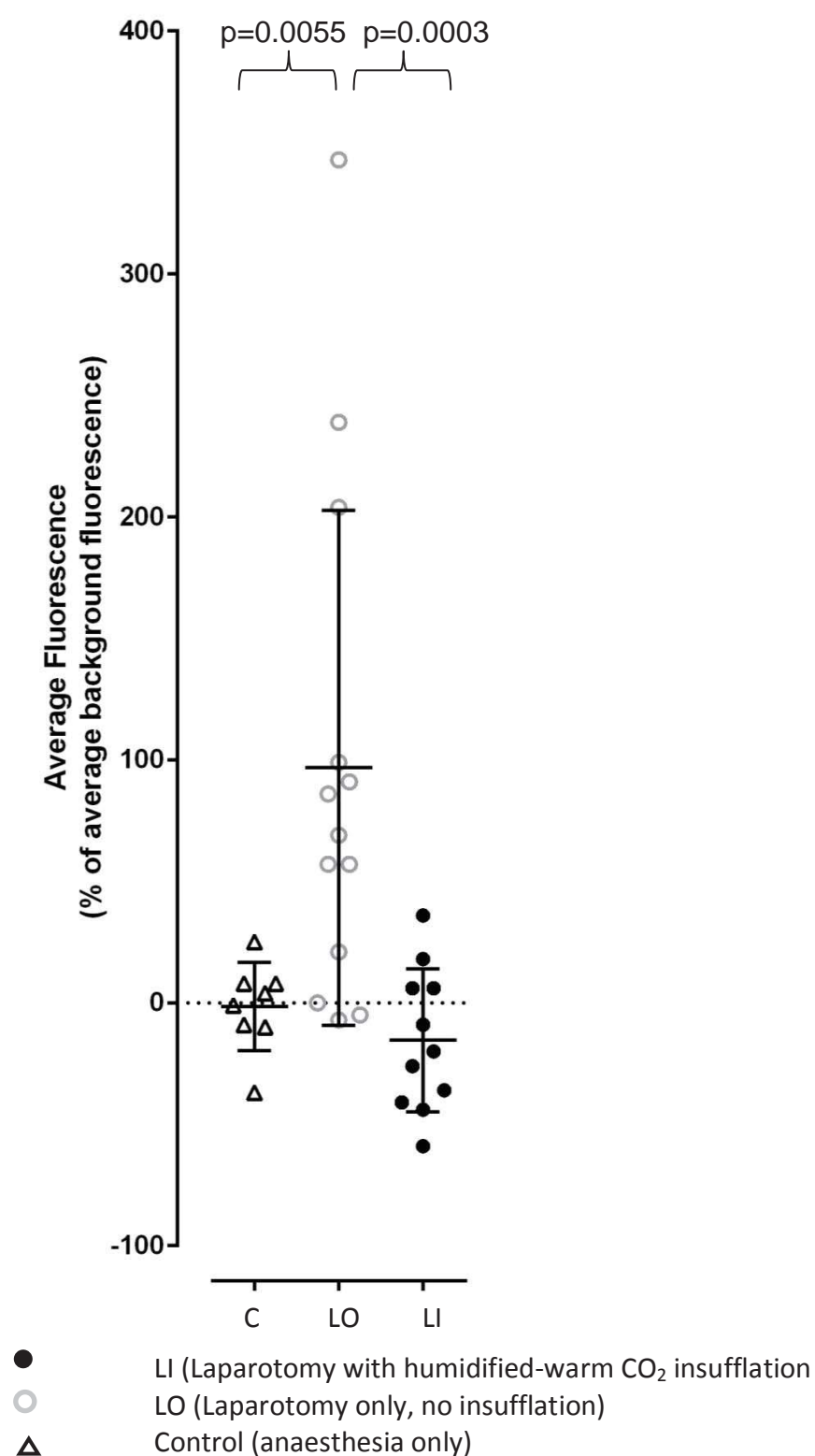


Figure 6-14: Average fluorescence along surface of TUNEL stained peritoneal tissue sections (exposed tissue only, visceral and parietal peritoneum combined) (Control: 2 rats; LO: 4 rats; LI: 3 rats. Four tissue samples were analysed per animal, three parietal and one visceral.)

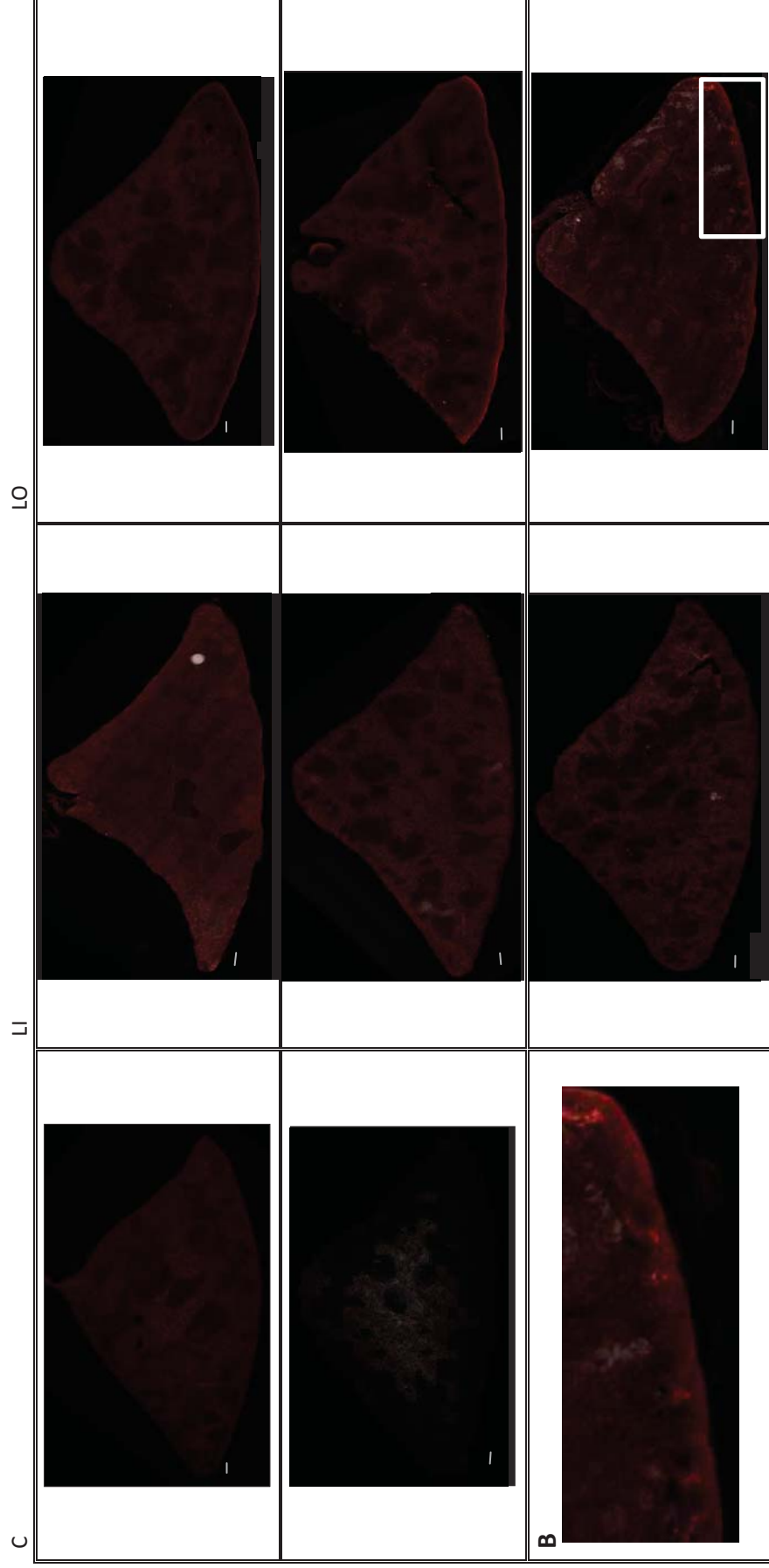


Figure 6-15: Fluorescent microscopy images of visceral peritoneal tissue stained with a TUNEL assay for the detection of apoptosis.

In all LI and LO images the lower, convex surface of the spleen was exposed during surgery. C and LI: Little fluorescent red TUNEL staining along all peritoneal edges. LO: Increase in fluorescent red TUNEL staining on the exposed peritoneal surface that is not observed on the non-exposed surface. Staining extends well below the mesothelium. (Note: one tissue sample from the LO group was discarded due to massive damage during processing). White bar = 200  $\mu$ m. B: An enlarged view of fluorescence present in the white box of the third LO tissue sample.

### 6.3.6 COX-2 expression

There was no significant difference in COX-2 expression between any of the groups, Kruskal Wallis test  $p = 0.46$  (C 2, 1.2-2.5; LO 1.8, 1.5-2.3; LI 1.8, 1.2-1.9), see Figure 6-16. Figure 6-17 illustrates COX-2 expression in a representative image.

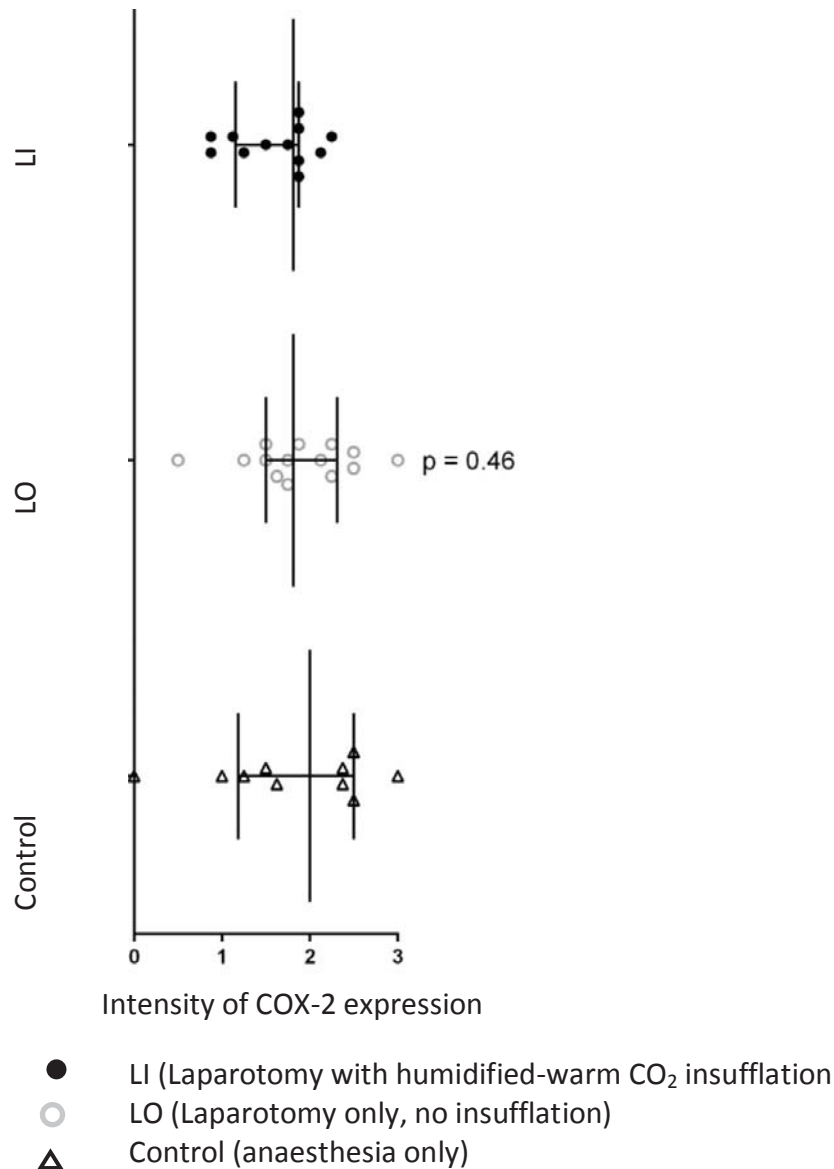
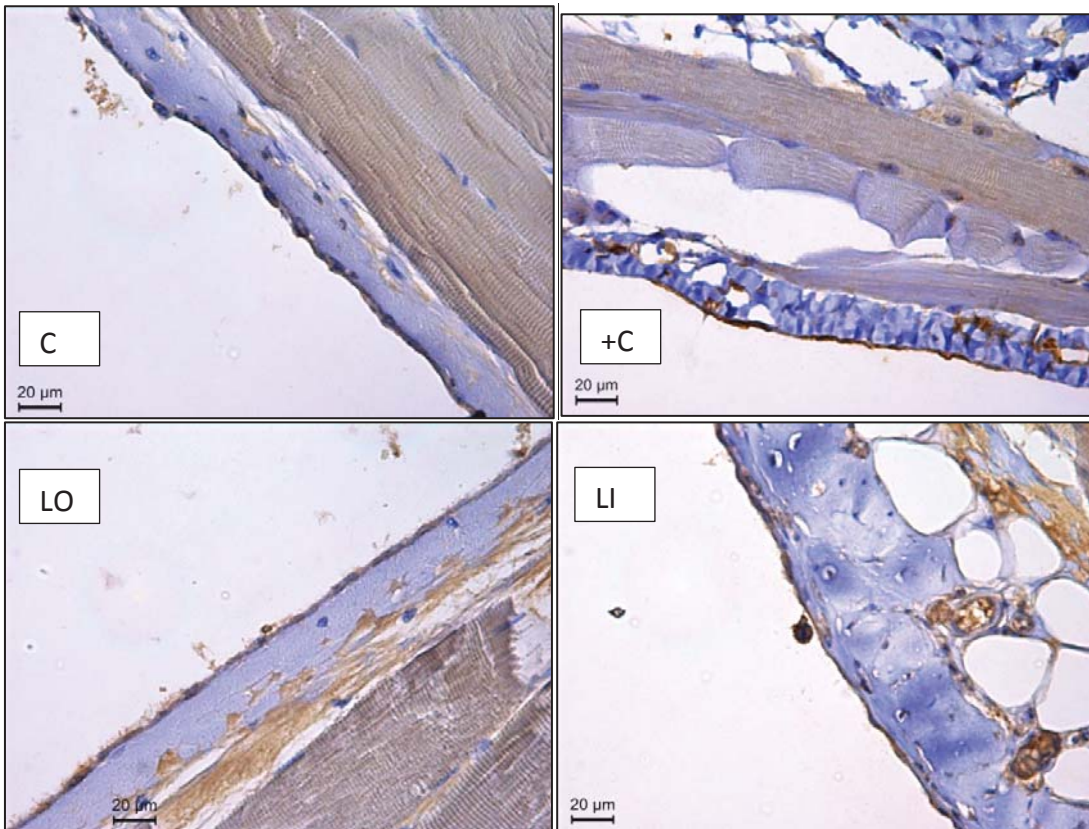


Figure 6-16: COX-2 expression in parietal and visceral peritoneum.

P-value shows result of Kruskal Wall test. Error bars show median and interquartile range. (Control: 2 rats; LO: 4 rats exposed; LI: 3 rats. Four tissue samples were analysed per animal. The whole length of the section was prepared. Four representative images were taken from each sample, in areas where mesothelial cells could be identified, if possible. The scores given to each of the four images from each section were averaged to give one score per tissue section.)



**Figure 6-17: Representative image of COX-2 expression showing brown COX-2 staining in parietal mesothelium and blue counterstaining of haematoxylin.**

**C: Experimental control (anaesthesia only); +C: Positive control for COX-2; LO: Laparotomy only, no insufflation; LI: Laparotomy with humidified-warm CO<sub>2</sub> insufflation.**

#### 6.3.7 Detection of intra-operative mesothelial damage by fluorescein

Rat one: (As part of a separate experiment, this rat had been anaesthetised for four hours and had undergone laparotomy with exposure of the left abdominal wall to both periods of dry-cold CO<sub>2</sub> insufflation and humidified-warm CO<sub>2</sub> insufflation.) Fluorescein was applied to the surface of the peritoneum on both the left and right abdominal walls for 20 seconds then rinsed away. Fluorescence was observed at each point of application (Figure 6-18). Note that when the 1 % fluorescein solution was applied to the tissue surface, it formed a 'bubble' over the area of application until rinsed away, explaining why fluorescence is observed in the specific area of application only. The spleen, which had been protected from gaseous exposure by overlying abdominal organ, was mobilised. After application of fluorescein, fluorescence was observed at an area where an inadvertent clamping injury had been made. Additional damage was made with the clamp and the again fluorescence was observed only at the site of injury.

Rat two: (As part of a separate experiment, this rat had been anaesthetised for four hours and had undergone laparotomy with exposure of the left abdominal wall to both periods of dry-cold CO<sub>2</sub> insufflation and ambient air.) The spleen, which again had been protected from gaseous exposure by overlying abdominal organ, was mobilised. Fluorescein was then immediately applied for 20 seconds then rinsed away. No fluorescence was observed on the peritoneal surface, see Figure 6-19 A. Fluorescein was reapplied following 2 minutes of exposure to air and fluorescence still was not observed, see Figure 6-19 B. The heat lamp was then turned on to accelerate

desiccation. Following a further 9 minutes, fluorescein was reapplied and distinct fluorescence was observed in the area of fluorescein application, see Figure 6-19 C. Similar results were seen when fluorescein was applied to the abdominal right wall, converse to the results with rat one in which fluorescence was observed following the initial application fluorescein.

Rat three: (As part of a separate experiment, this rat had been anaesthetised for 5 hours and had undergone laparotomy with exposure of the left abdominal wall to both periods of dry-cold CO<sub>2</sub> insufflation and ambient air). A small distal portion of the spleen had been exposed during the experiment. The spleen was retracted, exposing the remainder of the spleen to humidified-warm CO<sub>2</sub>, and fluorescein was applied. The distal portion of spleen that had been exposed prior to humidified-warm CO<sub>2</sub> insufflation showed fluorescence on initial application of fluorescein while there was no fluorescence on the remaining, proximal surface of the spleen, see Figure 6-20. Following 10 minutes of exposure to humidified–warm CO<sub>2</sub> insufflation, still no fluorescence was observed on the proximal surface. Humidified-warm CO<sub>2</sub> insufflation was turned off and the abdomen was exposed to ambient air, without a heat lamp. After 10 minutes of exposure to ambient air, still no fluorescence was observed.

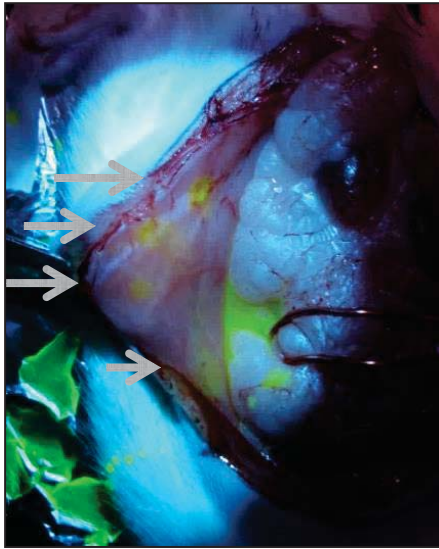


Figure 6-18: Fluorescence at points of fluorescein application on the surface of the parietal peritoneum (arrows) in rat one.

Note that arrows are offset to the left of the staining in order to avoid obscuring the image. Also note the desiccation of the fatty tissue on the right of the illuminate portion of the image.

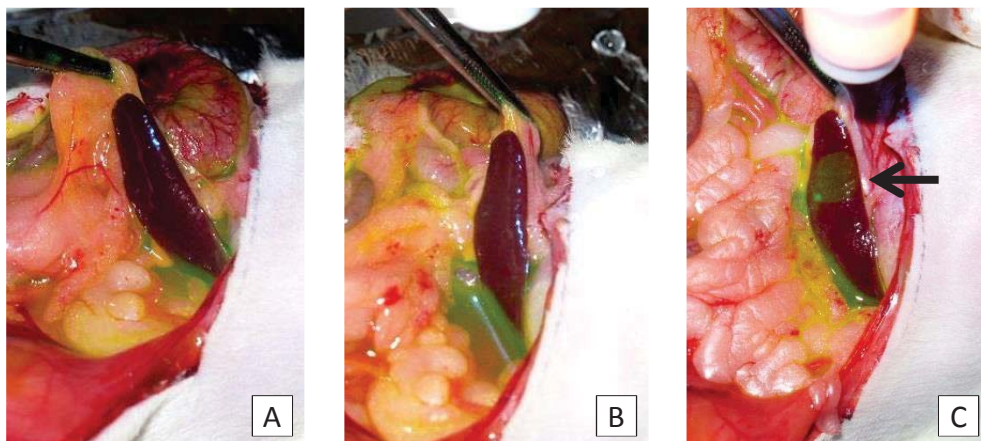
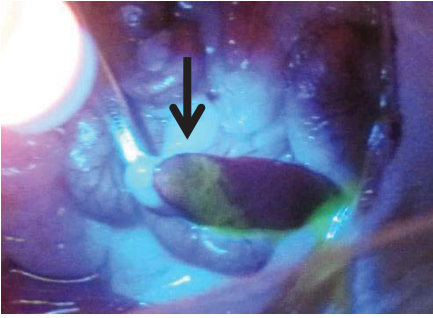


Figure 6-19: Fluorescein staining, green, on the surface of the spleen in rat two.

Image A and B show no fluorescence on the peritoneal surface immediately after mobilisation of the spleen (A) and after reapplication of fluorescein following 2 minutes of exposure to air (B). Image C shows distinct fluorescence (arrow) at the area of fluorescein application when reappplied after 11 minutes of exposure to air with an overhead heat lamp on for the final 9 minutes.



**Figure 6-20: Fluorescein staining on the surface of the spleen in rat three.**

Fluorescein was applied over the entire surface, but preferential adhered to the distal portion of the spleen (arrow) that was exposed to a desiccating environment during a preceding experiment.

## 6.4 Discussion

This study has for the first time established that simply exposing the peritoneum to conditions that replicate recommended air flow within an operating room causes sufficient desiccation to result in inadvertent loss of parts of the mesothelium. The mesothelial loss was consistent in peritoneal tissue independently analysed by both scanning electron and light microscopy. An extraordinary 25 % of the peritoneum sampled had more than half its surface area desquamated of mesothelial cells.

Furthermore, significant loss of mesothelium was only observed on the peritoneum exposed to the air flow and not on the contralateral abdominal wall. This suggests that the inadvertent loss of mesothelium was caused by exposure to the air flow, rather than the large surgical incisions made in the abdominal wall. Evidence was also found to support the second hypothesis that inadvertent loss of mesothelium by desiccation can be prevented with humidified-warm CO<sub>2</sub> insufflation into the open abdominal cavity.

Furthermore, the results suggest that this loss of mesothelium following laparotomy is accompanied by signs of inflammation in the mesothelial cells that remain adherent to the peritoneum, illustrated by increased bulging of parietal mesothelium both on exposed parietal peritoneum and parietal peritoneum that was not exposed, and also an increase in sub-mesothelial thickness following laparotomy (Whitaker and Papadimitriou 1985; Mutsaers, Whitaker et al. 1996; Mutsaers, Whitaker et al. 2000; Mutsaers, Whitaker et al. 2002). Humidified-warm CO<sub>2</sub> insufflation significantly reduced bulging of mesothelial cells and sub-mesothelial thickness. In contrast to the

predominantly flat mesothelial cells of the parietal peritoneum, visceral mesothelium of the spleen is normally characterised by cuboidal mesothelial cells (Michailova and Usunoff 2006) and therefore bulging of cells was expected in un-damaged mesothelium of the spleen. Of interest, there was a significant decrease in bulging in tissue exposed to the ambient environment compared to tissue that was exposed to humidified-warm CO<sub>2</sub>, and this difference was only seen in exposed tissue. It may be possible that reduction of a bulged appearance in normally cuboidal cells represents a deleterious change in function to those cells caused by exposure to ambient air. However, the expression of the inflammatory marker COX-2 was the same in all groups.

Unexpectedly, COX-2 expression was high in the control group. This may suggest that in the current rat model COX-2 is insensitive to the insult of laparotomy compared with anaesthesia alone. Alternatively, the lack of difference may be because of the use of tissue sections, in which only a single layer of mesothelial cells could be viewed. It should be acknowledged that the analgesia used in the current rat model was a non-steroidal anti-inflammatory drug. While non-steroidal anti-inflammatory drugs are used to relieve inflammation by inhibiting the action of COX-1 and COX-2, not the expression of COX itself, research suggests that in specific cells non-steroidal anti-inflammatory drugs can enhance expression of COX-2 (Pang, Nie et al. 2003).

Peritoneal damage was further illustrated by a marked increase in apoptosis, measured by DNA fragmentation, on the surface of exposed peritoneum in the LO group. It is therefore probable that some of the remaining mesothelial cells in the laparotomy group were undergoing apoptosis and quantification of mesothelial cell

loss would have increased if tissue samples were taken at a later time point. The current model is not designed to quantify mesothelial apoptosis. Apoptosis is a process with different cells at different phases of apoptosis at any one time, and therefore quantification would require time course measurements. This model was able to show that 12 hours after abdominal surgery some of the remaining mesothelium cells have DNA fragmentation that is indicative of the end stages of apoptosis. The image analysis used in the current investigation was limited by difficulty in identifying the mesothelial cells in the tissue section, due to inadequate counterstaining. To reduce bias during the analysis, the peritoneal surface was analysed regardless of the presence of mesothelial cells and therefore in areas of mesothelial cell loss DNA fragmentation in the adjacent connective tissue would have been analysed. No fluorescence in connective tissue was observed, and therefore this approach is expected to have underestimated fluorescence in the LO group in which significant mesothelial cell loss occurred. In addition to fluorescence on the surface of the peritoneum, a wide band of DNA fragmentation was observed underlying the exposed surface of the spleen that was in striking contrast to the adjacent non-exposed surfaces. This observation suggests that apoptosis caused by desiccation may not be limited to the mesothelium as hypothesised, damage may also occur in the underlying parenchymal tissue. Humidified-warm CO<sub>2</sub> insufflation significantly reduced DNA fragmentation, supporting the hypothesis that mesothelial cells that remain adherent in the LI group are not undergoing apoptosis.

The observed loss of peritoneal mesothelial following exposure to controlled air flow during laparotomy is consistent with previous investigations into the effect of desiccation. Experimental damage to the peritoneum, in order to study mesothelial healing and adhesion formation, has previously been induced with a “gentle stream” (Ryan, Grobéty et al. 1971; Ryan, Grobety et al. 1973) and 30 L/min (Burns, Skinner et al. 1995) of dry, compressed air for just 5 mins. Furthermore, the observed results are consistent with animal models of endoscopic surgery that have shown that loss of mesothelium by exposure to dry CO<sub>2</sub> can be reduced by humidification of the CO<sub>2</sub> gas (Mouton, Bessell et al. 1999; Erikoglu, Yol et al. 2005; Binda, Molinas et al. 2006; Peng, Zheng et al. 2009; Davey, Hayward et al. 2013). The observed increase in sub-mesothelial thickness is consistent with reports of sub-mesothelial oedema following desiccation of the peritoneum in laparoscopic surgery (Davey, Hayward et al. 2013) and following experimentally induced injury to the mesothelium in a murine model (Mutsaers, Whitaker et al. 2000). It may be possible that an even larger increase in sub-mesothelial thickness would be seen if the tissue samples were taken later than 12 hours following surgery. Results from a murine model suggest that oedema and inflammatory cell infiltration cause a significant increase in sub-mesothelial thickness 2 days following mesothelial injury, peaking at 6 days (Mutsaers, Whitaker et al. 2000).

Damage to the peritoneum during laparotomy is inevitable as often large surgical incisions are required and organs must be manipulated to achieve the surgical objective. However, the current research shows that additional, inadvertent damage to the parietal and visceral mesothelium is caused by desiccation simply by opening and

exposing the abdominal cavity. This may have important clinical implications as uninjured mesothelial cells surrounding surgically damaged mesothelium are essential for adhesion-free healing and prevention of peritoneal tumour implantation (Gillett, James et al. 1994; Mutsaers, Whitaker et al. 2000; Mutsaers and Wilkosz 2007; Hellebrekers and Kooistra 2011). Surgical practice has long recognised the need to prevent intra-abdominal desiccation (DeWilde, Trew et al. 2007; Schnuriger, Barmparas et al. 2010; Koninckx, Ussia et al. 2012; The Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society of Reproductive Surgeons 2013). However, surgeons are faced with the problem of how to prevent damage to the peritoneal mesothelium caused by desiccation during a time of growing criticism against the unnecessary use of intra-peritoneal lavage (Mutsaers and Wilkosz 2007; Hellebrekers and Kooistra 2011; The Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society of Reproductive Surgeons 2013; National Institute for Health and Care Excellence 2014) and the knowledge that rubbing the delicate peritoneum with wet packs likely also causes mesothelial damage (Van Den Tol, Van Stun et al. 1997; von Ruhland, Newman et al. 2003). The innovation of intra-abdominal insufflation of humidified-warm CO<sub>2</sub> is that it reduces desiccation invisibly, with no effort from the surgeon and without the risks to the mesothelium associated with unnecessary intra-peritoneal lavage or rubbing the peritoneum with wet packs.

Ideally these results will now be extended to include human data. However, the current research design would be difficult to repeat in human subjects due to the need

to delay collection of peritoneal tissue samples after the completion of surgery to allow for inflammatory changes to occur, which is likely un-ethical in humans. Research in laparoscopy suggests that tissue samples taken at the time of surgery will show an intact mesothelium under scanning electron microscopy, even under conditions that have been shown to result in cell loss when sample collection is delayed (Volz, Koster et al. 1999). However, a recent study has used transmission electron microscopy to show that apoptotic bodies are present in human mesothelial cells of peritoneum taken at the end of laparoscopic surgery, supporting previous animal results (Tarhan, Barut et al. 2013). The use of transmission electron microscopy requires ultra-thin sections and therefore is able to represent only a small number of mesothelial cells compared with en-face analysis, however, the results are promising. Preliminary results from the current investigation suggest that topical fluorescein may be an interesting visual illustration of peritoneal damage during surgery as fluorescein appears to adhere preferentially to desiccated or mechanically damaged areas of peritoneum, both on the visceral peritoneum of the spleen and on the parietal peritoneum. Collection of more extensive information of processes within the mesothelium may be possible by looking at expression of important genes, such as those relevant to adhesion formation and inflammation (Matsuzaki, Jardon et al. 2012) or apoptosis and necrosis. It may also be useful to extend the current animal model to look at other outcomes, and to investigate some of the consequences of mesothelial damage; these broader future research directions are discussed in the concluding chapter of the thesis, chapter 7.

In conclusion, the current study has shown that, in a rat model, exposing the peritoneal mesothelium to conditions that replicate recommended air flow within an operating room causes inadvertent loss of mesothelium that can be prevented by insufflating humidified-warm CO<sub>2</sub> into the open abdominal cavity. This finding suggests that humidified-warm CO<sub>2</sub> provides a simple method to reduce desiccative damage to the peritoneal mesothelium without the need for intra-peritoneal irrigation or wet packs.

## 7 Discussion and Conclusions

### 7.1 Introduction

This research developed an animal model to investigate two un-tested mechanisms of action by which humidified-warm CO<sub>2</sub> insufflation during open abdominal surgery may reduce the risk of post-operative complications. The investigations discovered that insufflation of humidified-warm CO<sub>2</sub> increased local tissue oxygen partial pressure (PtO<sub>2</sub>) and decreased loss of peritoneal mesothelium caused by exposure to the ambient gaseous environment. This research has important implications for complication-free post-surgical recovery and wound healing. This fills a significant gap in the literature that was confirmed by narrative and systematic review of the current state of knowledge.

### 7.2 Summary and discussion of main findings

The rat model developed was able to successfully illustrate effects of gaseous exposure during open abdominal surgery. In separate investigations, the current research has shown that insufflation of humidified-warm CO<sub>2</sub> following open abdominal surgery in a rat model increases intra-operative PtO<sub>2</sub> and decreases loss of peritoneal mesothelium. Insufflation of humidified-warm CO<sub>2</sub> into the abdominal cavity during open abdominal surgery caused an immediate and clinically significant increase in local PtO<sub>2</sub> compared with exposure to ambient air (average increase 29.8 mmHg or 96.6 %).

This is consistent with previous findings that dry CO<sub>2</sub> pneumoperitoneum during laparoscopic surgery increases PtO<sub>2</sub> in humans (Gianotti, Nespoli et al. 2011) and mice (Bourdel, Matsuzaki et al. 2007). The current research was the first to investigate the effect of humidified-warm CO<sub>2</sub> on PtO<sub>2</sub> and to attempt to elucidate the relative effect of dry versus humidified-warm CO<sub>2</sub>. A series of randomised cross-over experiments showed the increase in PtO<sub>2</sub> was an additive result of the delivery of CO<sub>2</sub> and avoidance of evaporative cooling via the delivery of the gas humidified at body temperature. With respect to loss of peritoneal mesothelium, a systematic review found a significant gap in current knowledge as to whether gaseous exposure of the peritoneum during laparotomy causes loss of peritoneal mesothelium. To address that gap, this research provides the first evidence that simply exposing the peritoneal mesothelium to conservative air flow for 1 hour caused inadvertent loss of mesothelium that was not observed on the contralateral, un-exposed abdominal wall. Furthermore, as hypothesised, mesothelial cell loss was prevented by insufflating humidified-warm CO<sub>2</sub> into the open abdominal cavity. This supports previous evidence that humidified-warm CO<sub>2</sub> during endoscopic surgery prevents mesothelial loss (Mouton, Bessell et al. 1999; Erikoglu, Yol et al. 2005; Peng, Zheng et al. 2009; Davey, Hayward et al. 2013).

It is likely that the two mechanisms tested in this research interact. An increase in local PtO<sub>2</sub> has been shown to decrease the risk of post-operative adhesion formation (Molinas, Mynbaev et al. 2001; Matsuzaki, Canis et al. 2007). This suggests that increased PtO<sub>2</sub> protects against loss of peritoneal mesothelium, and therefore it is

probable that the increase in  $PtO_2$  observed during exposure to a humidified-warm  $CO_2$  environment also contributed to observed protection against mesothelial loss. Conversely, low tissue oxygenation upon exposure to the ambient air of the operating room likely contributed to the loss of peritoneal mesothelium observed following laparotomy without  $CO_2$  insufflation. It is also possible that the concomitant increase in sub-mesothelial thickness observed 12 hours following laparotomy without  $CO_2$  insufflation may lead to a decrease in sub-mesothelial  $PtO_2$  due to larger diffusion distance (Tsai, Johnson et al. 2003). This post-operative effect is unlikely to have contributed to the intra-operative  $PtO_2$  changes observed in the current model. However, by avoiding sub-mesothelial oedema, the use of humidified-warm  $CO_2$  may result in higher tissue oxygenation post-operatively. It is also likely that an increase in local  $PtO_2$  and protection against loss of peritoneal mesothelium will interact to prevent post-operative infections. The role of high  $PtO_2$  in avoiding wound infections is well established (Hopf and Rollins 2007), however, it is likely that maintenance of an intact mesothelial layer may further decrease the risk of infection, as the mesothelial layer provides both a physical barrier and also plays an active role in the immune response (Mutsaers and Wilkosz 2007). Signalling by the intact mesothelial will likely attract more to phagocytes to utilise the increase in available oxygen.

The results of this research provide evidence to support the hypothesised mechanisms of action by which insufflation of humidified-warm  $CO_2$  may reduce post-operative complications (Persson and van der Linden 2008; Persson and van der Linden 2009).

Previous research has shown that insufflation of humidified-warm CO<sub>2</sub> during open abdominal surgery decreases airborne contamination (Persson and Van Der Linden 2004; Persson and van der Linden 2004), decreases the growth of *Staphylococcus aureus* (Persson, Svenarud et al. 2005; Persson and van der Linden 2008), improves core and wound temperature maintenance (Frey, Svegby et al. 2010; Frey, Janson et al. 2012; Frey, Janson et al. 2012) and decreases the risk of hypothermia (Frey, Janson et al. 2012). With the addition of the significant results presented in this thesis, there is building evidence that insufflation of humidified-warm CO<sub>2</sub> is a simple therapy with a multi-pronged mechanisms to reduce post-operative complications. Furthermore, the therapy presents a solution to the lack of practical advice on how to achieve surgical practice recommendations that state during open abdominal surgery tissue oxygenation should be maintained (Sessler 2006; Hopf and Rollins 2007; Yoshida, Nabeshima et al. 2007; Hunt, Gimbel et al. 2008; Davis and Rivadeneira 2013) and desiccation avoided (DeWilde, Trew et al. 2007; Schnuriger, Barmparas et al. 2010; Koninckx, Ussia et al. 2012; The Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society of Reproductive Surgeons 2013).

### 7.3 Significance of the research

This research provides a significant body of research regarding the effects of gaseous exposure of the peritoneum during open abdominal surgery. The experimental animal model developed by this research provides a controlled model in which to study these

effects in an optimised gaseous environment, and has allowed investigation into the mechanisms of action of insufflation of humidified-warm CO<sub>2</sub> during open abdominal surgery.

This research is important as there has been a call for additional technologies to reduce the on-going burden of post-operative complications (Yoshida, Nabeshima et al. 2007; Alpay, Saed et al. 2008; Aimaq, Akopian et al. 2011). Insufflation of humidified-warm CO<sub>2</sub> into the peritoneal cavity has been identified as a promising innovation to reduce post-operative infection and adhesion formation (Persson and van der Linden 2008; 2009; Persson and van der Linden 2009), despite gaps in understanding of its mechanism of action. These are common and expensive complications of open abdominal surgery (Klevens, Edwards et al. 2007; Fleming, Kim et al. 2010; Howard, Datta et al. 2010; Schnuriger, Barmparas et al. 2010; Utsumi, Shimizu et al. ; Aimaq, Akopian et al. 2011; Suh, Jeong et al. 2012; Okabayashi, Ashrafian et al. 2013). The results of this research are a critical step towards understanding a therapy that has the potential to make a vast impact on modern surgery.

#### 7.4 Limitations

Limitations specific to the individual investigations have been presented in each of the respective chapters. However, limitations are discussed here that are applicable to more than one chapter.

Firstly, the current research is limited by being carried out in an animal model. Applicability to clinical practise is therefore inferred. Nonetheless, the relevance of results from a carefully designed animal model is supported by a growing body of evidence successfully translating the results of rodent models of the effect of surgery on the peritoneal mesothelium to humans (Matsuzaki, Botchorishvili et al. 2011; Matsuzaki, Jardon et al. 2012; Koninckx, Corona et al. 2013). In one series of investigations, similar changes in markers of the risk of adhesion formation following abdominal surgery were measured in peritoneal samples taken in parallel from humans and mice (Matsuzaki, Botchorishvili et al. 2011). Furthermore, interventions that were identified to reduce adhesion formation in a large series of murine investigations (Binda and Koninckx 2009) were shown to significantly reduce adhesion formation in women following gynaecological surgery (Koninckx, Corona et al. 2013). With regard to tissue oxygenation measurements there is some evidence that the  $PtO_2$  response is similar between species, as similar increases in  $PtO_2$  have been reported in rodent (Bourdel, Matsuzaki et al. 2007) and human (Greif, Akça et al. 2000) surgical models in response to increased fraction of inspired oxygen.

A further possible limitation to the application of the current research is that the two primary outcomes were mechanistic and no clinically relevant endpoints were measured. In review of the literature prior to conducting the current research, it was determined that mechanistic studies were lacking in support of the lofty hypotheses about the potential benefits of humidified -warm  $CO_2$  insufflation during open

abdominal surgery. Such mechanistic studies are important to ensure that the therapy is acting as expected before more clinically relevant investigations are completed. This is especially true considering the large sample sizes that will be required in such investigations due to variability in extent and duration of mesothelial exposure during surgical procedures, see discussion in future research directions below. Furthermore, the outcomes used in the current research were carefully chosen to ensure they relate to important mechanisms of post-operative complications.

Finally, the rat model designed in the current research may be viewed limited insofar as the surgical insult was limited to a large (60 x 40 mm) abdominal incision and movement of the abdominal contents to the right side of the cavity. Although the incision was large relative to the abdomen of the rat, open abdominal surgery would always involve further surgical incisions than the abdominal incision alone. The model was designed with an abdominal incision alone so as to enable the investigation of inadvertent damage to the peritoneal mesothelium. Damage to the peritoneum during surgical manipulation of the abdominal contents are inevitable, however, the presence of neighbouring mesothelial cells is essential to allow the mesothelium to heal adhesion free (Gillett, James et al. 1994; Mutsaers and Wilkosz 2007; Hellebrekers and Kooistra 2011). It is therefore not surprising that inadvertent denudation of adjacent healthy mesothelium, in addition to surgical incision, has been shown to be necessary for adhesion formation (Gillett, James et al. 1994). The current model design was able to demonstrate that inadvertent denudation to the parietal and visceral mesothelium

is caused when desiccation of the peritoneum is allowed. It is possible that the inflammatory burden of additional surgical incisions may overshadow the decreased signs of mesothelial inflammation observed following surgery with humidified-warm CO<sub>2</sub> insufflation compared with surgery without. Inflammation of the mesothelial distal to surgical insult has been observed both in the current research and in previous reports (Mutsaers, Whitaker et al. 1996). However, there is no evidence to suggest that the additional inflammation caused by further incisions would cause loss of distal mesothelial cells, as was observed following desiccation in the current model. It is also unlikely that additional, distal surgical incisions would influence the local sub-mesothelial PtO<sub>2</sub> measurements or their interpretation.

Limitations aside, the strengths of the current research are the development and utilisation of the first controlled animal model of gaseous exposure during open abdominal surgery. The model included careful exposure of the peritoneum to controlled conditions that replicated recommended air flow within an operating room, and when necessary exposure to high CO<sub>2</sub> concentration. The rats were intubated and mechanically ventilated, insensible fluid loss was replaced, and depth of anaesthesia was continuously monitored by a dedicated anaesthetist. Tissue oxygen partial pressure was measured directly using the gold standard for oxygenation measurement (Govinda, Kasuya et al. 2010), with complete assurance that the PtO<sub>2</sub> probe remained in place and that the overlaying tissue remained free of surgical fluids, blood or bowel. Investigation of mesothelial loss and inflammation was supported by multiple

independent measures, and both within and between subject comparisons. Protocols for collecting, fixing and processing the delicate peritoneal tissue samples were carefully developed after thorough review of published literature and conducting optimisation experiments. Finally, the technology investigated is simple, safe, and addresses problems that have been identified in surgery but had thus far been without solution.

## 7.5 Future research directions

### 7.5.1 Tissue oxygenation

Future research should confirm the increase in  $PtO_2$  upon insufflation of humidified-warm  $CO_2$  during open abdominal surgery in humans. This could be conducted using the cross-over design utilised in the current study. Based on the current results of 29.8 mmHg (SD 13.3) increase in  $PtO_2$ , and assuming just one cross-over per patient, the predicted sample size would be as few as 4 patients (assuming  $\alpha=0.05$ ,  $\beta=0.8$ ). If the effect size observed in human surgery was half that observed in the current rat model, in light of reduced control of surgical conditions, the sample size would increase to 9. The protocol could be further improved by the addition of concomitant measurement of  $P_aCO_2$ . Minute ventilation should be adjusted to maintain a constant  $P_aCO_2$ , so as to confirm that the increase in  $PtO_2$  is not due to an increase in  $P_aCO_2$  alone.

### 7.5.2 Surgical site infection

A similar increase in  $PtO_2$  to that observed in the current study following the insufflation of humidified-warm  $CO_2$  has been shown to predict a 30 % drop in surgical site infection rate (Hopf, Hunt et al. 1997). In addition to the effect of an increase in  $PtO_2$ , prevention of damage to the mesothelial layer may further assist in the prevention of deep infection, as the mesothelial layer provides both a physical barrier and also plays an active role in the immune response (Topley 1995; Mutsaers and Wilkosz 2007). Therefore, future research should consider the impact of humidified-warm  $CO_2$  on the rate of surgical site infection. Assuming a patient group can be selected in which surgical site infection rates in the control group are 12 % or 20 % (Pinkney, Calvert et al. 2013), and that humidified-warm  $CO_2$  insufflation could drop infection by 30 % (assuming  $\alpha=0.05$ ,  $\beta=0.8$ ), a total sample size of 1764 or 450, respectively, would be required for a randomised control trial.

### 7.5.3 Wound healing

An increase in  $PtO_2$  by insufflation of humidified-warm  $CO_2$  may also improve wound healing (Hopf and Rollins 2007). For abdominal surgery, the primary outcome of interest should be the rate of wound, or anastomotic, rupture. This could be measured in a large randomised controlled trial, discussed below. However, prior to such an endeavour this ability of intra-abdominal  $CO_2$  to increase  $PtO_2$  near to a bowel anastomosis should first be established to allow appropriate sample size calculations. Measurement of  $PtO_2$  near to a bowel anastomosis could be achieved using a cross-

over animal model. In a randomised trial it would also be possible to continue measurement of PtO<sub>2</sub> post-operatively, and to measure intestinal anastomotic healing via intestinal burst pressure (Glatz, Boldt et al. 2014). Future research should also consider intra-operative measurements of perfusion in humans. There are increasing reports of the clinical utility of intra-operative fluorescent near infra-red spectroscopy for the visualisation of perfusion to bowel anastomoses (Diana, Noll et al. 2014; Ris, Hompes et al. 2014).

A study to investigate a reduction in bowel anastomotic rupture in a human model would require a large sample size. Patients that experience anastomotic leak have significantly lower bowel PtO<sub>2</sub>, approximately 20 mmHg lower, and bowel PtO<sub>2</sub> < 20 mmHg is predictive of anastomotic leak (Sheridan, Lowndes et al. 1987). Assuming that the 29 mmHg increase in PtO<sub>2</sub> measured in the current study could drop leakage rate from 10 % (Sheridan, Lowndes et al. 1987; Buchs, Gervaz et al. 2008) to 5 %, the required total sample size for a trial to compare anastomotic leak rate in patients with and without intra-operative humidified-warm CO<sub>2</sub> would be approximately 398 patients (assuming  $\alpha=0.05$ ,  $\beta=0.8$ ). The surgical technique in such a study would need to be carefully controlled.

If an improvement in wound healing is found, it is likely that the cosmetic result is also improved. This could be relevant to healing of the mid-line incision following laparotomy. However, improvement in wound cosmesis is likely to be of greater

interest to surgical disciplines outside of abdominal surgery, such as plastic surgeons. Future research could also investigate the application of topical humidified-warm CO<sub>2</sub> therapy to wound healing in burns victims.

Wound healing could also be investigated through measurement of surrogate markers, such as angiogenesis and collagen deposition. Previous research has shown that post-operative exposure to CO<sub>2</sub> for just 10 minutes a day increases angiogenesis (Irie, Tatsumi et al. 2005), therefore exposure to CO<sub>2</sub> during surgery that may be several hours could result in a significant increase in angiogenesis. PtO<sub>2</sub> also predicts collagen deposition (Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992), which is extremely important for wound strength (Hopf and Rollins 2007). Caution should be taken in adopting previous protocols investigating post-operative collagen deposition as these took measurement at a surrogate wound in the upper arm (Jonsson, Jensen et al. 1991; Hartmann, Jonsson et al. 1992). Insufflation of humidified-warm CO<sub>2</sub> to the abdominal cavity is not predicted to have a systemic effect and therefore measurement in a surrogate wound would not be relevant. Collagen deposition near to an intestinal anastomosis would be most relevant. However, this would likely be limited to animal investigations and would be limited by the size of the measurement catheter relative to the anastomosis. Collagen deposition at a surrogate wound in a location that was exposed to the humidified-warm CO<sub>2</sub> environment, such as on the abdominal wall, may be an appropriate compromise.

#### 7.5.4 Adhesion formation

The current rat model could be extended to investigate the incidence and tenacity of post-operative adhesion formation, based on animal models of laparoscopy have shown that reduction of desiccation by humidification of intra-abdominal CO<sub>2</sub> reduces post-operative adhesion formation (Binda, Molinas et al. 2006; Peng, Zheng et al. 2009). Furthermore, it may be possible to investigate some of the consequences of post-operative adhesion formation such as adhesion related hospital readmissions (Parker, Wilson et al. 2005) or adhesive bowel obstruction. Although the mortality of adhesive bowel obstruction is high, the incidence of adhesive bowel obstruction is approximately 8 % following open colo-rectal procedures (Reshef, Hull et al. 2013). A randomised control trial on this topic would therefore require a very large sample size. Investigation of the presence of adhesion in cases with a planned second surgery could be conducted (Koninckx, Corona et al. 2013), however, there is no means of determining whether such adhesions are clinically relevant.

#### 7.5.5 Intra-peritoneal tumour dissemination

Previous research using mouse models of laparoscopic surgery has shown that preventing desiccation of the peritoneal mesothelium, by humidification of insufflation CO<sub>2</sub>, reduces intra-peritoneal tumour dissemination (Binda, Corona et al. 2014; Carpinteri, Sampurno et al. 2015). This is likely due to reduction in loss of peritoneal mesothelium, which exposes the basement membrane resulting in preferential areas for tumour implantation (Volz, Koster et al. 1999), and may also be due to

maintenance of free hyaluronan available to bind to intra-peritoneal tumour cells (Mutsaers and Wilkosz 2007). The results of the current study, that insufflation of humidified-warm CO<sub>2</sub> during open abdominal surgery prevents loss of peritoneal mesothelium, warrant the investigation of the effect on intra-peritoneal tumour dissemination. This should be conducted in an animal model in the first instance. Translation to humans can then be begun by investigating mechanisms of action, such as loss of peritoneal mesothelium and peritoneal inflammation. Longitudinal investigation of the incidence of intra-peritoneal tumour dissemination following surgery has been conducted using retrospective data from databases (Segelman, Granath et al. 2012; Kerscher, Chua et al. 2013). In patients with colo-rectal cancer, 4 % (Segelman, Granath et al. 2012) to 6 % (Kerscher, Chua et al. 2013) of patients were diagnosed with metachronous peritoneal tumour dissemination a median of 16 months after the initial cancer diagnosis (Segelman, Granath et al. 2012). The peritoneum was the sole site of metastasis in > 50 % of patients with metastatic disease (Segelman, Granath et al. 2012) and such metastasis remains fatal (Kerscher, Chua et al. 2013). Despite the devastating consequences, the disease incidence and length of follow-up dictate that the scope of a randomised control trial to show that humidified-warm CO<sub>2</sub> reduces intra-peritoneal tumour dissemination is likely unfeasible.

## 7.6 Conclusion

This thesis presents a body of work investigating mechanisms by which insufflation of humidified-warm CO<sub>2</sub> into the abdominal cavity during open abdominal surgery may become a simple, but important adjunct to clinical surgical practise. Achievement of a local increase in PtO<sub>2</sub> by insufflation of humidified-warm CO<sub>2</sub> tackles the long standing problem of how to delivery oxygen to the tissue of surgical patients who are so often mal-perfused. In addition, primary evidence has been provided to show that surgeons can cause inadvertent loss of peritoneal mesothelial simply by allowing the mesothelium to be desiccated by the operating room environment. Insufflation of humidified-warm CO<sub>2</sub> is the first practical solution to prevent intra-operative mesothelial cell loss by desiccation that avoids further damage to the mesothelium and is effortless to the surgeon. These mechanisms show that insufflation of humidified-CO<sub>2</sub> may play an important role in the prevention of the processes of post-operative surgical site infection, bowel anastomotic leakage, adhesion formation, and intra-peritoneal tumour dissemination.

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# **Appendix 1: Instructions for the Analysis of Scanning Electron and Light Microscopy Images for Mesothelial Cell Loss and Bulging.**

## **Scanning electron microscopy image analysis instructions**

1. Before reading this document, look at the scanning electron microscopy image examples from previous publications in order to familiarise yourself with scanning electron microscopy images of mesothelium.
2. Each set of images for analysis, and the examples in this document, is made up of a set of three images. Each assessment must utilise all three images, as different characteristics are often visible at different magnifications. The imaging location was held constant during image capture; therefore the high magnification image represents the centre portion of the low magnification image.
3. Review each example in this document and the grade it has been given for absence of mesothelial cell (MC) and bulging. Also note the examples of stereographic analysis using a counting grid.
4. Install the counting grid on your computer and set up the counting grid properties for stereographic analysis: Grid 50\*50 squares. Line width 2. Form opacity approximately 70 %. Grid intersections over image – 96. Ensure 12 x 8 points overlay the image.

5. Analyse each set of images for absence and bulging of mesothelial cells and record assessments in the spread sheet provided. Refer to these example images throughout your assessment, and note an example image # in the appropriate column of the spread sheet, to justify each of your assessments. Rotate the image to improve your depth perception. Use the different magnification images in the following way:
- i. 2,000 x: Identify presence of microvilli and cell borders
  - ii. 650 x: Confirm consistency of microvilli and cell borders
  - iii. 300 x: Use grid to count areas without mesothelial cells (MC), based on information from 2000 x and 650 x images.

### Light microscopy image analysis instructions

- A. Before reading this document, look at the light microscopy images examples from previous publications in order to familiarise yourself with LM images of mesothelium.
- B. Analyse each image for the MC absence and bulging. Record your assessments in the spread sheet provided.

### Brief definitions

Feature	Value	Definition
Absence of MC	0-2	Area of tissue surface that is not covered by a MC. See detailed definition below.
Presence of microvilli	n/a (used to identify MC)	Identification, or not, of microvilli on the surface of MC. See detailed definition below.

Feature	Value	Definition
Visibility of cell borders	n/a (used to identify MC)	The outline of MC can be delineated on at least some of the tissue surface. See detailed definition below.
Bulging	0-2	Protrusion of normally flat MC into the luminal side of the peritoneal cavity. See detailed definition below.
P exposed	Number of points (0-96)	Number of points on the stereology counting grid NOT covered by MC, excluding un-analysable points described below.
P un-analysable	Number of points (0-96)	Number of points on the counting grid that could not be analysed, this may be due to coverage by debris.

### Assessment of mesothelial cell absence

0: None or slight absence	0-10 % of surface absent of mesothelial cells
1: Moderate absence	20-40% of surface absent of mesothelial cells
2: Marked absence	50-100 % of surface absent of mesothelial cells

### Identification of mesothelial cells in scanning electron microscopy images

Identify MC by the presence of microvilli, cell borders and also by their size. Utilise the definitions of “Presence of microvilli”, “Cell borders visible” below.

#### Presence of microvilli

Microvilli are clearly visible on at least some of the tissue surface in the 2000 x and 650 x images, with either an interlocking mesh like appearance, or as short, stubby protrusions. The density of microvilli on the surface of MC cells is not uniform, and may be sparse or dense (Baradi and Rao 1976; Slater, Raftery et al. 1989).

#### Visibility of cell borders

Cell borders are clearly visible on at least some of the tissue surface in the 2000 x and 650 x images, of appropriate cell diameter.

Cell borders may be identified by one of the following features:

- Change in density of microvilli at cell edges (Michailova and Usunoff 2006).
- Change in density of microvilli of adjacent cells (Michailova and Usunoff 2006).
- Changes in tissue height at point of overlapping of MC (Mutsaers and Wilkosz 2007). MC boundaries are tortuous and MC overlap; this can create a change in tissue height that helps to visualise cell borders.
- Obvious clefts between cuboidal cells on tissue surface.
- Regular arrangement of bulged cells and/or intercellular clefts.

#### Cell size

Normal parietal mesothelium is predominately composed of a sheet of flat, squamous-like mesothelial cells approximately 6-25  $\mu\text{m}$  in diameter (Michailova and Usunoff 2006; Mutsaers and Wilkosz 2007; Du, Yu et al. 2011). The diameter of cells will change depending on apical profile, which can be hexagonal, round or elongated (Michailova and Usunoff 2006). However, regardless of apical profile, or whether the cells are flat or cuboidal, MC should be  $> 4 \mu\text{m}$  along the longest axis, and the diameter of the longest axis will be larger for elongated cells.

#### Identification of mesothelial cells in light microscopy images

Identify MC as a mono-layer of nucleated cells on the surface of the peritoneum. In the presence of mesothelial cells, cell nuclei stained purple by haematoxylin will be seen

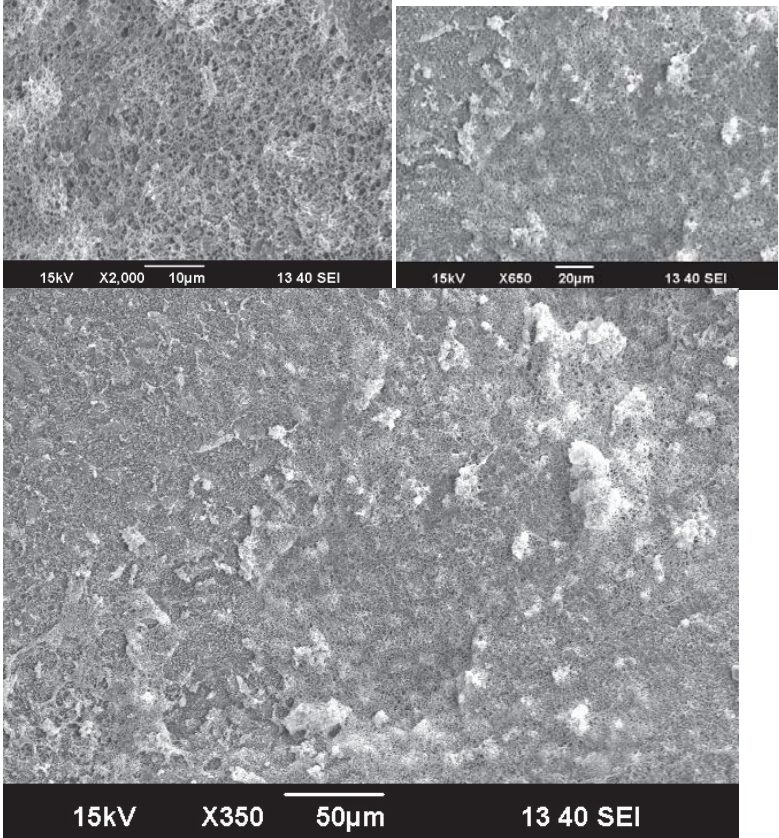
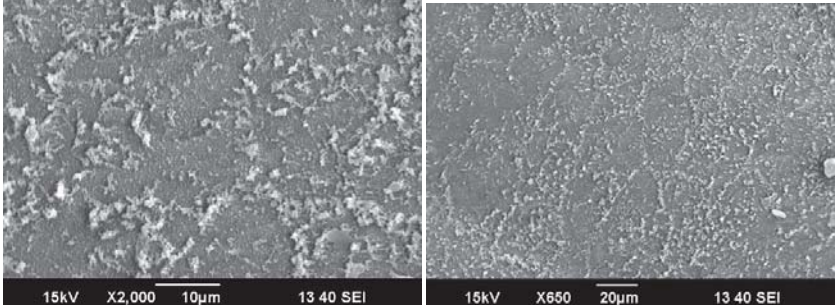
regularly along the surface of the peritoneum, superficial to the underlying connective tissue. In some areas the section will not have cut through the mesothelial cell nuclei. In these areas the cell cytoplasm retains the same tissue height as cells that have visible nuclei.

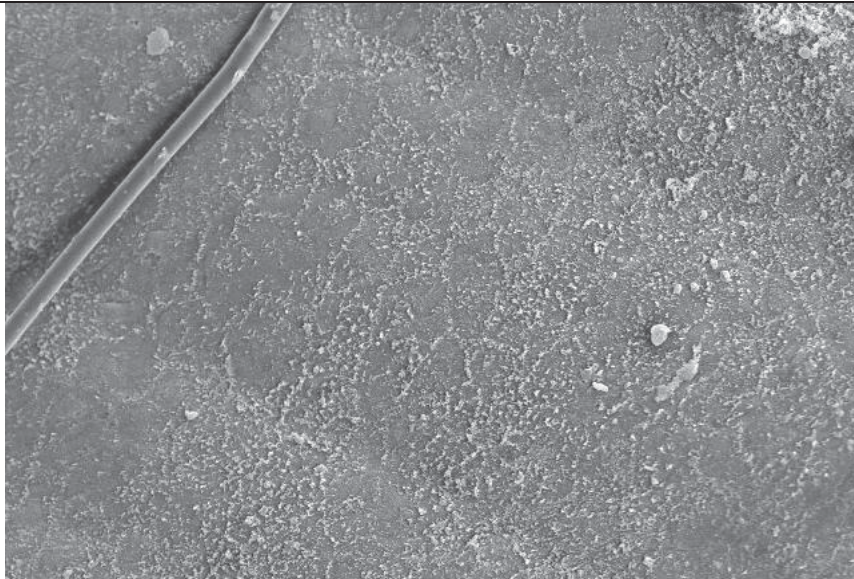
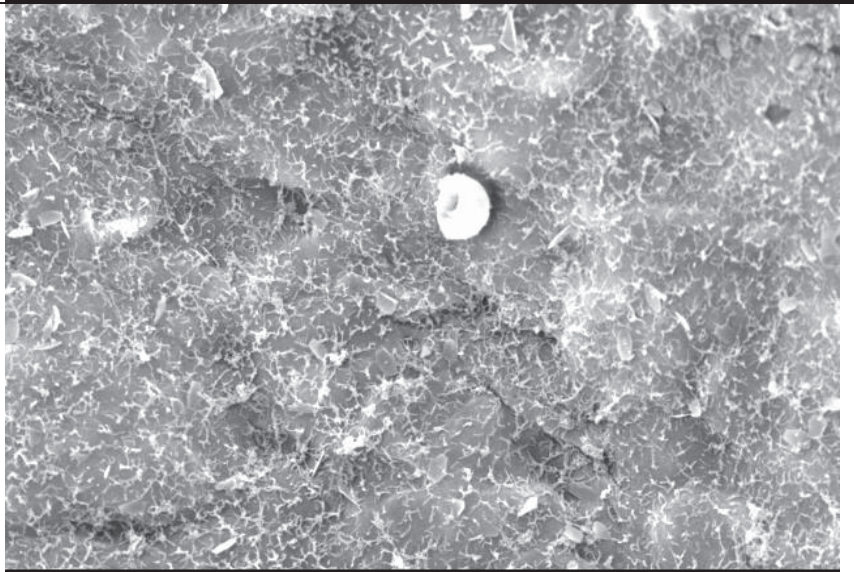
### Assessment of mesothelial cell bulging:

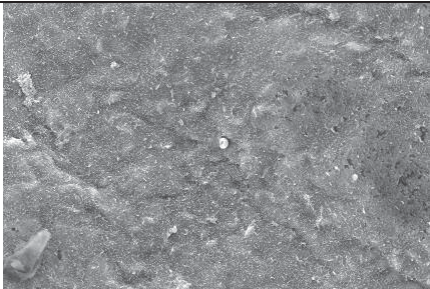
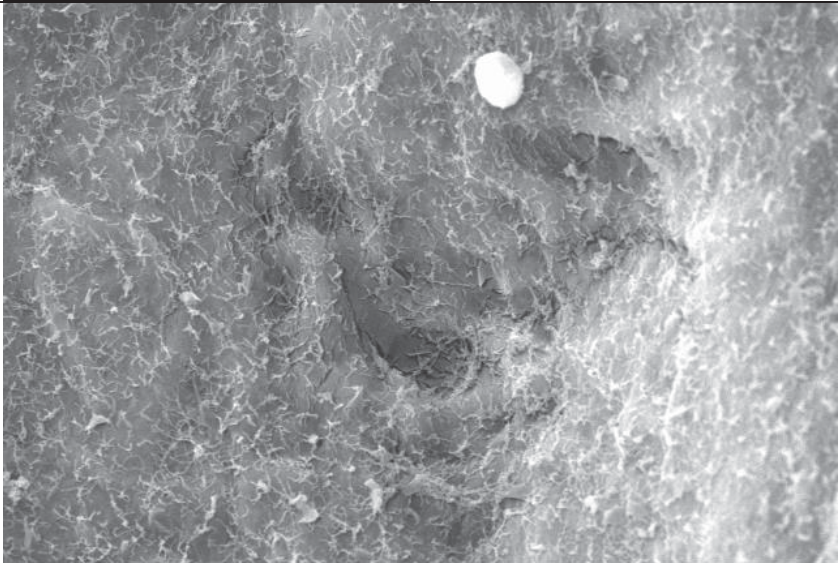
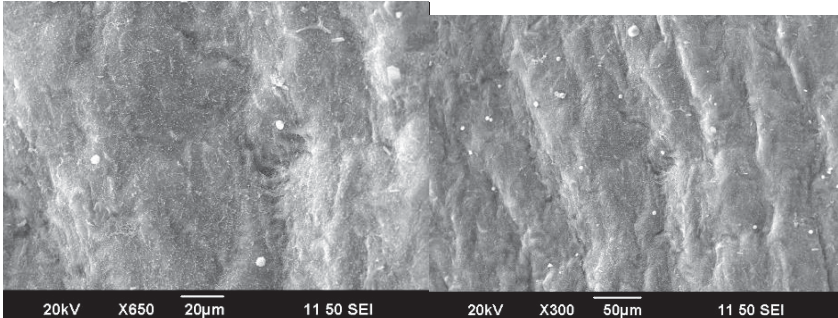
The cytoplasm of normal MC may appear raised over a central round or oval nucleus and mesothelial cells can overlap at tortuous cell-cell boundaries (Mutsaers and Wilkosz 2007). Conversely the nuclei indentations may be present (Michailova, Wassilev et al. 1999). When the cells become more metabolically active, such as during inflammation, the cell volume can increase as the cells change from flat cells, to smaller diameter cells that are characterised by increased cellular organelles (Mutsaers, Whitaker et al. 1996; Mutsaers 2002; Mutsaers 2004). Increase in cell volume can also occur during phases of cell death (Kaufman, Rostovshchikov et al. 1983). As the cell volume increases, the cell begins to protrude into the luminal side of the peritoneal cavity, and the cell takes on a bulged or rounded appearance. As the cell volume continues to increase, the cells become cuboidal (Mutsaers and Wilkosz 2007), or spherical (Rosario, Ribeiro Jr et al. 2006).


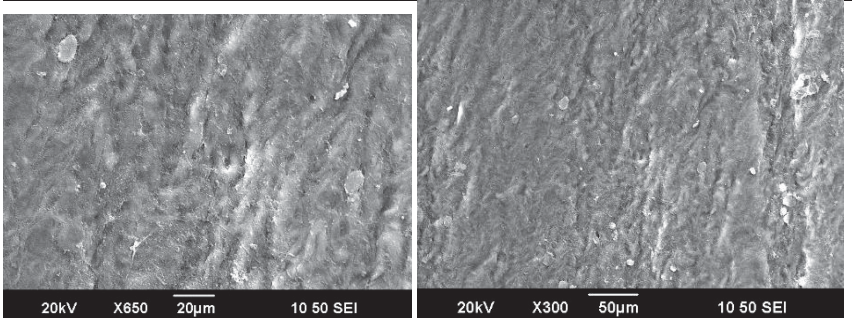
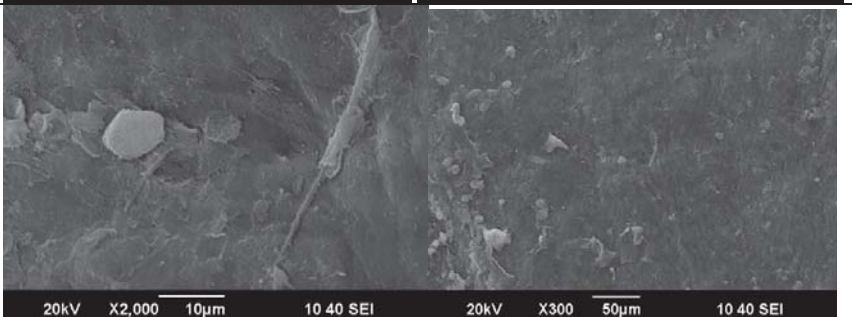
0: None or slight bulging	Flat, squamous- like MC. The cytoplasm may be raised in an area confined to a central round or oval nucleus (Mutsaers and Wilkosz 2007). The tortuous boundaries between mesothelial cells may show overlapping MC (Mutsaers and Wilkosz 2007). Narrow ruffles (diameter ~ 2 µm) may be present on the surface of MC, and are ignored. Undulations of the tissue, not related to individual MC bulging, are also ignored. “Moderate” or “marked” bulging may be present on up to 30 % of the 300 x image.
1: Moderate bulging	At <u>2000</u> x MC are rounded up from the basement membrane, increasing the volume of the MC. The rounding is in addition to any raised area that is confined to a central nucleus. Predominately, the cells are <u>not</u> cuboidal or spherical, although “marked bulging” may be present on up to 30 % of the 300 x image. At 650 x and 300 x the bulging portion of the cells creates a repeating and obvious pattern, seen over 40-100 % of the 300 x image, or the total of areas of “moderate” and “marked” bulging together accounts for 40-100 % of the 300 x image. Narrow ruffles on the surface of MC are ignored. Note: magnification relates to scanning electron microscopy image sets and can be ignored for light microscopy images.
2: Marked bulging	Cuboidal or spherical MC protrude from the basement membrane over 40-100 % of the 300 x image.

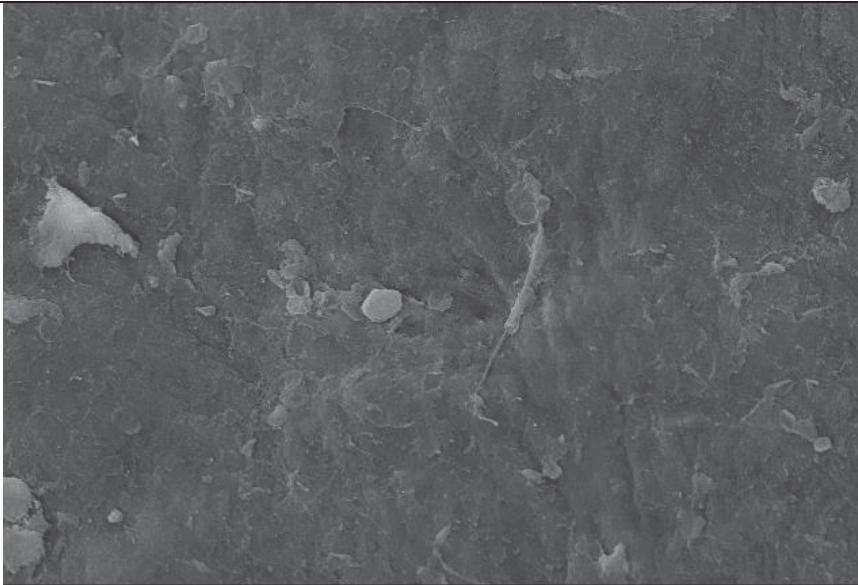
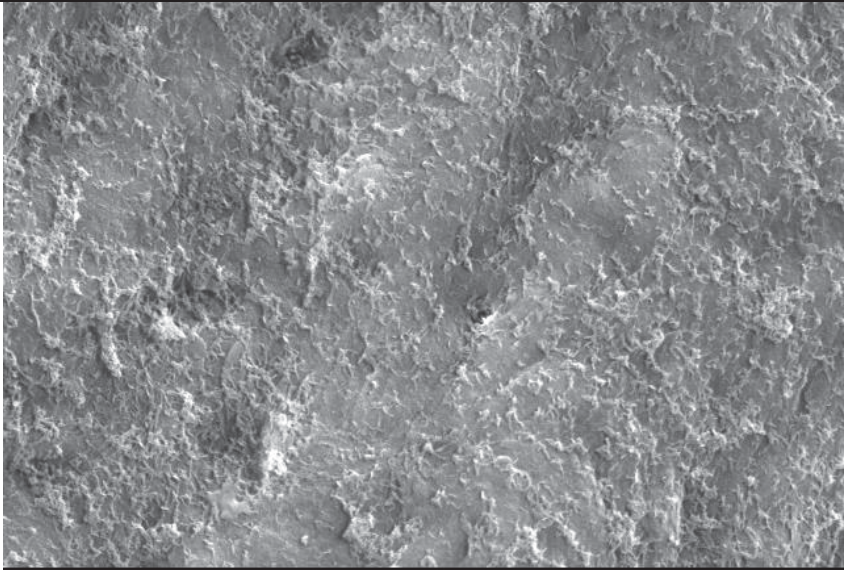
## Example images

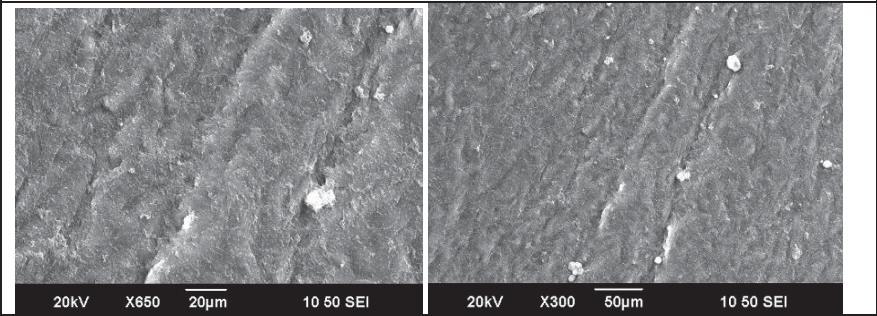
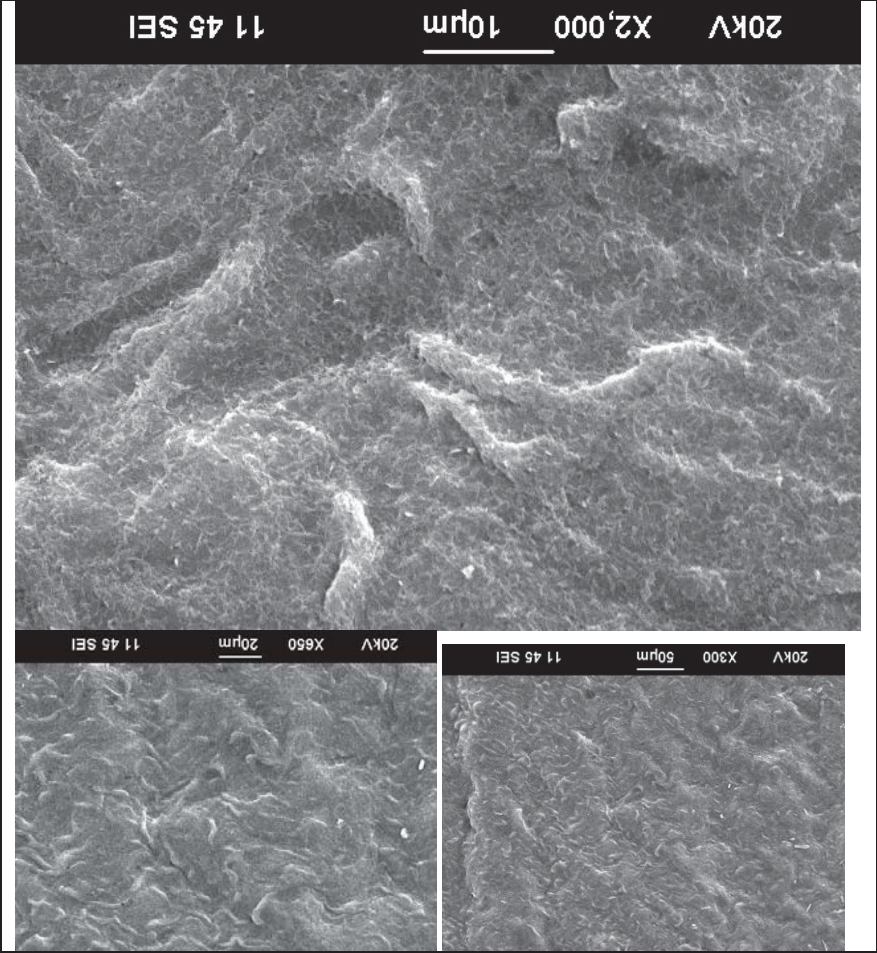
Bulging	Absence of MC	Comments	Example
0	0	None/slight MC bulging. 100 % covering of MC.	
0	0	Microvilli present, cell borders visible due to increased concentration of microvilli.	

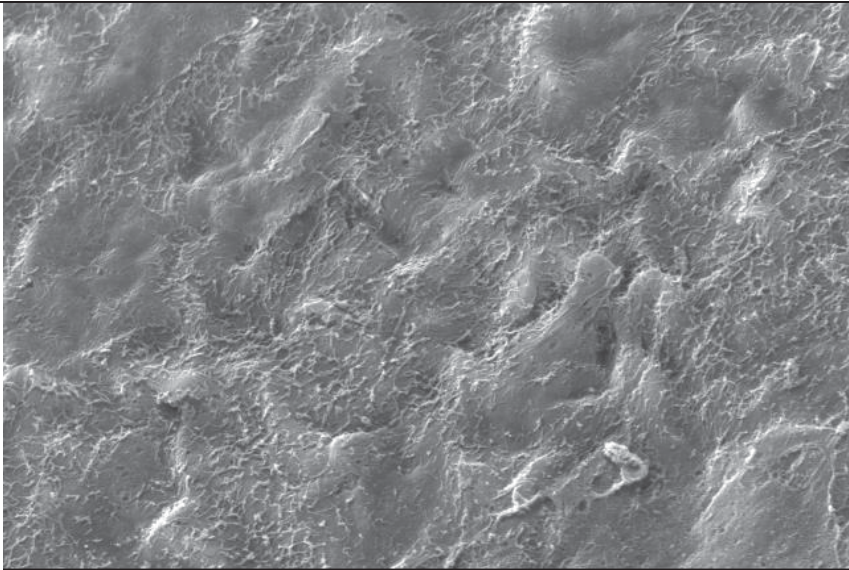
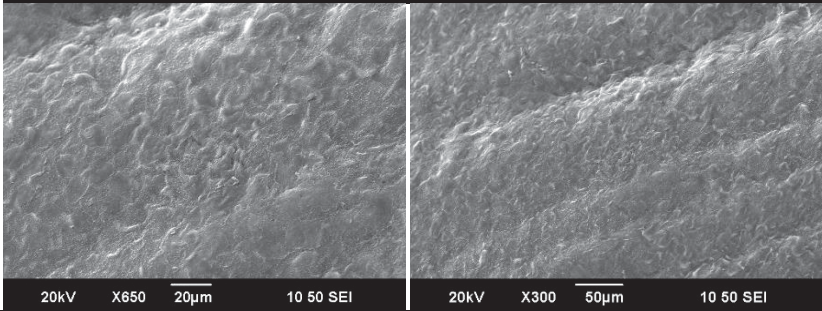
Bulging	Absence of MC	Comments	Example
			 <p data-bbox="507 907 1369 972">15kV X350 50µm 13 40 SEI</p>
0	0	Microvilli present, convoluted cell borders. 100 % MC coverage.	 <p data-bbox="507 1541 1369 1597">20kV X2,000 10µm 11 50 SEI</p>

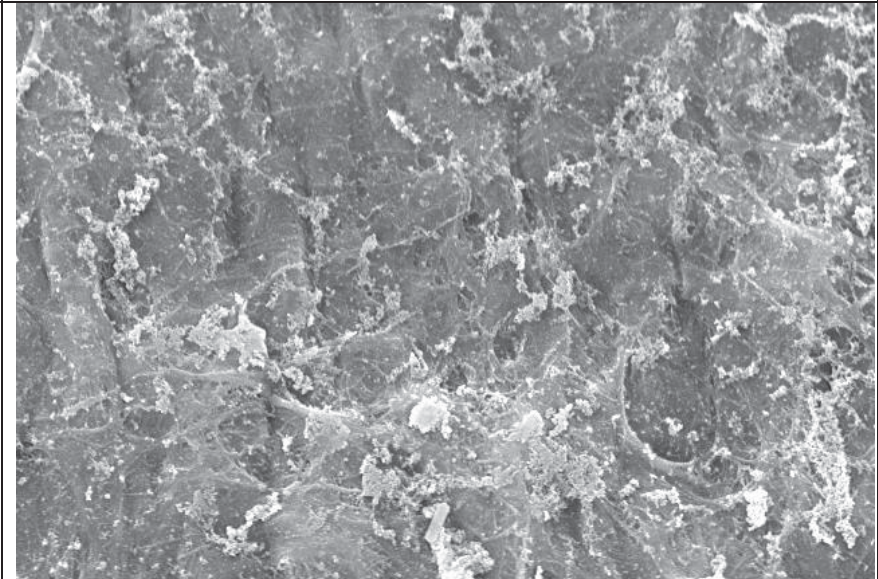
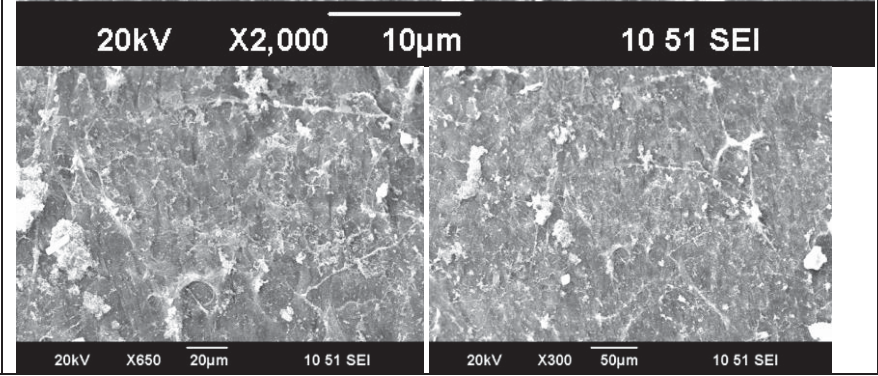
Bulging	Absence of MC	Comments	Example
			 <p>20kV X650 20µm 11 50 SEI</p>
0	0	<p>100 % MC coverage. Microvilli present, convoluted cell borders. None/slight bulging and intercellular clefts. No exposure of basement membrane</p>	 <p>20kV X2,000 10µm 11 50 SEI</p>  <p>20kV X650 20µm 11 50 SEI      20kV X300 50µm 11 50 SEI</p>

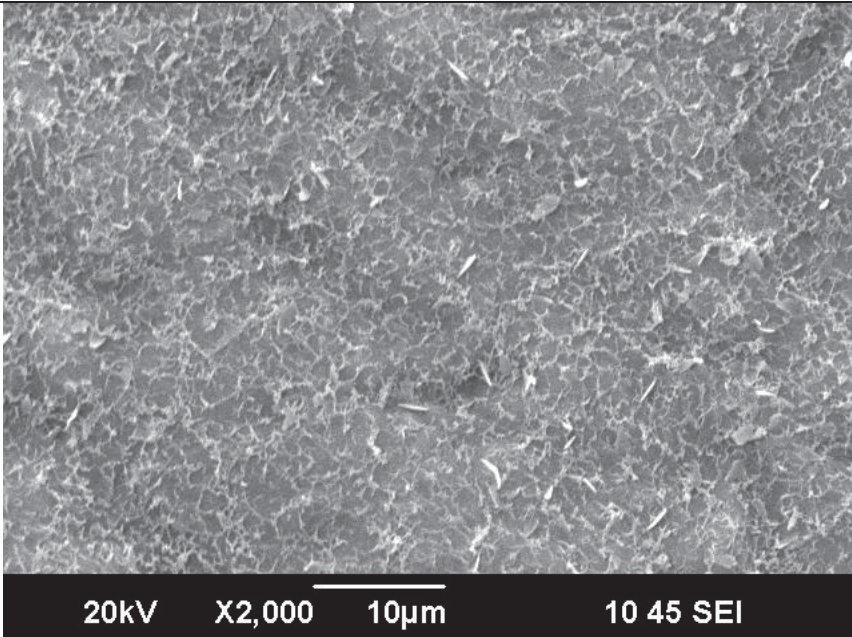
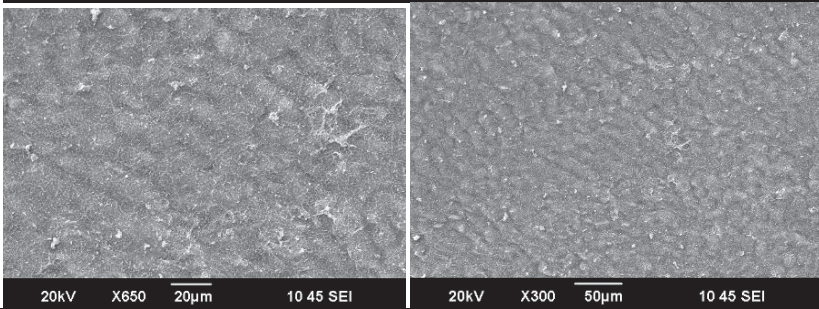
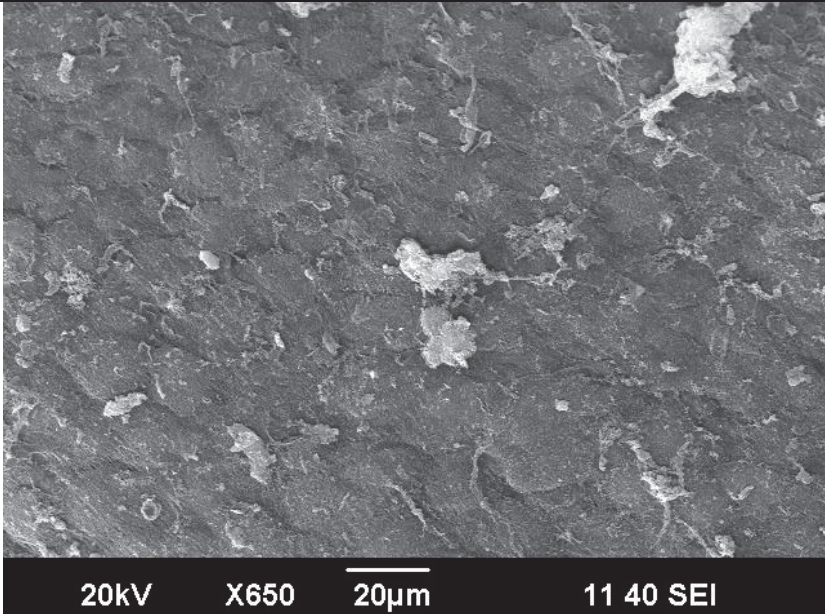
Bulging	Absence of MC	Comments	Example
0	0	Microvilli appear present although flattened; cell borders appear to be present but are not sharply defined. No evidence of connective tissue. Very low analysis confidence.	 
0	0	Cell borders are visible at all magnifications. Short stubs of microvilli may be visible, and may be concentrated around the border of some cells in 650 x image. Completed covered in MC.	

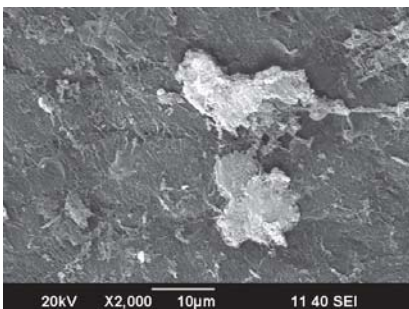
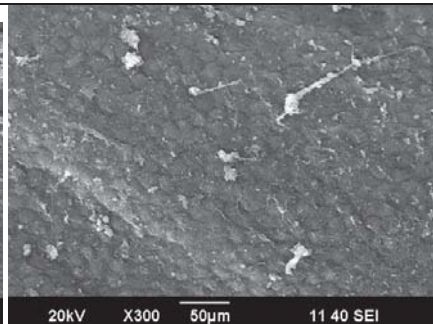
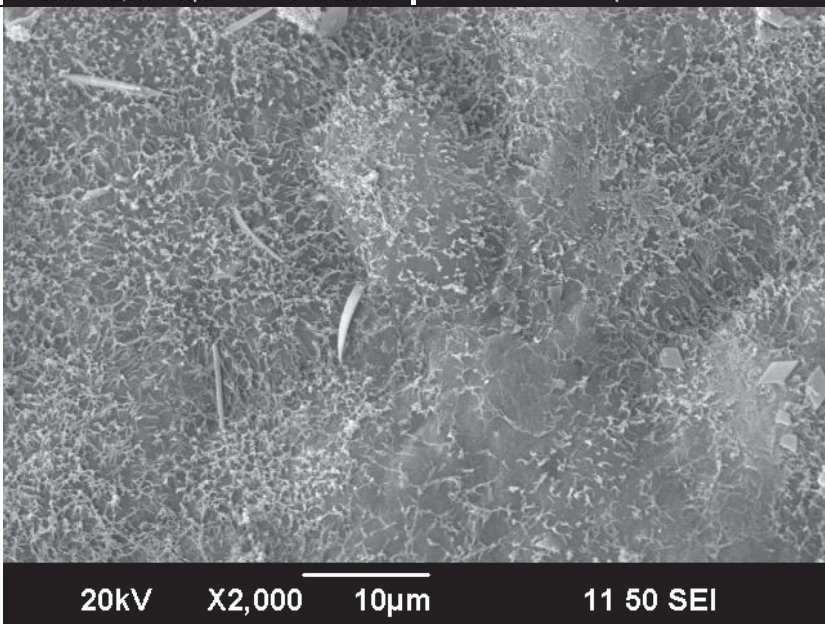
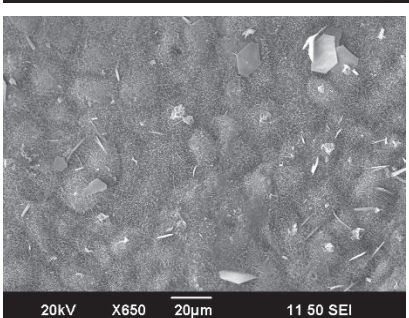
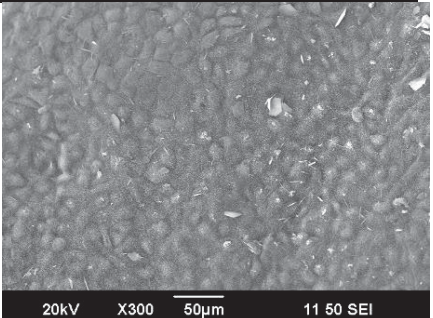
Bulging	Absence of MC	Comments	Example
			 <p>20kV X650 20µm 10 40 SEI</p>
0	0	100 % MC coverage. No exposure of basement membrane. Dense microvilli, cell borders visible, slight bulging.	 <p>20kV X2,000 10µm 10 50 SEI</p>

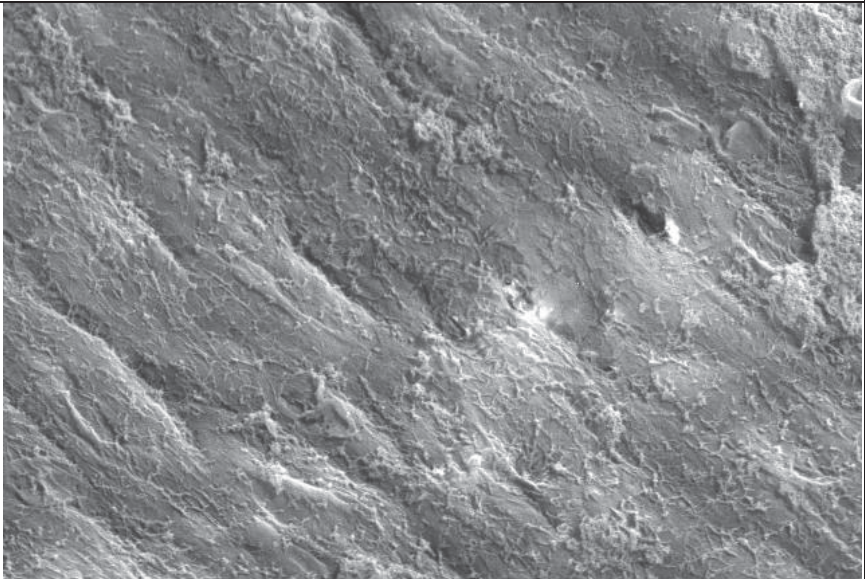
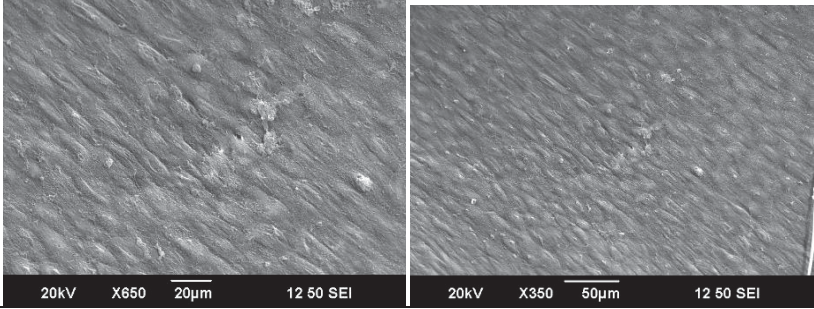
Bulging	Absence of MC	Comments	Example
			
0	0	100 % MC coverage. Microvilli present, cell borders not visible.	

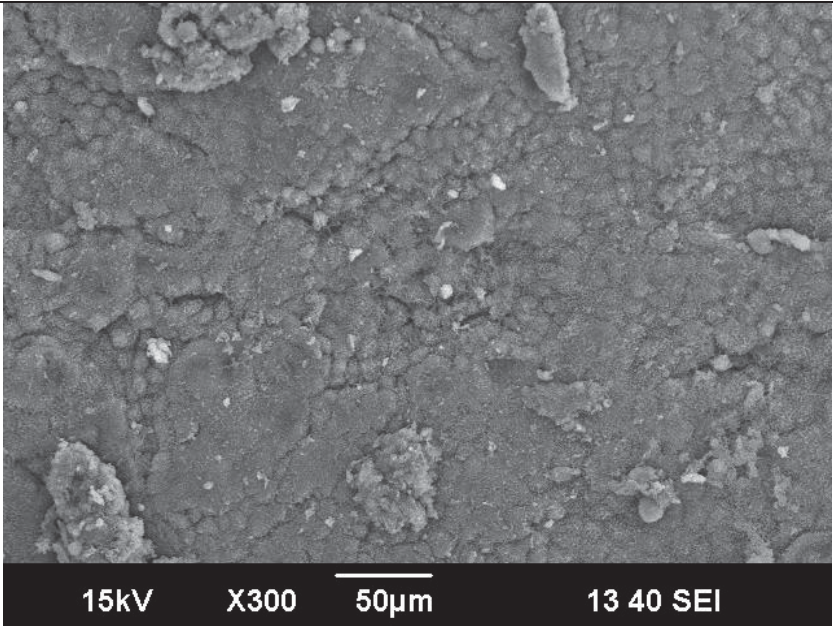
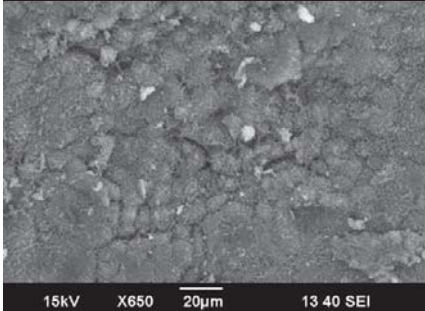
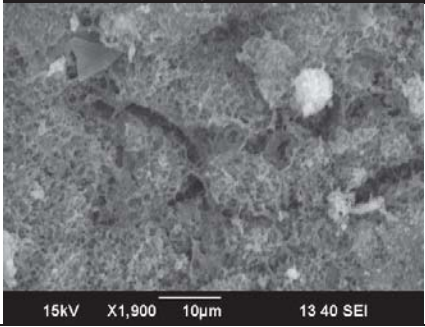
Bulging	Absence of MC	Comments	Example
0	0	100 % MC. Microvilli present and concentrated at cell edges. Wrinkling with slight bulging, no intercellular clefts.	 

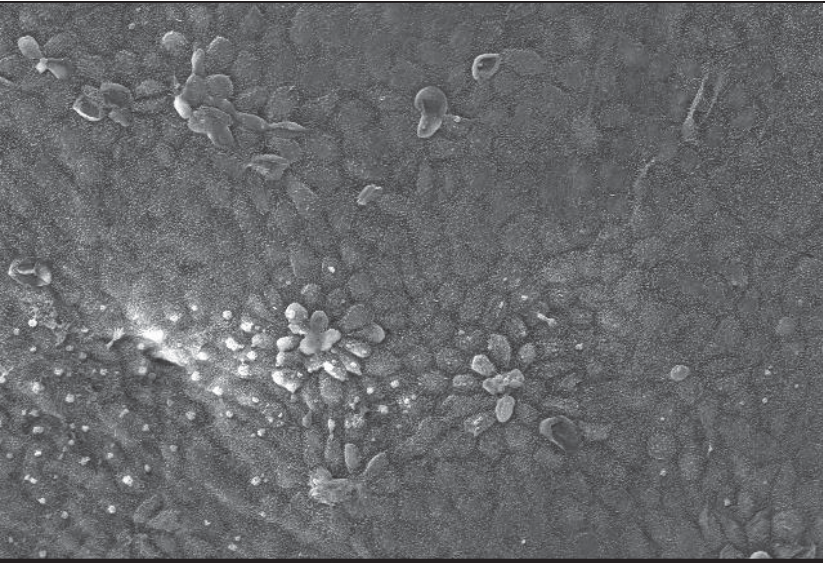
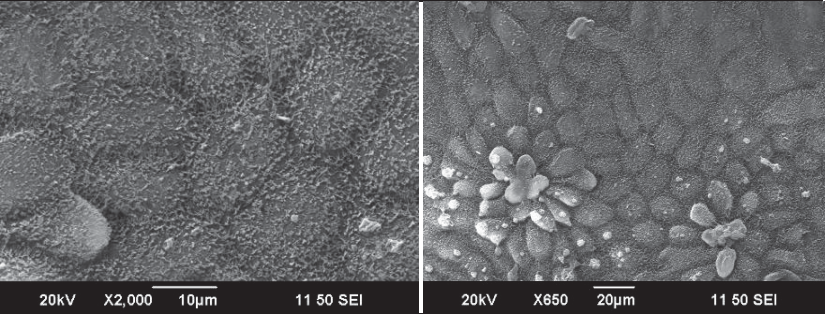
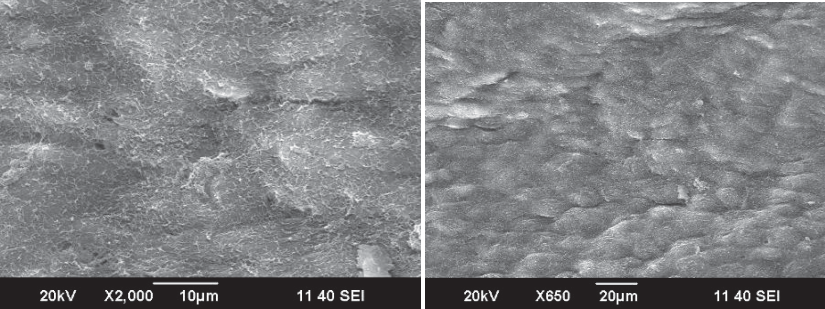
Bulging	Absence of MC	Comments	Example
0	0	Cell borders present. Microvilli as both mesh and stubs may be present.	 <p>20kV X2,000 10µm 10 51 SEI</p>  <p>20kV X650 20µm 10 51 SEI      20kV X300 50µm 10 51 SEI</p>

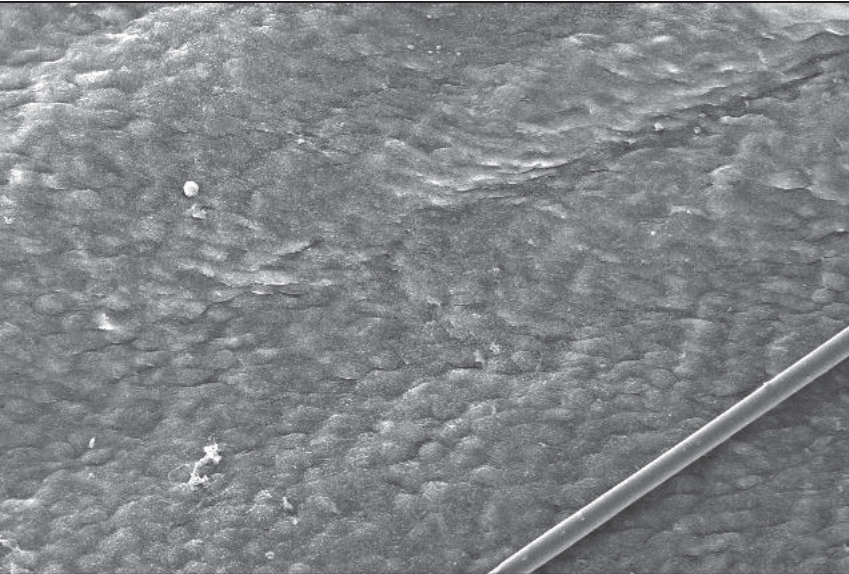
Bulging	Absence of MC	Comments	Example
1	0	100 % MC coverage. Microvilli present, cell borders visible, moderate bulging and intercellular clefts.	 
1	0	100 % covered in MC Moderate bulging.	

Bulging	Absence of MC	Comments	Example	
			 	
1	0	100 % MC coverage. No exposure of BM. Dense cilia, cell borders visible, moderate bulging.	  	

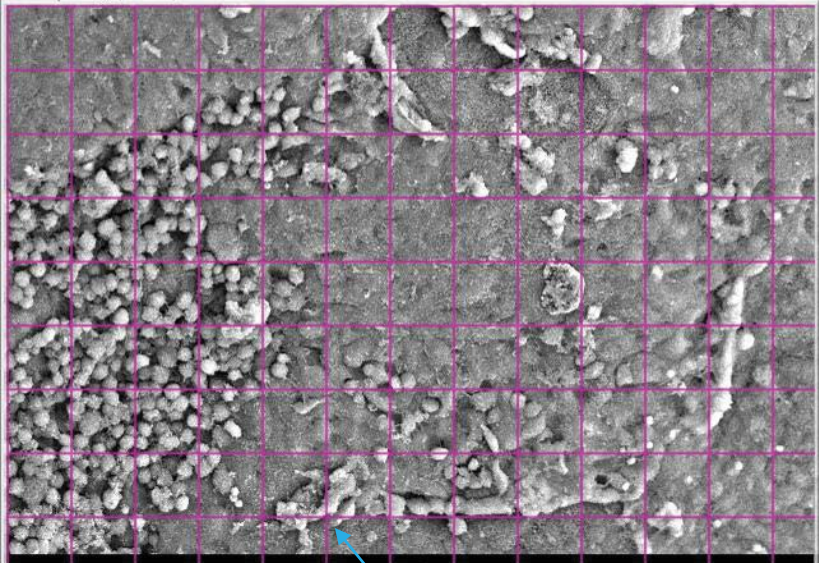
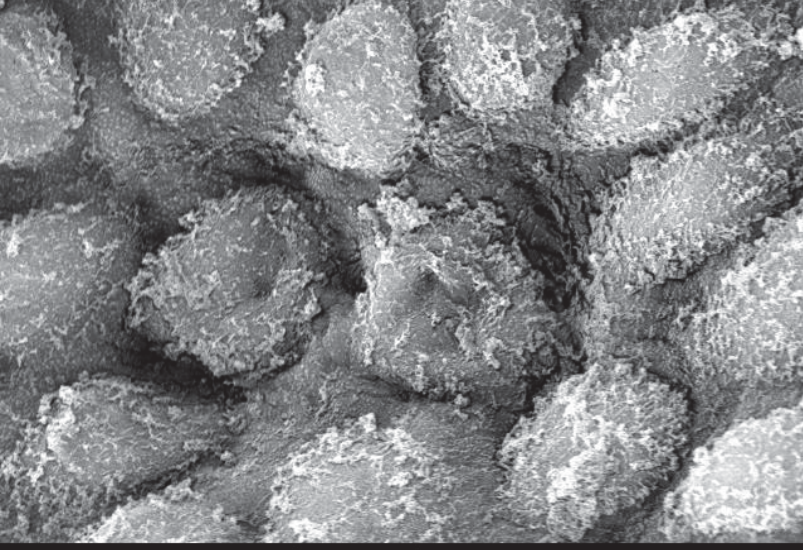
Bulging	Absence of MC	Comments	Example
1	0	100 % MC coverage. Microvilli present, cell borders visible. Moderate bulging of cells, moderate intercellular clefts.	 <p>20kV X2,000 10µm 12 50 SEI</p>  <p>20kV X650 20µm 12 50 SEI      20kV X350 50µm 12 50 SEI</p>

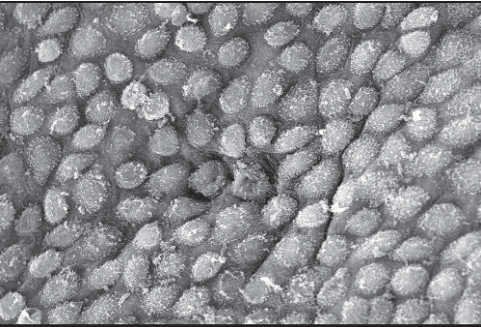
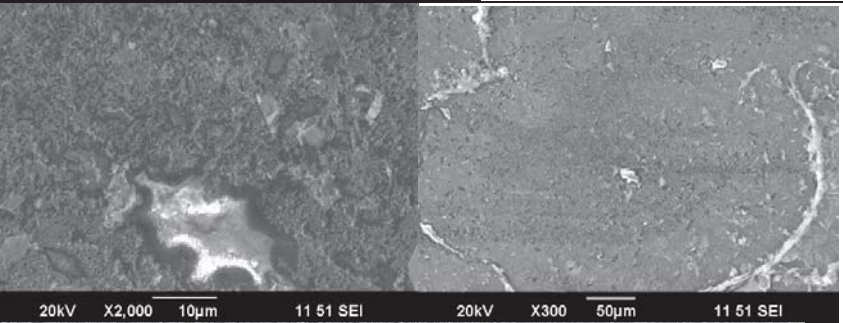
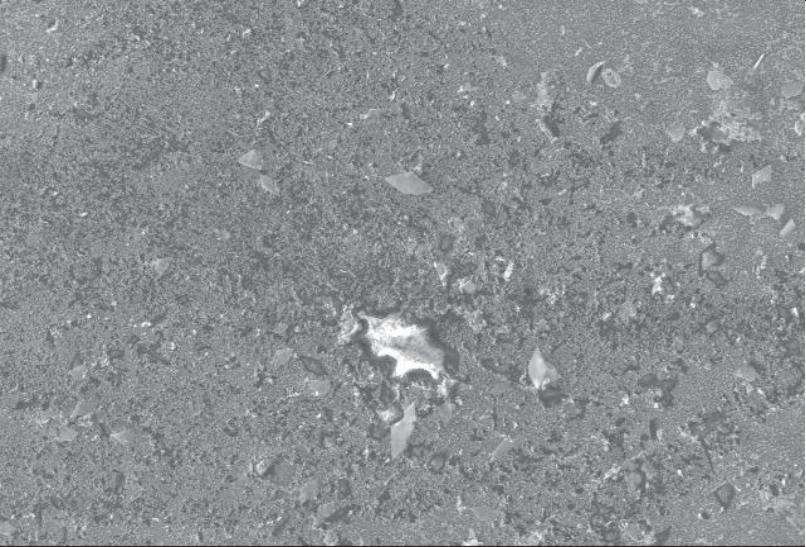
Bulging	Absence of MC	Comments	Example
2	0	Using grid <40 % of image has intercellular clefts.	  

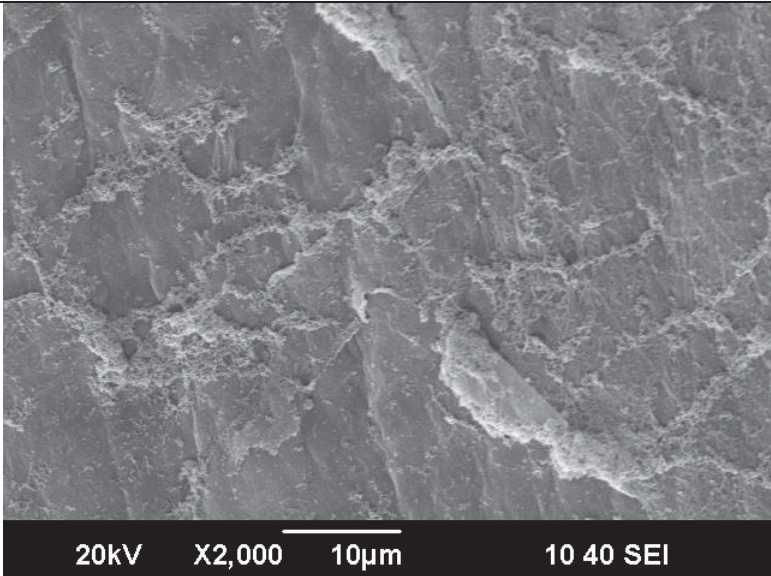
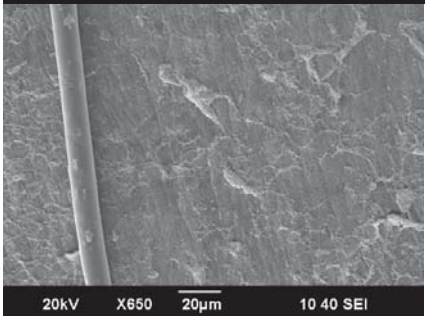
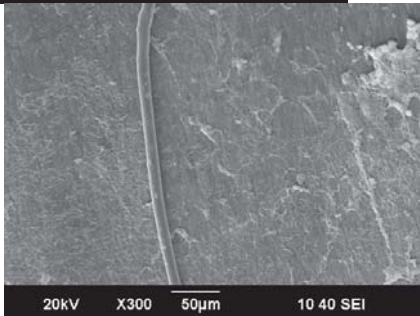
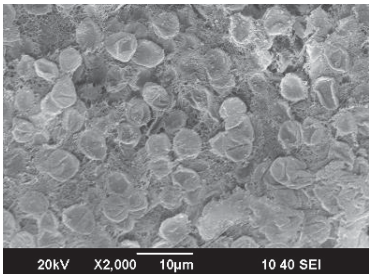
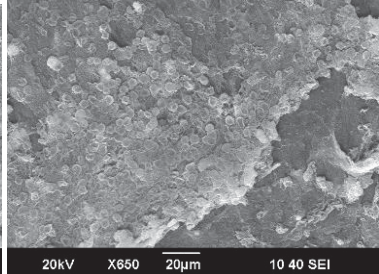
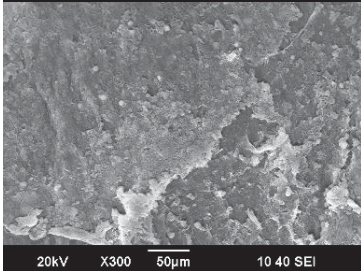
Bulging	Absence of MC	Comments	Example
2	0	100 % MC coverage. Microvilli present, cell borders clearly visible. Marked bulging, moderate intercellular clefts.	 
2	0	100 % MC coverage. Microvilli present, cell borders clearly visible. Marked bulging, moderate intercellular clefts.	

Bulging	Absence of MC	Comments	Example
			 <p data-bbox="587 920 1257 958">20kV X300 50μm 11 40 SEI</p>

Bulging	Absence of MC	Comments	Example
1	0	Microvilli and cell borders visible. One un-analysable point (blue arrow). Cells appear to be starting to detach, but there is no evidence of areas where MC are absent.	
2	0	Microvilli and cell borders visible. One un-analysable point (blue arrow). Marked bulging. There appear to be some areas where a singular cell may have been lost as the space left is	

Bulging	Absence of MC	Comments	Example
		regular, however none of those areas fall on the grid.	 <p>15kV X300 50µm 13 40 SEI</p>
2	0	Red arrows – exposed. Marked visibility of cell borders; marked intercellular clefts; marked bulging. Microvilli appears to be present between bulges, suggesting that there is no absence of MC.	 <p>20kV X2,000 10µm 12 51 SEI</p>

Bulging	Absence of MC	Comments	Example
			 <p>20kV X650 20µm 12 51 SEI</p>
n a	2	No MC No evidence of microvilli or cell borders.	 <p>20kV X2,000 10µm 11 51 SEI      20kV X300 50µm 11 51 SEI</p>  <p>20kV X650 20µm 11 51 SEI</p>

Bulging	Absence of MC	Comments	Example
na	2	No MC No evidence of microvilli or cell borders. Very low confidence in analysis.	  
na	na	Un-analysable Obscured by blood.	  

Bulging	Absence of MC	Comments	Example
na	na	Un-analysable. Obscured with debris (drawn on wrong side of tissue)	