The Evaluation of Colorectal Anastomotic Leak and Novel Prevention Strategies

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Abstract

Experimental strategies to reduce the incidence of anastomotic leak (AL) have failed to establish efficacy when translated into clinical practice. There is an increasing interest in the use of stem cell therapies with a focus on the potential repair capability of mesenchymal stem cells (MSCs). However, there remains uncertainty around the fundamental mechanistic process of bowel healing. It is hypothesised that a novel source of MSCs, derived from omentum harvested at laparotomy, can be delivered to an anastomosis to promote healing and prevent AL.

A large scale *in-vivo* murine study provides a compelling case that the alginate gel delivery vehicle chosen may reduce the severity of anastomotic leak and could provide a practical therapeutic delivery option in humans. The stromal vascular cell fraction is similar across two murine strains, regardless of sex, and contains factors conducive to wound healing. There was a signal that mesenchymal stem cells contained within the stromal vascular cell fraction might reduce anastomotic leak. By combining all the known literature, a novel anastomotic healing model has been developed that provides a way for researchers to investigate the histological changes at the anastomotic line in pre-clinical studies. My findings indicate that healing at the colonic anastomotic line appears to follow the same broad biological steps as documented in the skin healing literature. These findings can now be used to inform pre-clinical testing of other therapeutic interventions for anastomotic leak. My research concludes at the stage where gel/mesenchymal stem-cell-based interventions are ready to be taken into the clinical setting.

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List of Abbreviations and Definitions

ADRC	Adipose Derived Regenerative Cells
AL	Anastomotic Leak
B6	C57BL/6 (Mouse)
BAR	Bio-fragmentable Anastomotic Ring
bFGF	basic Fibroblast Growth Factor
CAL	Colorectal Anastomotic Leak
DAB	3,3' Diaminobenzidine
ESCP	European Society of Coloproctology (EAGLE)
ESCP	Safe-anastomosis Programme in Colorectal Surgery
FPA	Fluorescent Perfusion Angiograph
GAGs	Glycosaminoglycans
GI	Gastrointestinal
H&E	Haematoxylin & Eosin
HEAP	Health Economic Analysis Protocol
ICG	Indocyanine Green
ISREC	International Study Group of Rectal Cancer
LOS	Length of Stay
LGI	Lower Gastrointestinal
MMP-9	Matrix Metalloproteinase-9
MSC	Mesenchymal Stem Cell
ODRC	Omental Derived Regenerative Cell
OPA	Oral Antibiotic Preparation
PFA	Paraformaldehyde
PDGF	Platelet Derived Growth Factor
PROMS	Patient Reported Outcomes
RCT	Randomised Control Trial

SVCF	Stromal-vascular Cell Fraction
TBS	Tis-Buffered Saline
TBST	Tis-Buffered Saline w/ 1% Tween
TIMPs	Tissue inhibitors of matrix metalloproteinases
TGFβ	Transforming Growth Factor Beta
UK	United Kingdom
VEGF	Vascular Endothelial Growth Factor

1. Introduction

Anastomotic leak is a common, but poorly understood, complication of colorectal surgery for both benign and malignant conditions. Strategies to address anastomotic leak have largely failed, due to limited standardisation of techniques, heterogeneity of study design, and a lack of understanding of the mechanisms underpinning anastomotic healing. A multifaceted approach to investigate and overcome these issues is required. This chapter describes the current understanding of the aetiology underlying anastomotic leak. A commentary on the normal wound healing processes of colonic anastomoses is provided and compared to normal skin healing, which is thought to involve analogous mechanisms. The evidence supporting current and novel interventions is discussed in the context of its impact on patient outcomes. Finally, a structure for my doctoral investigations will be outlined, including the development of a novel intervention to prevent anastomotic leak and an animal model to facilitate laboratory investigations of anastomotic leak and the temporal biology of anastomotic healing.

1.1 The Clinical Problem - Colorectal Anastomotic Leak

The prevention of anastomotic leak (AL) remains one of biggest challenges in gastrointestinal surgery with ongoing controversy regarding the best strategy to reduce this serious post-operative complication. (5) AL is the cause of significant morbidity and mortality. (6,7) Research into the development of AL, its management, short- and long-term costs, and impact on patients and society has received relatively little attention. (8) Attempts to reduce the incidence and impact of AL, including advances in surgical technique and perioperative care, have shown promise in pre-clinical studies, but have failed to demonstrate efficacy in clinical practice, with the incidence of AL remaining the same as it was half a century ago. A better understanding of why AL occurs is required to enable novel interventions to be developed. In addition, an understanding of

the true cost of AL is essential if we are to accurately assess the value of new interventions and their potential translation into routine clinical care.

1.1.1 Definition and Grading

There remains no universally accepted definition of AL (9) with the majority of studies using a combination of clinical features and radiological findings for diagnosis. In 2001, Bruce *et. al.* described 56 separate definitions of AL in a systematic review of 97 studies. (10) Until 2016, there was no core outcome set that existed for AL precluding accurate comparison of leak rates between institutions and outcomes in interventional studies. (11) In 2020, following a modified Delphi exercise, Van Helsingen *et. al.* published an international consensus document with recommendation to adopt the International Study Group of Rectal Cancer (ISREC) definition of AL which defines leak as "*a defect of the intestinal wall at the anastomotic site (including suture and staple lines of neorectal reservoirs) leading to a communication between the intra- and extraluminal compartments."* (12) The same group validated a severity grading system in 746 patients who underwent sphincter preserving surgery and described a severity rating based on intervention requirement; Grade A (no change in management), Grade B (active therapeutic intervention) and Grade C (requiring re-laparotomy or laparoscopy) with leak rates of 16%, 23% and 61% respectively. (13) These consensus grading systems are based on evaluation of leaks in rectal anastomoses but are often extrapolated to other gastrointestinal anastomoses. In 2020, the TENTACLE-Rectum group began recruitment to an international multicentre retrospective cohort study to investigate which factors contribute to AL in rectal anastomoses and how best to manage leak based on clinical and radiological characteristics; the results are awaited. (14)

1.1.2 Incidence

The incidence of AL is variably reported between 5%-19% depending on the site of the anastomosis in the gastrointestinal tract (15) with the largest studies reporting incidences of 6% after both right and left sided colonic cancer surgery (16) and 8% following right hemi-colectomy. (17) In a recent meta-analysis of 84 studies including 45 randomised controlled trials (RCTs) and 24,845 anastomoses, the incidence of AL following rectal cancer surgery was estimated at 11%. (18) Smaller studies have demonstrated colo-colonic leak rates of 0-2% and ileocolonic leak rates from 0-4%. (19–22) There has been no reported difference in AL rates between laparoscopic or robotic resections when compared to open procedures or between stapled and hand sewn anastomoses. (23–26) There is some evidence to suggest that intra-corporeal anastomoses confer a lower risk of AL, however, this is disputed with several systematic reviews providing opposing evidence. (27,28)

1.1.3 Risk Factors & Risk Predictions Scores

Many studies have identified risk factors for AL and multiple preoperative and intraoperative AL prediction scores have been proposed (29–31) with reasonable predictive value as described by Dekker *et. al.* (30) However, a recent systematic review by Venn *et. al.* demonstrated poor reporting strategies and concluded methodological shortcomings have limited the wider adoption of these tools. Prediction scores appeared to perform well in discriminating those at highest from low risk, but the authors reported concerns of bias in the 34 included studies. (32)

A review of the literature shows that risk factors for AL are often classified as technical *versus* patient / perioperative factors or modifiable *versus* non-modifiable factors (Table 1).

Table 1 | Risk factors for anastomotic leak identified compiled from current currently available literature.

	Modifiable	Non-Modifiable
Technical Factors	▪ Intraoperative Blood Loss (>100-500mls) (28) ▪ Number of Stapler Firings (>2-3) (19,33–41) ▪ Duration of Surgery (>2-4 Hours) (33,42–44) ▪ Operating Field Contamination (42) ▪ Preoperative Transfusion (15,45,46) ▪ Epidural (47) ▪ Vasopressors (47) ▪ Extra-corporal Anastomosis (28)11/10/24 11:31:00 AM	▪ Emergency Surgery (15) ▪ Non-specialised Surgeon (48,49) ▪ Centre Case Load (50)
Patient or Peri-operative Factors	▪ Poor Nutrition (Low Albumin) (15) ▪ Anaemia (<10.5g/dL) (42) ▪ Peri-operative Hypothermia (51) ▪ Antibiotics (52,53) ▪ Bowel Preparation (54–56)	▪ Sex (Male) (15,33,43,44,57–61) ▪ Smoking (15,43,58,62,63) ▪ Obesity (15,33,43) ▪ Diabetes Mellitus (42,43) ▪ Renal Disease (15) ▪ Coronary Heart Disease (62) ▪ Previous Neoadjuvant Therapy (15,33,44,60,64–68) ▪ Tumour Size >2.5-5cm (15,33,43) ▪ Tumour Stage (T3/T4) (57) ▪ ASA Score (II-IV) (15,57) ▪ Alcohol Excess (15,63) ▪ Immunosuppressants (15) ▪ Pulmonary Disease (45)

1.1.4 Anastomotic Healing

Two explanations are usually put forward to explain the mechanism underlying AL:

(1) patient-related or perioperative factors, for example, baseline nutritional status, smoking history, or the use of pre-operative mechanical bowel preparation
(2) surgical technique, for example, ischaemia and tension at the anastomotic line or stapling device failure.

However, without reviewing the macroscopic and microscopic appearances of the anastomosis it is often impossible to determine the exact cause of anastomotic failure. (69)

Within the literature, studies commonly investigate single causative factors for AL without attempt to standardise or control for other causative factors which could confound the results. In addition, these studies almost unanimously fail to attribute differences that occur within treatment groups. This has led to a current evidence base built on between-group comparisons where conclusions are more likely to be associative than causative, with claims often caveated with the (likely) multifactorial causation of AL. (70)

The molecular and cellular events of wound healing at the anastomotic line and the underlying risk factors for anastomotic leak (Table 1) remain poorly understood. (71–74) The observation that AL can still occur in a technically perfect anastomosis and optimised patient, suggests that additional underlying factors have not been defined. (75–77). Previous doctrine on the construction of a secure anastomosis dictates that there should be: (A) sound surgical anastomotic technique (B) adequate blood supply at the anastomotic line (C) tension-free anastomotic join (D) good approximation of tissues (E) absence of obstruction distal to the anastomosis, and (F) absence of disease (e.g. malignancy or local sepsis) at the anastomotic site. (78)

During acute intestinal inflammation (e.g. inflammatory bowel disease), neutrophils and monocytes (later macrophages) migrate into the local tissues and secrete a variety of oxygen radicals and enzymes, which degrade the tissue. Pro-inflammatory cytokines and chemotactic and cell-activating peptides are also secreted or released from the stromal matrix. In severe tissue damage, migration of myofibroblasts occurs to the area of insult with the production of new extracellular matrix and ultimately contraction of the wound. (79)

1.1.4.1 Response to Tissue Injury

Wound repair is a regulated process which occurs immediately following an insult that disrupts the normal structure and function of tissue. (80) The primary purpose is to restore the integrity, function and tensile strength of the injured tissue. The process has been extensively studied in human skin (81–83) and follows three distinct but overlapping stages:

I. Inflammation: Platelet aggregation and vasoconstriction at the site of injury results in the formation of a fibrin-based clot to secure haemostasis. (84) Increased vascular permeability surrounding the injured site results in an efflux of inflammatory cells into the wound which utilise the fibrin clot as a provisional matrix. Neutrophils, the initial dominant cell type, protect the wound from microbe invasion through the removal of foreign particles and bacteria. At 48 hours following insult, monocytes which differentiate into macrophages, are the predominant cell type, being recruited through release of growth factors and cytokines from platelets. Platelet derived growth factor (PDGF) and transforming growth factor beta (TGFβ) are two of the key mediators driving the repair process. These combine with plasma proteins such as fibrin, fibronectin and vitronectin to deposit provisional wound matrix and encourage cell migration. Macrophages phagocytose foreign organisms and secrete pro-inflammatory cytokines, including PDGF, which stimulate the next stage of healing with granulation tissue formation. (84–86)

II. Proliferation: By day 4, fibroblasts predominate and their influx into the wound is regulated by various growth factors including basic fibroblast growth factor (bFGF), PDGF and TGFβ. Fibroblasts substitute the initial wound repair matrix established in the inflammation phase with collagen rich granulation tissue. (87) Proteases such as matrix-metalloproteinases (MMPs) are released to degrade the extracellular matrix and

aid the migration of fibroblasts into the wound. New extracellular matrix is formed, consisting of fibrin, fibronectin and proteoglycans, including hyaluronic acid, which are gradually replaced through collagen deposition. This is initially collagen III which is then gradually replaced by collagen II. The process is modulated by several factors including vascular endothelial growth factor (VEGF), which also induces angiogenesis. A proportion of fibroblasts take up a myofibroblast phenotype with alpha smooth muscle actin (α-SMA) myofilaments which allows the wound to contract. (86,88)

III. Remodelling: newly formed granulation tissue undergoes remodelling with an observed reduction in the density of fibroblasts and macrophages. Collagen fibres undergo cross linkage and are converted to thick collagen bundles and contractile units through the synergistic action of MMPs and tissue inhibitors of MMPs (TIMPs). Endopeptidases also are also secreted by cells such as fibroblasts, and macrophages. Through this process, a proportion of cells within the ECM undergo apoptosis however remodelling continues and scar tissue (with a reduced cellular and vascular component) forms surrounding uninjured, healthy tissue. (86,87)

1.1.4.2 Physiology of Anastomotic Healing

The colonic wall is made up of four layers, from innermost (luminal) to outermost (serosal) layers: i) mucosa and lamina propria, ii) submucosa, iii) muscularis mucosa (circular and longitudinal muscle), and iv) serosa with peritoneal lining. In adults, the colon can be distinguished from the small intestine histologically by the absence of villi, plicae circularis, and Paneth cells. The crypts of Lieberkühn are deeper in the colon and goblet cells become more abundant. (Figures 1 & 2). The strength of an anastomosis is reliant on the serosa supported by the submucosa which contains blood vessels, lymphatics and nerve fibres along with the majority of bowel wall collagen; Type I (68%), Type III (20%) and Type V (12%). (89,90) Smooth muscle cells intertwine

with a collagen network to make up the muscularis propria which is covered by the thin serosa – the external most layer of the bowel wall which includes the peritoneum (Figures 1 & 2).

Figure 1 | Schematic representation of the normal structure of the large intestine created with Biorender.com

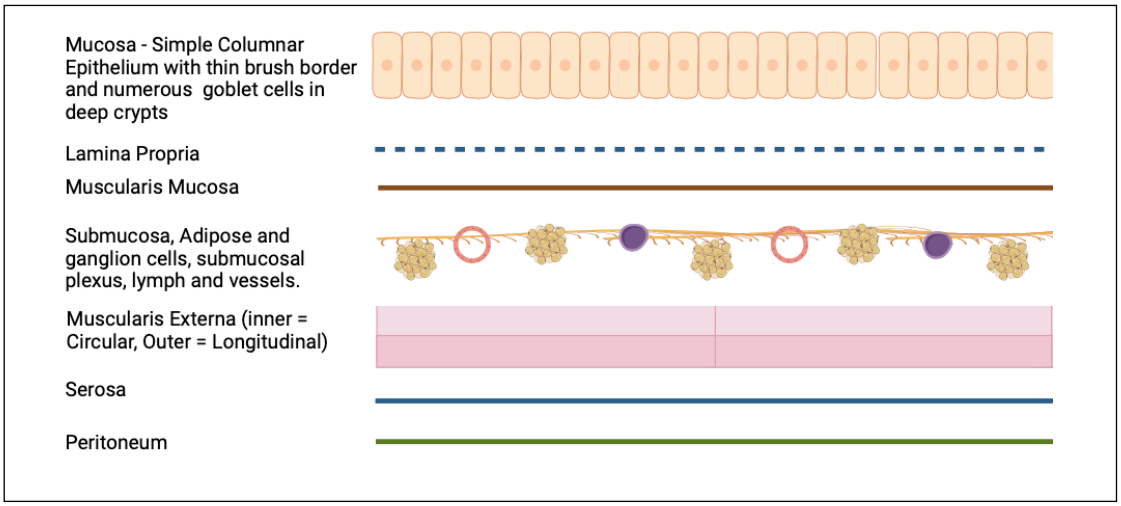
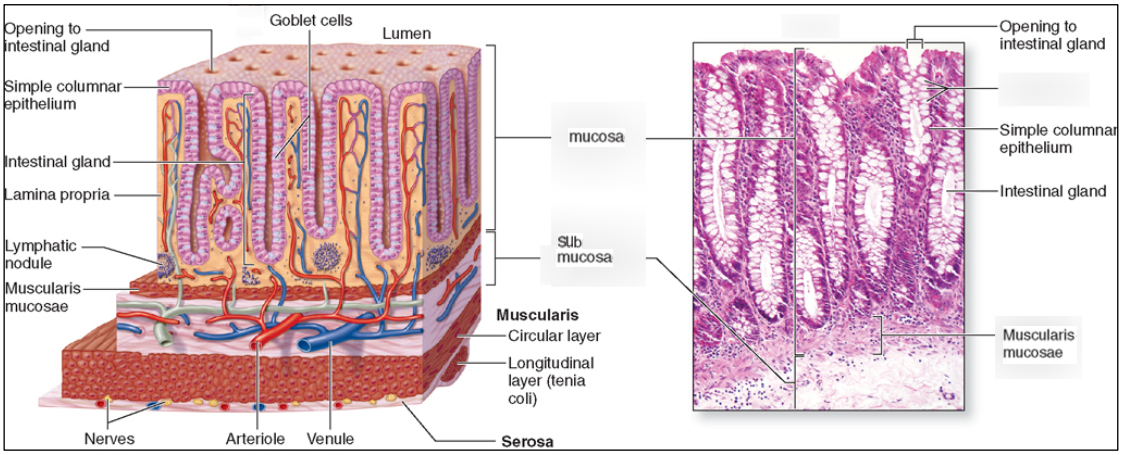


Figure 2 | Schematic human representation (a) and Haematoxylin and Eosin stain of large intestine of the normal structure of the large intestine adapted from Mescher *et. al.* (91)



During the formation of an anastomosis, all four layers of the bowel wall are transected. Throughout the 1800's, approximation of bowel wall layers included either the serosa alone (92) the serosa and the mucosal layers (93) or submucosa. (94) By the late 20th century studies favoured single layer anastomosis. (94–96) Findings from a recent systematic review including 13 experimental studies suggested that double-layer anastomoses were inferior to single-layer anastomoses due to an observed reduction of perfusion and increased inflammation at the anastomotic line. (97) It is also highlighted that single layer anastomoses take less time to construct and are less costly. (98) It has been demonstrated in *ex vivo* animal and human studies that sutures which incorporate the mucosal layer of the bowel wall do not contribute to anastomotic strength; with the strength of the join lying within the serosa and collagen and elastin rich submucosa. (80) Although practice varies and is subject to personal preferences of the surgeon, a single-layer anastomoses, not including the mucosa in the suture, has been broadly adopted. (25,26) The evidence base surrounding advantage of stapled versus hand-sewn anastomoses is discussed in detail in section 1.3.3.

Anastomotic healing is a dynamic process involving the interplay of degradative and reparative processes. It is a fine balance where factors may tip the balance in favour of degradation and anastomotic breakdown (e.g. local sepsis at the anastomotic line), whilst excess repair will lead to fibrosis and stricture formation. It is believed that bowel wall healing broadly follows the same processes as wound healing in human skin. (81–83) Although many of the cellular and molecular processes are common to all tissue healing, anastomotic healing environment differs markedly (Table 2). (89) The technical and biological processes involved make it challenging to reproduce anastomotic healing *in vitro* (99) and hence the translation of wound healing models in skin to the GI tract is extremely challenging.

Table 2 | Major differences between skin and anastomotic healing. Adapted from Morgan *et. al.* 2022 (100)

	Skin Wound	Intestinal Anastomosis
Time to Full Strength (~80%)	Gradual up to 6 Months	Rapid ~ 1 month
Strength Layers	Dermis	Submucosa & Serosa
Collagen Producing Cells	Fibroblasts	Fibroblasts and SMCs
Main Collagen Subtypes	Type I 80% / Type III 20%	Type I 68% / Type III 20% / Type V 12%
Collagenase Activity	Minimal	Increased in inflammatory phase (colon> small bowel)
pH	Stable	Wide range based on gastrointestinal tract location
Microbiome	Aerobic skin flora, infections generally treated topically, rare instances of bacteraemia	Gut microbiome with aerobic and anaerobic bacteria, potential for intra-peritoneal infection in the setting of AL
Mechanical Stress	Potential tension with movement depending on location, minimal sheer stress	Persistent sheer stress from intraluminal contents and peristalsis
Perfusion	Remains reasonably constant, but subject to temperature changes	Perfusion dependant on splanchnic perfusion which varies with physiological state

When bowel mucosa is breached, repair is undertaken by migration and hyperplasia of epithelial cells along with platelet aggregation and clot formation. This closes the defect, generates a partition to luminal bacteria and stimulates initial repair over a 72 hour period. (101) During the first few days following anastomosis creation, collagen degradation occurs with breakdown of damaged ECM regulated through several growth factors, cytokines, and collagenases at the site of injury which results in low anastomotic strength. (102) In the presence of sepsis, the reduction in tissue collagen content is exaggerated. (103) Extracellular MMPs are the main group of enzymes responsible for anastomotic healing, with some evidence suggesting overexpression of MMP-9 may increase the risk of AL through increased collagen degradation. (104)

Anastomosis integrity is therefore reliant on the ability of the collagen ratio present to hold the suture or staple material until new deposition can occur.

Three subtypes of collagen are produced by both fibroblasts and smooth muscle cells in anastomotic healing, whereas collagen is only synthesised by fibroblasts in skin healing. (105,106) Limited evidence suggests that there is an increased risk of AL in patients with specific extracellular matrix compositions. This includes a reduced collagen type I/III ratio and an increased expression of collagenase subtypes in the mucosa and submucosa. (107) Aprotinin (a collagenase inhibitor that promotes collagen formation) has been shown to increase anastomotic burst pressure and breaking strength in a rat model. (108) In humans a reduction of clinical and radiological AL was observed in a single RCT with application of aprotinin at the time of surgery (n=100). (109)

1.1.4.3 Anastomotic Vascularity

Adequate blood supply to the anastomotic line is essential for healing, with impaired tissue perfusion being a recognised risk factor for AL. Impaired perfusion can be secondary to systemic hypoperfusion (hypovolaemia), global reduction in bowel perfusion (macro- or microvascular disease, vasoconstriction to support other vital organs in states of shock), or a local reduction in blood supply as a consequence of bowel resection and tissue injury. Tissue oxygenation must be adequate to ensure optimal healing and is reliant on three factors; vascularity, vasomotor control, and arterial oxygen tension. (110,111)

Intraoperatively, bowel perfusion is assessed by palpation of pulsatile of mesenteric vessels, visibly assessing the colour of the bowel, and any active (or lack of) bleeding

at the wound edges of the bowel transection. More complex means of assessing tissue perfusion and oxygenation have been trialled and are discussed in section 1.3.2.3.

1.1.4.4 Influence of Microorganisms and The Microbiome

There is a growing literature in support of the gut microbiome being implicated in AL based on the hypothesis that leakage is an infective complication of an anastomosis. Recent studies in rat models have demonstrated that anastomotic injury results in a change in microbiota at the anastomosis with a 500-fold and 200-fold relative increase in the mucosal prevalence of *Enterococcus* and *Escherichia/Shigella* species respectively, with no change in the luminal prevalence. In rats that developed an AL, there was an observed increase in the production of cytotoxic necrotic factors and extracellular matrix degrading enzymes. (112) In a further rat study, it was demonstrated that *Enterococcus Faecalis* contributed to AL through the upregulation of collagenase activity and activation of MMP-9. (113) This has been further supported in animal models of low anterior resection with rectal anastomoses (114) which demonstrated changes of rectal flora observed following radiotherapy. (115)

In humans, *Enterococcus faecalis* and *Pseudomonas aeruginosa* are pathogens which have been implicated in AL (116) Alverdy *et al*. suggest that a decrease in microbial diversity following bowel resection and anastomosis encourages overgrowth of certain opportunistic species with a transition to a pathological phenotype with increased proteinase production leading to disruption in anastomotic healing. However an understanding of ‘normal’ microbiota and implications for gut function remain broadly unknown. (117)

1.1.4.5 Bowel Mobility

An overlooked area of potential influence on anastomotic integrity is the shear stress exerted by bowel peristalsis and luminal faecal transit. In a *post hoc* analysis of a randomised control trial, post-operative ileus was significantly associated with a higher prevalence of AL and an increased day-2 serum C-reactive protein post colorectal resection (n=43). (118) However, it is important to consider that the converse could also be argued and that early AL could in-fact cause ileus and a raised serum C-reactive protein.

1.2 The Economic Problem[1]

Anastomotic leak poses a significant financial burden on patients, hospitals and society. The evidence on the true cost of AL is limited and varies internationally, with differences in the methodology of economic analysis, health care systems, cost coding, and reporting of outcomes. (8) In the UK, current evidence suggests that NHS hospitals underestimate the true cost of AL, leading to inadequate reimbursement. (8) Little is known about the direct or in-direct financial costs in terms of reduced quality of life for patients and decreased productivity for society. There is also a need to better understand what factors influence these costs (119) to inform improved health economic modelling.

[1] *The following are the abridged findings of the systematic review undertaken as part of the preparatory work for this thesis published as: Khalil M, Burke JR La Raja C, Quyn A, Wargos-Palacios A, Meads D, Jayne D. The economic burden of colorectal anastomotic leak: A systematic review. (2022) The Annals of The Royal College of Surgeons of England Volume 105, Number S1, doi: 10.1308/rcsann.2022.0159 (Peer Reviewed Abstract)* (3)

1.2.1 Economic Burden

A recent systematic review investigated the economic burden of anastomotic leak in 10 studies across Europe and North America (8 studies in the USA, 1 study in the UK, and 1 study in Italy). Of the 486,444 patients included, 27,338 (5.62% [4.45%-12.30%]) had AL within 30 days of colorectal surgery. Reported total hospital costs ranged between $15,545 - $72,905 (AL) versus $7491 - $30,409 (Non-AL). Median length of hospital stay after AL was increased in 8 of the 10 studies by an average of 9.96 days (AL) [11.88 days - 30.28 days] as compared to the average hospital stay with no AL 5.0 days [3.46-13.93]. In the UK, the estimated cost of AL is £17,000, equating to an additional annual cost of £1-3 million when extrapolated nationally. (8) In Europe, the estimated additional cost of an AL is around 12,000 EUR with an added length of stay of 20 days (120). In the US, the figures are an additional $24,000 per AL and an extra 7 days stay. (121). Given that AL rates remain constant and the incidence of colorectal cancer is increasing in the younger population there is concern that this problem, and the associated costs, will only escalate. (122)

1.2.2 The Environmental Impact

The treatment of AL carries an environmental impact. A recent environmental impact assessment by Biscofberger *et. al.* using activity and carbon emission data for a typical AL care pathway demonstrated an average climate, water and waste impact of 1303 kg CO_2-eq, 1803 m^3 of water, and 123kg of waste per patient. (123) The greatest contributors to this were grade C leaks (see section 1.1.1). Building environmental impact into the rationale for allocation of research funding is slowing starting to take hold and is an important consideration. (124)

1.3 Current Strategies to Prevent Colorectal Anastomotic Leak

The mainstay of attempts to reduce AL are based on altering modifiable risk factors and reducing the impact of non-modifiable risk factors (Table 1). (125) These can be broadly classified into 5 categories:

1. Preoperative interventions
2. Intraoperative checks
3. Anastomotic construction
4. Anastomotic reinforcement
5. Anastomotic protection

1.3.1 Preoperative Interventions

1.3.1.1 Mechanical Bowel Preparation

The use of oral mechanical bowel preparation prior to colorectal resection and anastomosis remains contentious (126–128) with clear disadvantages documented including hypovolaemia and metabolic disturbance. (55,129) Mechanical bowel preparation does confer the ability to perform intra-operative endoscopic investigation and helps to avoid faecal loading of the colon. Until recently the effect of mechanical bowel preparation and its effect on the gut microbiome had not been considered. (130) A recent meta-analysis of 60 randomised controlled trials, including 16,314 patients, demonstrated no significant difference in AL when using mechanical bowel preparation in the absence of intravenous and oral antibiotics. However, it is thought that mechanical bowel preparation does reduce septic complications including abdominal and pelvic collections which are likely related to subclinical leaks. (131)

1.3.1.2 Preoperative Antibiotics

Preoperative non-absorbable oral antibiotic preparations (OAP) have been shown to reduce the risk of AL following elective gastrointestinal surgery, supported by a recent systematic review. (132) Administering OAPs with mechanical bowel preparation may allow increased delivery of OAP to the colonic mucosa through reduction of faecal loading within the bowel lumen. More recently Koskenvuo *et. al.* demonstrated that mechanical bowel preparation and preoperative oral antibiotics significantly reduced anastomotic dehiscence's (5.8% vs 13.5%, OR, 0.39 [95% CI, 0.21-0.72]) in a 565 patient randomised controlled study. (126,133–135) The previous systematic review of Tan *et. al* demonstrated marginally lower AL rates with intravenous antibiotics and oral antibiotics (OR 0.63 (95 per cent CI 0.44 to 0.90)) and intravenous antibiotics and oral antibiotics and mechanical bowel preparation (OR 0.62 (95 per cent CI 0.41 to 0.94)) compared with intravenous antibiotics alone. (131)

1.3.2 Intraoperative Checks

1.3.2.1 Air Leak Test

The air leak test is a safe, rapid, and inexpensive check for anastomotic integrity that involves submerging the anastomosis under saline and carefully infiltrating air into the distal bowel lumen by means of a syringe or endoscope per rectum. (136) In those patients with a positive test, suture repair of the defect alone was associated with a higher AL rate and it was recommended that the anastomosis either be refashioned or a diverting stoma performed. 25% of air leaks were detected in patients that underwent the test. After their operation 4% suffered clinical leaks in the 'test' group and 14% in the 'no test' group (P = 0.043). There were 11% radiological leaks in the 'test' group and 29% in the 'no test' group (P = 0.006). These principles have been applied to anastomoses throughout the large intestine (137)

1.3.2.2 Intraoperative Endoscopy

Endoscopic evaluation of an anastomosis allows for a more accurate assessment of the anastomotic line including integrity of the join, the identification of intraluminal bleeding and the detection of additional pathology. (137,138) Its benefit in routine use has yet to be demonstrated. The largest study by Shamiyeh *et. al.* (n=338) demonstrated minimal AL pick up rates between routine (1.2%) and non-routine endoscopy (1.5%) after the formation of an anastomosis. (139)

1.3.2.3 Bowel Perfusion Assessment

Recent advancements in fluorescent perfusion angiography (FPA) have allowed surgeons to subjectively assess local tissue perfusion, identify optimal bowel segments for anastomosis and to check anastomotic perfusion afterward joining. This is achieved through systemic injection of a fluorophore (e.g. indocyanine green, ICG) followed by visualisation using a near-infrared camera. (140,141) When administered intravenously, ICG binds with high affinity to plasma proteins and remains largely within the intravascular space. It has a short plasma (3-5 minutes) and intravascular (15-20 minutes) half-life , which allows for multiple dose administration. (142)

The use of near-infrared indocyanine green (NIR-ICG) intraoperatively to assess anastomotic perfusion is safe and feasible (142,143), however, no system is yet able to objectively quantify tissue perfusion and the adequacy of the blood supply remains reliant on the operating surgeon's judgement. There have been numerous systematic reviews on the use of bowel perfusion assessment in colorectal surgery, based on observational studies but few randomised controlled trials. The latest review by Renna *et. al.* (2023) investigated the assessment of bowel perfusion with fluorescence angiography as well as hyperspectral and laser speckle contrast imaging with all modalities demonstrating comparable results. The pooled leak rate following an

anastomosis was 0.05 (95 per cent CI 0.04 to 0.07) as compared to 0.10 (0.08 to 0.12) without, across 66 eligible studies involving 11,560 patients. (144) In 2017, a large RCT in the USA evaluating ICG-NIR in rectal surgery was terminated due to slow recruitment. (145) Following this review the EssentiAL trial published its results in May of 2023. This was a randomised open-label phase 3 trial including 850 patients across 41 hospitals in Japan. Designed as a non-inferiority study; the observed reduction AL rate in the ICG+ group was lower than expected leading the authors to conclude that ICG-FI was not superior to white light. Despite a statistically significant 4.2% reduction in AL seen in the ICG+ group the study was not deemed significant overall because It failed to reach the prescribed non-inferiority boundaries. (146) The MRC/NIHR IntAct trial is an ongoing European RCT comparing surgery with NIR-ICG against white light laparoscopy in the prevention of AL with results anticipated in 2024. (147)

This use of light spectroscopy and modified pulse oximetry has demonstrated a reactive rise in tissue oxygen tension in the proximal anastomotic segment in a single study, highlighting a possible tissue level compensatory mechanism. (148–150) A further study has demonstrated increased bowel blood flow and volume through CT perfusion scanning in patients undergoing chemoradiotherapy at the site of rectal cancer. (151) This has implications for assessing AL risk particularly after neo-adjuvant therapy where there is a perceived increased risk of AL despite a recent meta-analysis indicating the opposite. (152) The AVOID Study (Trial registration: NCT0471203) which is a phase III, multicentre, randomised controlled trial (n=978) using indocyanine green for the prevention of anastomotic leakage in colorectal surgery is due to report out in the coming months.

1.3.3 Anastomosis Construction

1.3.3.1 Stapled Versus Hand-sewn Anastomoses

Findings from a number of Cochrane reviews and meta-analyses have not demonstrated superiority for either stapled or hand-sewn anastomoses at any site of the large intestine and rectum. (97,153–155) However stapled anastomoses are preferred in many centres due to speed, ease and reproducibility. There are several studies where findings indicate that in rectal anastomoses, the number of staple firings in double stapled anastomoses increase AL rates in the distal rectum with no difference in single over double-stapled low rectal anastomoses. (19,34–37)

1.3.3.2 Compression Anastomoses

Various innovative methods for constructing anastomoses have been tried, including the use of anastomotic compression devices (first described 200 years ago) (156), and then in the 1980s using the AKA-2™ device (Seidel Medipool, Munich, Germany). This device was made from two plastic rings which compressed inverted bowel edges that released spontaneously at day 4 to 6. The ring device results in simultaneous necrosis and healing, but the device can only be applied during open surgery. (157,158) A similar bio fragmentable anastomotic ring (BAR) (Valtrac, Davis & Geck, Wayne, NJ, USA) was devised, which when closed left a small window between the two rings to reduce tissue necrosis. The patient then passes the rings per rectum 2-3 weeks later. Although described as a “suture-less” anastomosis, it requires sutures to secure the device in place. (159,160) In 2011, the NiTi ColonRing™ (NiTi Surgical Solutions, Chesterfield, USA) introduced compression rings that had shape memory properties, with claims of a more uniform tissue compression at the anastomotic line. Findings from a large multicentre study (n=1180) demonstrated a leak rate of 3% in mostly end-to-end laparoscopic, left sided anastomoses, but like all other compression devices it has yet to be broadly adopted. (161)

1.3.4 Anastomotic Reinforcement

The challenge of AL has attracted substantial industry attention resulting in several diverse products being brought to the market. However, few strategies have enjoyed commercial longevity, largely through failure to establish efficacy in wider clinical practice. At the other end of the spectrum, simple staple line reinforcement with bio absorbable materials such as a buttress sutures to improve tissue apposition and increase tensile strength has shown no benefit in three large randomised studies. (162–164)

1.3.4.1 Intra-luminal Stents / Bypass Technology

Various intra-luminal colonic stents and anastomotic line bypass technologies have been trialled to protect colorectal anastomoses. Clinical testing has shown the devices to be safe, but lacking in efficacy. (165–168) Findings from the most recent pilot studies of Colospan (Colospan Ltd. Kfar-Saba, Israel) and Safeheal Colovac (Colovac, Safeheal, France) have shown them to be safe and are currently undergoing efficacy trials. (169,170)

1.3.4.2 Mesenteric Flap

A single study has reported the use of a pedicled mesenteric flap 1-2cm distal to the site of anastomosis to auto-buttress the join line following left sided colectomies with no difference in AL rates in 65 patients. This has yet to be the subject of a comparative study and hence findings of this single study are to be interpreted with caution. (171)

1.3.4.3 Defunctioning stoma

In many centres the routine use of a defunctioning stoma upstream from the anastomosis is used. The rationale that this may reduce reoperation rate in the event of AL (due to stool already being diverted) or reduce the severity (reducing the amount of

effluent passing through any mucosal breach) appears to have some merit. (172) The suggestion that a defunctioning stoma reduces the risk of leak by around 10% is evidenced in a recent meta-analysis of 8000 patients, however this is disputed. (173–175)

1.3.4.4 Trans-anal / -rectal Drainage Tube

A trans-anal drainage tube is a silicon tube placed per rectum with the tip beyond / above the joint line and the distal end secured to the thigh or buttock following an anastomosis. Seven trials, with over 1600 participants, were examined in a recent meta-analysis which demonstrated a lower AL rate in the trans-anal tube group (RR 0.38; 95 % confidence interval (CI) 0.25-0.58; P < 0.0001), as well as a reduced reoperation rate (RR 0.31; 95 % CI 0.19-0.53; P < 0.0001) and a shorter hospital stay (mean = -2.59 days; 95 % CI -3.69 to -1.49; P < 0.0001). (176)

1.3.5 Tissue Adhesives and Sealants

1.3.5.1 Fibrin Glue

The use of fibrin-based products to ‘seal’ an anastomosis has been investigated for the best part of three decades. Fibrin is a polymer protein which is the final product following lysis of plasma fibrinogen and is readily available from donated plasma. Although findings from some animal studies have demonstrated a benefit in reducing AL, a systematic review by Vakalopoulos *et. al.* in 2013 found no benefit in AL rate from the use of fibrin glue across all human studies. (177,178)

1.3.5.2 Platelet Rich Concentrates[2]

There are two main types of platelet concentrates: platelet-rich plasma (PRP) and platelet-rich fibrin (PRF). PRP was the first to be developed and reported by Marx *et al.* in 1998. It is produced using a two-step centrifugation process with biochemical additives, such as anticoagulants or bovine thrombin. (179) In contrast, PRF does not require additives and is produced using a single centrifugation step. This is carried out immediately after blood is drawn to prevent coagulation. (180)

The main difference between the two types is their fibrin architecture, where PRF has a higher density of fibrin. (181) During production of PRF, in the absence of an anticoagulant, the fibrin network gradually forms during centrifugation. This results in a three-dimensional fibrin scaffold with associated platelets and leukocytes, which has the advantage of releasing high quantities of growth factors over a sustained period. Findings from previous studies have shown that PRF releases a significant amount of platelet derived growth factor (PDGF), fibroblastic growth factor (FGF) and vascular endothelial growth factor (VEGF) for up to 2 weeks after centrifugation. (182,183)

Platelet rich concentrates have been used in clinical trials for arthroscopic rotator cuff repair, and a recent meta-analysis suggests the PRP-infused collagen matrices may reduce recurrence rates in selected circumstances. (184) Delivery methods vary but it is feasible to create a PRP mesh which could theoretically be sutured externally to the bowel wall. (4) Findings from rat and pig models have shown mixed benefits in

[2] *The following are the abridged findings of the feasibility study and literature review undertaken as part of the preparatory work for this thesis published as: Burke JR, Helliwell J, Kowal M, Jayne D. Characterisation of a Platelet Rich Fibrin Membrane and Formation of an Autologous Fibrin Mesh. British Journal of Surgery, Volume 108, Issue Supplement_6, September 2021 doi: 10.1093/bjs/znab259.198 (Peer Reviewed Abstract)* (4)

anastomotic bursting pressure, but have not shown any difference in AL rates. (185–187) PRP has yet to be investigated for efficacy in clinical trials for colorectal AL. (188)

1.3.5.3 Cross Linking Gelatins

Cross-linking Gelatins are cow-derived sealants that are currently under investigation in clinical studies. Early animal studies suggest that these sealants induce less tissue inflammation than fibrin glue after it is exposed to high-frequency light which induces polymerisation. (189,190)

1.3.5.4 Polyglycolic acid

Seamguard® (Gore & Associates Inc, Flagstaff, Arizona, USA) is a polyglycolic acid-based material used to buttress and provide additional extraluminal strength to stapled anastomoses. Efficacy is reported in bariatric surgery, but a recent randomised controlled trial in colorectal surgery failed to show a benefit in AL reduction. (163,164,191)

1.3.5.5 Alginate Gel

The most notable ongoing trial is currently investigating LifeSeal™ (LifeBond, Caesarea Industrial Park, Israel) and its effect on overall AL rates in subjects undergoing low anterior resection with an anastomosis less than 10 cm from the anal verge, in a prospective, multi-centre, multinational randomised, single-blind, double armed study. It is a gelatin based product which aims to seal the anastomotic line. (192) Findings from a recently published retrospective study of 356 patients demonstrated a lower incidence of AL in a group of left sided colon cancers (n=43) after propensity score matching in LifeSeal (2.3% AL) versus control (13.4% AL) p=0.042. (193)

1.3.5.6 Butyrate

In animal models, Butyrate has been used in 19 studies and demonstrated significantly increased anastomotic strength, collagen synthesis and collagen maturation but there remains no current use in human clinical studies. Butyrate is thought to exert its function by acting as a histone deacetylase (HDAC) inhibitor or signalling through G-protein coupled receptors at the anastomotic line. (194)

1.3.6 The Role of Adhesions

Bowel adhesions are irregular bands of scar tissue that form between two abdominal structures that are not normally bound together. Little is known about the role of adhesions in anastomotic leak. Intuitively, adhesions could play a role in contributing to leak through their theoretical external tension on an anastomotic line or be protective as a “natural sealant” for breaches in the anastomosis. There are few animal studies investigating this concept. (113)

1.4 The Use of Omentum

1.4.1 Omental Wrapping (Omentoplasty)

The greater omentum is a fatty, double layered membrane that hangs from the greater curvature of the stomach and is the first structure encountered during a laparotomy. Its role in localising abdominal inflammation through adhesion formation, its angiogenetic properties, and its ability to absorb fluid and particulate matter and present them to macrophages and immunocompetent cells suggest it might be a viable source of protection for bowel anastomoses. (195). The deliberate wrapping of vascularised greater omentum around an anastomosis (Omentoplasty) may enhance healing (196). However, the evidence is not strong enough to recommend routine adoption in colorectal surgery. There is some evidence of its use in the prevention of oesophageal

leak which may reflect the early evidence of differing microbiome between the two sites and its effect on AL. (195) It is safe and technically easy to perform and may help fill dead spaces (such as the pelvis) after anterior resection and so could theoretically contain low volume leaks. (15,197)

1.4.2 Mesenchymal Stem Cells

Clinical interest in stem cell therapy for wound healing has risen exponentially over the last decade with a particular focus on the capability of adipose-derived mesenchymal stem cells and their ability to promote human tissue regeneration and repair. (198) (199). First described by Becker *et. al. in 1963,* (200) stem cells can be classified broadly as Embryonic or Adult Mesenchymal and are characterised by their ability to self-renew and differentiate into multiple cell lineages. (201) Mesenchymal stem cells (MSCs) are usually harvested from the stromal-vascular cell fraction (SVCF) of subcutaneous fat or bone marrow with established minimum criteria for defining them in humans. (199,202) They have multipotent capability to differentiate into several cell types and produce angiogenic and anti-inflammatory factors that promote wound healing. They can be isolated or delivered within a mixed cell population to a site of interest through a variety of delivery mediums making them a potential prophylactic intervention at the site of an anastomosis. Autologous adult stem cells, such as MSCs are more readily available than embryonic stem cells and have none of the ethical or immune-reactive constraints. (199,203,204)

Minimal criteria to characterise human MSCs have been defined (Appendix 1). These criteria, proposed in 2006 by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, aimed to promote standardisation in MSC research findings. (202) Notably, both in humans and in mice, radiotherapy resistant

phenotypes have been described. In 2015, Nicolay *et. al.* demonstrated that after high radiation doses MSCs were able to retain their stem cell characteristics which may be a result of their particular DNA repair pathways. (205) A proposed limitation to the therapeutic use of MSCs is the concern that they may promote abnormal growth of occult tumour cells. However, studies show conflicting results with the reporting of both promotion and suppression of tumour growth, which may be due to the different cancer types and treatment regimens. (206) Despite this, findings of a review by Klopp *et. al.* in 2011 found evidence of new tumour formation in over 1000 patients treated with MSCs for a variety of pathologies. (207)

1.4.3 Adipose Derived Regenerative Cells

MSCs have been isolated from various tissue types, including bone marrow, muscle, skin, and adipose tissue. In addition to its role as an energy store and thermal insulator, adipose tissue performs numerous endocrine functions. (208) Given the abundance and accessibility of adipose tissue, it is an ideal source of regenerative cells for therapeutic application. Adipose derived regenerative cells (ADRCs) is a term used to describe MSCs isolated from the SVCF of human adipose tissue. (209) ADRCs consist of a heterogenous mixed cell population including mesenchymal progenitor cells, pericytes, adipocytes, macrophages and T-cells that produce a rich milieu of cytokines and growth factors conducive to wound healing. (59,210,211)

1.4.4 Omental Derived Regenerative Cells

ADRCs are found in the SVCF of white adipose tissue which is present in subcutaneous fat and the omentum. (212) Current lipoaspiration techniques confer an abundance of ADRCs over bone marrow aspiration. (213) Procedures are less painful than harvesting bone marrow stem cells and with less ethical considerations, (201)

however, they are not without morbidity. (214–217) Other biological sources may therefore be more suitable and translatable for use in animal and human models of anastomotic leak. An ideal source for harvest in abdominal operations is the greater omentum. If omental derived regenerative cells (ODRCs) can be harvested through the modification of existing ADRC methods, they could be a powerful biological source for augmenting healing of gastrointestinal anastomoses. (218–221) MSCs have been used in a single human study demonstrating the feasibility of autologous delivery through mesenteric vessels to the site of 6 paediatric small bowel anastomoses following transplantation for short gut syndrome. (222) However, no clinical or histological efficacy was demonstrated.

1.5 Summary

Colorectal AL has been a longstanding complication of bowel surgery and the subject of clinical, academic, and industry interest. Improving outcomes of patients who undergo an anastomosis relies on a better understanding of anastomotic line healing and a standardisation of technique for those patients who are recruited into clinical trials. Innovative solutions, with the patient and surgeon at the centre of new developments, is imperative if we are to overcome the challenge of AL.

2. Thesis Aim and Objectives

2.1 Thesis Aim

The main aim of this PhD is to determine whether the application of omental derived regenerative cells to the anastomotic line improves healing and reduces AL in an animal model.

The completed programme of work will form an evaluation of the current literature on the clinical use of omental derived regenerative cells, the processes involved in normal anastomotic healing, and characterisation of omental derived regenerative cells (ODRCs) harvested from the Omental stromal vascular cell fraction. I will utilise different methodologies including systematic reviews, animal studies, and laboratory techniques to put forward the case to support the first in human clinical trial of ODRCs to reduce AL.

2.2 Thesis Objectives

I. To conduct a systematic review of the literature to identify how omental derived regenerative cells, as a source of mesenchymal stem cells, have been used to augment anastomotic healing (Chapter 3).
II. To investigate the characteristics of omental stromal vascular cell fraction and explore a suitable vehicle for their delivery to an anastomosis (Chapter 4).
III. To develop a reliable and reproducible animal model to study the biological mechanisms of anastomotic healing (Chapter 5).
IV. To evaluate the efficacy of an omental regenerative cell-gel implant to improve anastomotic healing and prevent leak in an animal model (Chapter 6).

The discussion in Chapter 7 explores how these models may be used as the standard to test new interventions for both histological (Chapter 5) and macroscopic or clinical outcomes e.g. leak vs. no leak (Chapter 6). If significant changes to AL rates are to be realised a standardised approach to preclinical testing of new interventions is required.

3. The Use of Mesenchymal Stem Cells in Animal Models of Anastomotic Leak – A Systematic Review[3]

Mesenchymal stem cells are the primary regenerative cell of interest within the human omentum. To date, there has been one human study of MSCs applied to small bowel transplant in six patients in a paediatric population. Animal models are required to test both the safety, feasibility and, if possible, efficacy of any novel intervention before any clinical evaluation is possible. A narrative meta-synthesis is conducted which analyses the key variables in animal studies of anastomotic healing. Given the possible cross-over, studies involving the upper gastrointestinal tract are included to present a full understanding of the topic. This chapter will inform both the selection of an appropriate animal model and the design of a randomised study to test the use of mesenchymal stem cells in the prevention of anastomotic leak.

3.1 Background

The fact that clinical studies do not include an accepted definition of AL precludes an accurate comparison of study outcomes. (223) It is suggested that studies should focus on understanding the biological processes that lead to AL prior to conducting human investigations. (224) Various preclinical models of AL exist, but Yauw *et. al.* concluded in a systematic review that the vast majority of studies were of poor quality and there was heterogeneity in animal models due to varying anatomy and physiology between

[3] *The following are the abridged findings of the systematic review undertaken as part of the preparatory work for this thesis published as: Burke JR, Helliwell J, Wong J, Quyn A, Herrick S, Jayne D. The use of mesenchymal stem cells in animal models for gastrointestinal anastomotic leak: A systematic review. Colorectal Disease. 2021 Dec;23(12):3123-3140. doi: 10.1111/codi.15864. Epub 2021 Aug 20. PMID: 34363723. (Peer Reviewed Full Manuscript)* (1)

species. (225) The introduction of the ARRIVE guidelines in 2010 (updated in 2020) improved the reporting of outcome measures in animals. (226) A 2016 International consensus statement (210) outlines clear recommendations and reporting standards for animal research into lower gastrointestinal AL.

Clinical interest in stem cell therapy for wound healing has risen exponentially over the last decade (198) with particular focus on the capability of adipose-derived mesenchymal stem cells and their use as a cell-based therapy to promote human tissue regeneration and repair. (199).

3.2 Aim

To investigate the evidence supporting the use of MSCs in animal models of anastomotic leak and whether the models and methodology used meet the minimum standard to justify their clinical application. (59,210)

3.3 Research Question

Primary Research Question:

- Do MSCs confer any benefit in preventing leakage when applied to animal models of gastrointestinal AL and how is efficacy assessed?

Secondary Research Questions:

- What methods are used to isolate and deliver MSCs to an anastomosis?
- Do the animal models comply with ARRIVE guidelines? (227)
- In those models with lower gastrointestinal anastomoses, do they comply with the 2016 International Consensus statement regarding the use of animal models for research in lower gastrointestinal tract anastomoses? (210)

3.4 Methods

3.4.1 Search Strategy

The protocol for this review was guided by the PRISMA reporting guidelines and was registered with PROSPERO (reg. number: 42020169057) and the SyRF (Systematic Review Facility) in line with The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). (228) The following electronic databases published between 1st January 1947 to 1st May 2020 were searched: MEDLINE, EMBASE, Web of Science, Cochrane Library and clinicaltrials.gov. To ensure literature saturation, citations and reference lists of selected studies were reviewed to identify any missed papers. All studies that explored the delivery of MSCs to or within a gastrointestinal anastomosis were considered. All studies which involved animals across all species that underwent surgery to model a gastrointestinal AL were considered.

Figure 3 | Search Strategy for OVID (MEDLINE, EMBASE & Web of Science)

1. Mesenchymal.mp
2. Stem.mp
3. Stromal.mp
4. Cell.mp
5. Anastomo*

1 OR 2 OR 3
4 AND 6
5 AND 6 AND 7

[Limit to Animals]
[Limit to English Language]

Cochrane Library Search Strategy
((((mesenchymal) OR stem) OR stromal)) AND (Cell) AND (Anastomo*))

Clinicaltrials.gov Search Strategy
Search “Anastomotic Leak” = 111 hits
Search “Anastomotic Leak” [Limit= studies which has results] = 2 hits

Studies where MSCs were applied to laboratory animals to demonstrate an effect on the healing of a gastrointestinal anastomosis (through any route or vehicle) were considered. These included, but were not limited to, autologous, allografts and xenograft cell transplants delivered locally or systemically:

Interventions:

(a) Mesenchymal Stem Cells alone

(b) Mesenchymal Stem Cell + Delivery Vehicle

Comparators:

(a) Placebo

(b) Control

(c) Placebo + other therapy

(d) Control + other therapy

The search was undertaken on 31st January 2020 and repeated on 1st May 2020. Results were limited to title and full abstract for the initial search. English-language, original articles identified through the database search were included. Duplicates and conference abstracts were removed. Selection was not limited to peer-reviewed publications and included grey literature. Reviews were not excluded.

3.4.2 Inclusion Criteria

- Investigates the application of mesenchymal stem cells to or within a gastrointestinal anastomosis
- Experimental Study
- English Language

3.4.3 Exclusion Criteria

- Study Protocols
- Non-English Language
- Conference abstracts, case reports, letters and chapters

3.4.4 Data Extraction and Analysis

Studies were selected using a staged review of titles and abstracts, followed by full text review by two independent reviewers; Joshua Burke (The Candidate) and Mr Jack Helliwell (NIHR Academic Clinical Fellow, Leeds, UK). Identified abstracts, and those from additional sources, were screened independently by the two reviewers to identify studies that potentially met the inclusion criteria. The full texts of potentially eligible studies were retrieved and independently assessed for eligibility by the two reviewers. Any disagreement over the eligibility of studies was resolved through discussion with the senior research team. Data were compiled in an Excel spreadsheet (Microsoft, CA, USA), which was tested initially in five studies to ensure clarity and completeness. Extracted data included study characteristics, animal breed, age, strain and weight, AL model, stem cell source, mode of delivery and vehicle. All experimental and histological outcomes were extracted.

3.4.5 Data Items

3.4.5.1 Compliance to ARRIVE Guidelines

The ARRIVE Guideline checklist was used to quantitatively assess compliance to reporting outcomes and the design of animal studies. Studies were scored independently by the two reviewers; Joshua Burke (The Candidate) and Mr Jack Helliwell (NIHR Academic Clinical Fellow, Leeds, UK) using the ARRIVE 2.0 38-point checklist with each item scored as 1 (compliant) or 0 (non-compliant). Each of the scores were then averaged and reported as a total percentage score (Appendix 2).

3.4.5.2 Compliance of Lower Gastrointestinal Models to International Consensus

All studies involving lower gastrointestinal anastomoses were reviewed against recent International Consensus criteria on the use of animal models for research on lower gastrointestinal tract anastomoses. Studies were scored independently by two reviewers across the 6 domains; selection of animal model, location and type of surgery, macroscopic outcome, histological assessment, mechanical and biochemical outcome measures, animal testing and welfare. Each domain was scored as 1 (compliant) or 0 (non-compliant) depending on alignment with the domain consensus statement. Each of the Scores was then averaged and reported as a total percentage score (Appendix 3). (229)

3.4.6 Risk of Bias in Individual Studies

Due to the heterogeneity of study designs, the use of a bias scoring tool was deemed inappropriate.

3.4.7 Types of Outcome Measures

All outcomes including experimental and histological were examined. Experimental outcomes included anatomical site, mortality, anastomosis closure rate, AL rate, systemic infection rate, abscess formation, adhesion formation and ileus. Histological outcomes included inflammatory cell infiltration, mechanical strength, neovascularisation, collagen deposition and the presence stem cells confirmed as residing at anastomosis.

3.4.8 Summary Measures

Binary outcome measures included leak rate reduction and odds ratios. Continuous outcomes included difference in means.

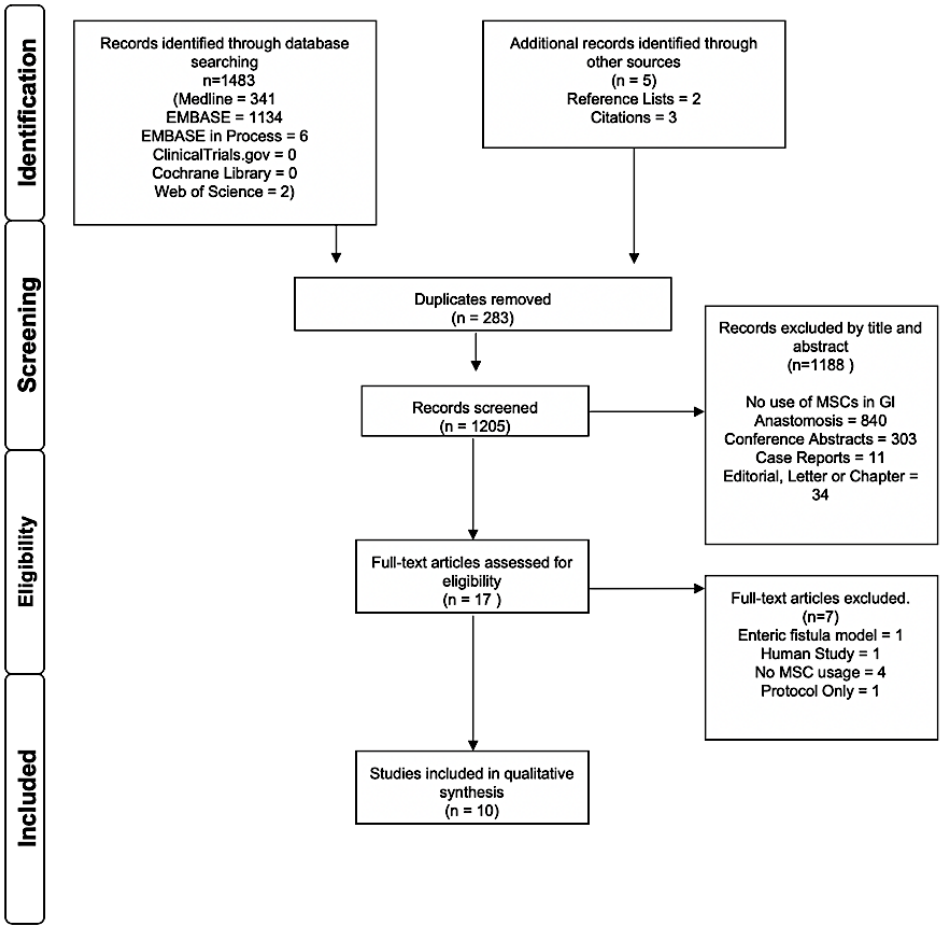
3.4.9 Synthesis of Results

Quantitative data synthesis was not attempted to avoid the anticipated effects of excess heterogeneity between studies. A descriptive approach was chosen to summarise the diverse range of selected studies in a structured manner, following the European Social Research Council Guidance on the Conduct of Narrative Synthesis in Systematic Reviews. (230) The results were tabulated to highlight important similarities and differences between the studies. The studies were also grouped by anatomical site and evidence synthesised to provide a narrative, relevant to the research questions.

3.5 Results

The initial search using all 3 search platforms returned 1483 articles. After duplicates were removed, 1205 results remained and the abstracts were compiled into a database and screened, leaving 17 abstracts for full-text assessment based on inclusion and exclusion criteria. Of these, seven studies were further excluded leaving 12 studies in the final analysis. (Figure 4)

Figure 4 | PRISMA flow diagram of search and eligibility process.



Do mesenchymal stem cells confer any benefit in preventing leakage when applied to animal models of gastrointestinal AL and how is efficacy assessed?

3.5.1 Anatomical Location

Gastrointestinal anastomoses were performed in 438 animals across 7 studies in 4 different species (rabbit, rat, pig and micro pig) with 58% being performed in rat colon (Table 3). The studies demonstrated variance in the anatomical site of gastrointestinal anastomoses (Oesophagus = 1, Stomach = 1, Bile Duct = 2, Small Bowel = 1, Large bowel = 7). Half were randomised studies and half were prospective cohort studies.

3.5.2 Use of Mesenchymal Stem Cells

Eleven studies utilised allogenic MSCs, and only Sukho *et. al.* delivered cultured cells using a xeno-transplant (231). Eight studies used subcutaneous adipose tissue as the source of MSCs. (232–238) A further three studies used bone marrow aspirate (232,239,240) and a single study used intrabdominal adipose tissue. (241) All cells were cultured to confluence, most commonly at third passage with a median of $2x10^6$ cells ($1x10^6$-$1x10^7$) applied to the anastomosis. In nine studies, MSCs were labelled prior to application to confirm that MSCs were present at the anastomotic site (Table 3). Three studies isolated MSC subtypes using a range of cell markers with immunofluorescence.

Table 3 | Study design, population, mesenchymal stem cell source, and data outcomes for the 12 included studies. SCA = Sub-cutaneous Adipose Tissue. BMA = Bone Marrow Aspirate. IAA = Intrabdominal Adipose Tissue.

#	Anatomical Location	Authors	Date	Study Type	N	Population	Stem Cell	Source	Method of Delivery	Vehicle	Passage	No of cells del./ dose	Labelled
1	**Oesophagus**	*Xue et. al. (China)* (232)	*2019*	*RS*	*21*	*Rabbit*	*Allogenic MSC*	*BMA*	*Local*	*Fibrin Matrix*	*3*	$2x10^6$	*Lenti.GFP*
2	**Gastric**	*Komiyama et. al. (Japan)* (242)	*2013*	*Cohort*	*40*	*Rat*	*Allogenic MSC*	*IAA*	*Local*	*None*	*3*	$1x10^7$	*CM-DiL*
3	**Bile Duct**	*Zhang et. al. (USA)* (243)	*2020*	*Cohort*	*9*	*Pig*	*Allogenic MSC*	*SCA*	*Local*	*Vicryl Mesh Stent*	*3/4*	$4x10^6$	*89Zr PET*
4	**Bile Duct**	*Hara et. al. (Japan)*(233)	*2020*	*Cohort*	*11*	*Pig*	*Allogenic MSC*	*SCA*	*Local*	*Cell Sheet*	*3*	$2x10^6$	*PKH26GL*
5	**Small Bowel**	*Maruya et. al. (Japan)* (234)	*2017*	*RS*	*7*	*Micro Pig*	*Allogenic MSC*	*SCA*	*Local*	*Cell Sheet*	*3*	$2x10^6$	*PKH26GL*
6	**Colon**	*Pascual et. al. (Spain)* (235)	*2008*	*RS*	*40*	*Rat*	*Allogenic MSC*	*SCA*	*Bio suture*	*Bio suture*	-	-	*copGFP*
7	**Colon**	*Adas et. al. (Turkey)* (240)	*2011*	*Cohort*	*40*	*Rat*	*Allogenic MSC*	*BMA*	*Local*	*Injection (PBS)*	-	$1x10^6$	*BrdU*
8	**Colon**	*Yoo et. al. (Korea)* (244)	*2012*	*RS*	*60*	*Rat*	*Allogenic MSC*	*SCA*	*Local*	*Fibrin Matrix*	*3*	$1x10^6$	-
9	**Colon**	*Adas et. al. (Turkey)* (239)	*2013*	*Cohort*	*40*	*Rat*	*Allogenic MSC*	*BMA*	*Systemic*	*NACL*	*0*	$1x10^6$	*BrdU*
10	**Colon**	*Van De Putte et. al. (Belgium)* (237)	*2017*	*Cohort*	*48*	*Rat*	*Allogenic MSC*	*SCA*	*Systemic*	*PBS*	-	$1x10^6$	*Crystal Violet*
11	**Colon**	*Sukho et. al. (Netherlands)* (231)	*2017*	*RS*	*57*	*Rat*	*Xenograft MSC*	*SCA*	*Local*	*Cell Sheet*	-	-	*SPIO*
12	**Colon**	*Alvarenga et. al. (Brazil)* (245)	*2019*	*RS*	*65*	*Rat*	*Allogenic MSC*	*SCA*	*Local*	*Injection*	-	$2x10^6$	-

Table 4 | MSC subtypes, characterisation markers, and method of characterisation used for the 12 included studies. SCA = Sub-cutaneous Adipose Tissue. IF = immunofluorescence. FACS = Fluorescence-activated cell sorting.

#	Anatomical Location	Authors	Date	Study Type	N	Population	Stem Cell	Source	Isolation of Subtype	Characterisation Markers Used	Method
1	**Oesophagus**	*Xue et. al. (China)*(232)	*2019*	*RS*	*21*	*Rabbit*	*Allogenic MSC*	*BMA*	*Adipocytes and Osteocytes*	*CD29 / CD44 / CD90 / CD45 / oil-red O / alizarin red*	*IF*
2	**Gastric**	*Komiyama et. al. (Japan)*(242)	*2013*	*Cohort*	*40*	*Rat*	*Allogenic MSC*	*IAA*	*N/A*	*CD90 / CD31 / CD34*	*FACS*
3	**Bile Duct**	*Zhang et. al. (USA)*(243)	*2020*	*Cohort*	*9*	*Pig*	*Allogenic MSC*	*SCA*	*N/A*	*CD34*	*IF*
4	**Bile Duct**	*Hara et. al. (Japan)*(233)	*2020*	*Cohort*	*11*	*Pig*	*Allogenic MSC*	*SCA*	*N/A*	*CD44 / CD31 / CD45*	*IF*
5	**Small Bowel**	*Maruya et. al. (Japan)*(234)	*2017*	*RS*	*7*	*Micro Pig*	*Allogenic MSC*	*SCA*	*Adipocytes and Osteocytes*	*CD29 / CD44 / CD90 / CD105 / CD31 / CD45*	*IF*
6	**Colon**	*Pascual et. al. (Spain)*(235)	*2008*	*RS*	*40*	*Rat*	*Allogenic MSC*	*SCA*	*N/A*	*Not Disclosed*	*N/A*
7	**Colon**	*Adas et. al. (Turkey)*(240)	*2011*	*Cohort*	*40*	*Rat*	*Allogenic MSC*	*BMA*	*N/A*	*CD29 / CD45 / CD90 / CD 31 / CD 34 / CD45 / CD71 / CD105 HRP conjugated antibodies*	*FACS / IF*
8	**Colon**	*Yoo et. al. (Korea)*(244)	*2012*	*RS*	*60*	*Rat*	*Allogenic MSC*	*SCA*	*Endothelial Cells*	*CD31*	*IF*
9	**Colon**	*Adas et. al. (Turkey)*(239)	*2013*	*Cohort*	*40*	*Rat*	*Allogenic MSC*	*BMA*	*N/A*	*CD29 / CD45 / CD54 / CD106*	*FACS*
10	**Colon**	*Van De Putte et. al. (Belgium)*(237)	*2017*	*Cohort*	*48*	*Rat*	*Allogenic MSC*	*SCA*	*N/A*	*CD90 / CD73 / CD45*	*FACS*
11	**Colon**	*Sukho et. al. (Netherlands)*(231)	*2017*	*RS*	*57*	*Rat*	*Xenograft MSC*	*SCA*	*N/A*	*CD34 / CD3 /. CD163 / CD31 /*	*IF*
12	**Colon**	*Alvarenga et. al. (Brazil)*(245)	*2019*	*RS*	*65*	*Rat*	*Allogenic MSC*	*SCA*	*N/A*	*CD90 / CD73 / CD34 / CD45*	*FACS*

3.5.3 Primary Outcome Measures (Operative)

Ten studies reported anastomotic healing or a defect closure rate through macroscopic observation. Eight reported on all-cause mortality of animals. Studies varied in their method of measurement of adhesions, abscess formation and ileus. Seven studies measured burst pressure of the healed anastomosis (Table 4).

Mortality and Anastomosis Leak / Closure Rate

Mortality was reported in 75% of studies and AL in 83% of studies. (Table 5) The study in rabbit oesophagus by Xue *et. al.* was the only study to demonstrate a significant leak rate reduction when MSCs were applied in the intervention group versus control with an odds ratio of 0.02 (95%CI 0.01-0.29 p<0.01) (Table 6). However, all-cause mortality was more than double that in the control group. Studies that measured leak and closure rate did so through macroscopic specimen evaluation, however all studies failed to report their definition of a 'leak'. Xue *et. al. also* examined healing rate in an oesophageal defect model through T2 weighted cervical magnetic resonance imaging. (232) Van de putte *et. al.* (paediatric endoscope) and Alvarenga *et. al.* (flexible bronchoscope) used endoscopic evaluation on rat models prior to euthanasia. (237,245)

Table 5 | Study design, operative and histological primary outcomes for the 12 included studies. A/CR = Anastomosis / Closure Rate. AB = Abscess M = Mortality. A = Adhesions, I = Ileus. BP = Burst Pressure. IN = Inflammation. N = Neovascularisation. C = Collagen Deposition. SCR = Stem Cell Resides.

#	Anatomical Location	Author / Country	Date	Study Design	N	Population	Primary Outcome Measures (Operative)						Primary Outcome Measures (Histological)			
							A/CR	AB	M	A	I	BP	IN	N	C	SCR
1	**Oesophagus**	*Xue et. al. (China)*	*2019*	*RS*	*21*	*Rabbit*	√	√	√	X	X	X	√	X	√	√
2	**Gastric**	*Komiyama et. al. (Japan)*	*2013*	*Cohort*	*40*	*Rat*	√	X	X	X	X	√	√	√	√	√
3	**Bile Duct**	*Zhang et. al. (USA)*	*2020*	*Cohort*	*9*	*Pig*	X	X	√	X	X	X	X	X	X	√
4	**Bile Duct**	*Hara et. al. (Japan)*	*2020*	*Cohort*	*11*	*Pig*	√	√	√	√	X	X	√	X	√	√
5	**Small Bowel**	*Maruya et. al. (Japan)*	*2017*	*RS*	*7*	*Micro Pig*	X	X	X	X	X	√	√	X	√	√
6	**Colon**	*Pascual et. al. (Spain)*	*2008*	*RS*	*40*	*Rat*	√	X	X	√	X	√	X	X	X	√
7	**Colon**	*Adas et. al. (Turkey)*	*2011*	*Cohort*	*40*	*Rat*	√	X	X	√	X	√	√	√	√	√
8	**Colon**	*Yoo et. al. (Korea)*	*2012*	*RS*	*60*	*Rat*	√	√	√	√	√	√	√	√	√	X
9	**Colon**	*Adas et. al. (Turkey)*	*2013*	*Cohort*	*40*	*Rat*	√	X	√	X	X	√	√	√	√	√
10	**Colon**	*Van De Putte et. al. (Belgium)*	*2017*	*Cohort*	*48*	*Rat*	√	X	√	√	X	X	√	√	X	√
11	**Colon**	*Sukho et. al. (Netherlands)*	*2017*	*RS*	*57*	*Rat*	√	√	√	√	X	√	√	√	√	√
12	**Colon**	*Alvarenga et. al. (Brazil)*	*2019*	*RS*	*65*	*Rat*	√	√	√	√	X	X	√	√	√	X

Table 6 | Study design, mortality, and leak rates for the 12 included studies.

#	Anatomical Location	Authors	Date	Study Design	N	Population	Established model	Mortality Rate vs. Control	Odds Ratio of Mortality vs. Control	Leak Rate Reduction	Odds Ratio of AL Versus Control
1	Oesophagus	*Xue et. al. (China)*	*2019*	*RS*	*21*	*Rabbit*	X	25% v 56 % (p=0.17)	0.26 (95%CI 0.04-1.70 p=0.16)	83% vs 33% (p=0.02)	0.02 (95%CI 0.01-0.29 p<0.01)
2	Gastric	*Komiyama et. al. (Japan)*	*2013*	*Cohort*	*40*	*Rat*	X	-	-	-	-
3	Bile Duct	*Zhang et. al. (USA)*	*2020*	*Cohort*	*9*	*Pig*	X	0% vs 33% vs 0%	-	-	-
4	Bile Duct	*Hara et. al. (Japan)*	*2020*	*Cohort*	*11*	*Pig*	X	0%	-	0%	1.0
5	Small Bowel	*Maruya et. al. (Japan)*	*2017*	*RS*	*7*	*Micro Pig*	X	-	-	-	-
6	Colon	*Pascual et. al. (Spain)*	*2008*	*RS*	*40*	*Rat*	X	-	-	-	1.0
7	Colon	*Adas et. al. (Turkey)*	*2011*	*Cohort*	*40*	*Rat*	X	-	-	0%	-
8	Colon	*Yoo et. al. (Korea)*	*2012*	*RS*	*60*	*Rat*	√ No leak Rate	13% vs. 10 % (p=0.6)	1.38 (95%CI 0.28-6.79 p=0.16)	13% vs. 13 % p=1.0)	1.0
9	Colon	*Adas et. al. (Turkey)*	*2013*	*Cohort*	*40*	*Rat*	X	0%	0%	0%	-
10	Colon	*Van De Putte (Belgium)*	*2017*	*Cohort*	*48*	*Rat*	X	30% vs 30%	1.0 (95% CI 0.10-9.61 p=1.0)	0%	-
11	Colon	*Sukho et. al. (Netherlands)*	*2017*	*RS*	*57*	*Rat*	X	0%	0%	0%	-
12	Colon	*Alvarenga et. al. (Brazil)*	*2019*	*RS*	*65*	*Rat*	X	30% vs 30% 30% vs 0%	1.0 (95% CI 0.10-9.61 p=1.0) 6.5 (95% CI 0.27-160.9 p=0.25)	0%	-

3.5.4 Adhesion and Abscess Formation

Eight studies macroscopically evaluated adhesion formation with four studies using adhesion scoring systems (Table 5). (233,235,237,238,240,245,246) Hara *et. al.* used a previously published classification of adhesion formation (grade 0: none, grade 1: film-like with no neovascularisation, grade 2: moderately thick with partial neovascularisation, grade 3: thick, solid adhesion with neovascularisation). (233,247)

Pascual *et. al.* adapted the adhesion index originally described by Garcia-Olmo *et. al.* in 1996 on a rat colon model. (235,248)

Five studies reviewed the macroscopic presence of abscess. Four studies failed to define their minimum criteria for abscess. Sukho *et. al.* utilised an adhesion scoring system identifying the size and quantity of abscess formation. (0, no adhesion; 1, mild adhesions, mainly between the lesion and omentum; 2, moderate adhesion, between the lesion and a loop of the small bowel and the omentum; 3, severe, extensive adhesion, including abscess formation in rat colon). (231,249) Yoo *et al.* evaluated adhesions using a scale reported by Van der Ham *et. al.* (0, no adhesion; 1, mild adhesions, mainly between the lesion and omentum; 2, moderate adhesion, between the lesion and a loop of the small bowel and the omentum; 3, severe, extensive adhesion, including abscess formation) in a rat colon model with vessel ligation. (244,250)

3.5.5 Ileus

Yoo *et. al.* defined ileus as small bowel dilatation ≥1.5 cm near the ileocecal junction and found a lower incidence of ileus in the control group (4/27) versus the mesenchymal stem cell group (0/26) in a rat colon model (p= 0.041) (244)

3.5.6 Burst Pressure

Burst pressure was measured in resected anastomoses in seven studies. Komiya *et. al.,* Maruya *et. al.* and Sukho *et. al.* used an under-solution, air leak pressure technique with a manometer. (231,234,242) Pascual *et. al.* and Sukho *et. al.* used methylene blue

injected intraluminally to observe leakage at the anastomotic line, (235,244) whereas Adas *et. al.* used a constant airflow pump to assess leakage. (239,240)

3.5.7 Primary Outcome Measures (Histological)

Inflammation, neovascularisation and collagen deposition were the three most common primary outcomes measures used as surrogate markers for anastomotic healing with five studies demonstrating efficacy in at least one of the outcome measures (Table 5).

3.5.8 Inflammation

Eleven studies measured Inflammation as a histological outcome by Haematoxylin and Eosin staining and light microscopy using different, non-validated, quantification scores. Van de Putte *et. al.* used ^{18}F-FDG-PET-CT to identify intramural inflammation but failed to demonstrate a significant difference between MSC exposed rat colon and controls. (237) Sukho *et. al. measured* the number of T-cells and M2 macrophages to evaluate the inflammatory response at the anastomoses in rat colon, but with no significant difference between intervention and control groups.(231)

Table 7 | Study design and surrogate markers for wound healing for the 12 included studies.

#	Anatomical Location	Authors	Date	Study Design	N	Population	No of Days Post Anastomosis	Inflammation MSC vs. Control	Inflammation S Assessment	Neovascularisation MSC vs. Control	Neovascularisation Assessment	Collagen Deposition MSC Vs. Control	Collagen Deposition Assessment
1	**Oesophagus**	*Xue et. al. (China)*	*2019*	*RS*	*21*	*Rabbit*	Week 5	Dispersive vs. Severe	-	-	-	No Difference ($p<0.05$)	-
2	**Gastric**	*Komiyama et. al. (Japan)*	*2013*	*Cohort*	*40*	*Rat*	Day 7	MSD	0: none, 1: slight; 2, moderate; and 3, dense	4d NSD 7d 2.7 vs. 1.9 $p<0.01$	0: none, 1: slight; 2, moderate; and 3, dense	4d NSD 7d 2.4 vs. 1.7 $p<0.01$	0: none, 1: slight; 2, moderate; and 3, dense
3	**Bile Duct**	*Zhang et. al. (USA)*	*2020*	*Cohort*	*9*	*Pig*	Day 30	-	-	-	-	-	-
4	**Bile Duct**	*Hara et. al. (Japan)*	*2020*	*Cohort*	*11*	*Pig*	Day 14	Gross Assessment	-	Gross Assessment	-	Gross Assessment	-
5	**Small Bowel**	*Maruya et. al. (Japan)*	*2017*	*RS*	*7*	*Micro Pig*	Day 5 Day 7	-	-	-	-	7d 4.91 ± 0.43 vs. 3.82 ± 0.83	Hydroxyproline Content µg/mg
6	**Colon**	*Pascual et. al. (Spain)*	*2008*	*RS*	*40*	*Rat*	Days 4,7,14 & 21	-	-	-	-	-	-
7	**Colon**	*Adas et. al. (Turkey)*	*2011*	*Cohort*	*40*	*Rat*	Day 4 Day 7	NSD	0: none, 1: slight; 2, moderate; and 3, dense	4d 2.5 vs. 1.4 $p<0.01$ 7d No Sig. Dif.	0: none, 1: slight; 2, moderate; and 3, dense	4d No Sig. Dif 7d 2.2 vs. 1.4 $p<0.05$	0: none, 1: slight; 2, moderate; and 3, dense
8	**Colon**	*Yoo et. al. (Korea)*	*2012*	*RS*	*60*	*Rat*	Day 7	1.50 vs. 2.88 ($p<0.001$)	0, no alteration; 1, mild alteration; 2, moderate alteration; 3, dense alteration; and 4, profuse alteration (CD31)	32.6 vs. 25.7 ($p<0.05$)	No/mm^2	0.76 vs. 0.35 ($p<0.05$)	0, no alteration; 1, mild alteration; 2, moderate alteration; 3, dense alteration; and 4, profuse alteration
9	**Colon**	*Adas et. al. (Turkey)*	*2013*	*Cohort*	*40*	*Rat*	Day 4 Day 7	NSD	scored from 0 to 3	4d 2.4 vs. 1.4 $p<0.01$ 7d NSD	0: none, 1: slight; 2, moderate; and 3, dense	4d 0.77 vs. 044. ($p<0.001$)	Hydroxyproline Content µg/mg
10	**Colon**	*Van De Putte et. al. (Belgium)*	*2017*	*Cohort*	*48*	*Rat*	Day 7	NSD	^{18}F-FDG-PET-CT	NSD	vWF vessels / sample	-	-
11	**Colon**	*Sukho et. al. (Netherlands)*	*2017*	*RS*	*57*	*Rat*	Day 3 Day 7	7d 508 vs 204 ($p<0.001$) /	CD3+ T cells/mm^2	NSD	Vessel Density	NSD	% Area
12	**Colon**	*Alvarenga et. al. (Brazil)*	*2019*	*RS*	*65*	*Rat*	Day 7	NSD	3 severe; 2 moderate; 1 mild; and 0 absent.	NSD	Gross Assessment	22% vs. 32.5% ($P<0.005$)	Density

3.5.9 Neovascularisation

Three studies measured neovascularisation using Masson Trichome staining and light microscopy. *Yoo et. al.* showed significantly higher microvascular density in the MSC group than in the control group as demonstrated by CD31 immunohistochemistry. They showed a greater number of vessels per high field view in the MSC group as compared to controls: 32.6 vs. 25.7 ($p<0.05$) on day 7 in an ischaemic rat colon model. (244). Adas *et. al.* has previously demonstrated a greater proportion of vessels at day 4 with no significant difference shown at day 7. This assessment was completed using a non-validated classification method: 0: none, 1: slight; 2, moderate; and 3, dense. (239,240)

3.5.10 Collagen Deposition

Collagen deposition was assessed using Masson Trichome staining in six studies, hydroxyproline content in two studies, picrosirius red staining in two studies, and Collagen-1 staining in one study. (Table 7) Two studies by Xue *et. al.* and Maruya *et. al.* distinguished between collagen sub-type using immunohistochemistry. (232,234) Xue *e. al.* demonstrated that collagen deposition between the submucosa layer and muscular layer was denser and the collagen fibres distributed more compactly and disorderly in a MSC exposed rabbit oesophagus model.(232) *Yoo et al* demonstrated that collagen deposition, as shown by Masson trichrome staining, was significantly more prevalent in an MSC group than in controls. (244)

What models and methodology are used to isolate and deliver MSCs to the anastomotic site?

3.5.11 Method of Delivery & Vehicle

Nine studies delivered MSCs locally, two delivered MSCs systemically, and one through cells cultured on bio sutures incorporated within the anastomosis (Table 8). A mesenchymal stem cell sheet (three studies) was the most common method of local delivery followed by use of a fibrin matrix (two studies) (Table 3).

3.5.12 Model of Anastomotic Leak

There was little consistency in the models of AL used. No studies used a model of AL with a known leak rate. All studies included a control/comparator group. Three utilised full anastomoses alone, four utilised a full anastomosis in combination with ischaemia / delayed wound healing - one in the presence of induced colitis and one post radiation of the abdominal cavity. The final two studies included a full anastomosis with stent insertion and an incision model (Table 3). All studies that disclosed detailed methods of anastomotic construction used an end-end anastomosis. Suture size ranged from 6/0 to 8/0. Nine studies opted for a monofilament suture. The six colorectal models all utilised a rat model with a complete anastomosis.

Table 8 | Study design, anastomosis model, and suture choice for the 12 included studies. CA = Complete Anastomosis, VL – Vessel Ligation.

#	Anatomical Location	Authors	Date	Study Design	N	Population	Model	Specific Site	Anastomoses	Suture No	Suture Size	Suture Material	Known Leak Rate?	Control
1	Oesophagus	*Xue et. al. (China)*	*2019*	*RS*	*21*	*Rabbit*	*2mm Defect in CA*	*Cervical Oesophagus*	*End-end*	*-*	*-*	*-*	X	√
2	Gastric	*Komiyama et. al. (Japan)*	*2013*	*Cohort*	*40*	*Rat*	*Repaired Incision*	*Greater Curvature*	*-*	*1*	*6/0*	*Polypropylene*	X	√
3	Bile Duct	*Zhang et. al. (USA)*	*2020*	*Cohort*	*9*	*Pig*	*CA with stent*	*CBD Midpoint*	*End-end*	*2 x cont.*	*7/0*	*PDS*	X	√
4	Bile Duct	*Hara et. al. (Japan)*	*2020*	*Cohort*	*11*	*Pig*	*CA*	*Proximal to duct*	*End-end*	*-*	*6/0*	*monofilament*	X	√
5	Small Bowel	*Maruya et. al. (Japan)*	*2017*	*RS*	*7*	*Micro Pig*	*Incision + VL + Mito-C*	*Multiple sites / subject*	*-*	*-*	*5/0*	*Vicryl*	X	√
6	Colon	*Pascual et. al. (Spain)*	*2008*	*RS*	*40*	*Rat*	*CA*	*5cm Distal to ICV*	*End-end*	*6*	*-*	*Vicryl*	X	√
7	Colon	*Adas et. al. (Turkey)*	*2011*	*Cohort*	*40*	*Rat*	*CA + VL*	*3cm Proximal perit. reflection*	*End-end*	*8*	*6/0*	*Polypropylene*	X	√
8	Colon	*Yoo et. al. (Korea)*	*2012*	*RS*	*60*	*Rat*	*CA + VL*	*Proximal 3cm of Dec. Colon*	*End-end*	*-*	*6/0*	*Prolene*	X	√
9	Colon	*Adas et. al. (Turkey)*	*2013*	*Cohort*	*40*	*Rat*	*CA + VL*	*3cm Proximal perit. reflection*	*End-end*	*8*	*6/0*	*Polypropylene*	X	√
10	Colon	*Van De Putte et. al. (Belgium)*	*2017*	*Cohort*	*48*	*Rat*	*CA + Radiation*	*4cm proximal to the rectum*	*End-end*	*2mm spacing*	*6/0*	*PDS*	X	√
11	Colon	*Sukho et. al. (Netherlands)*	*2017*	*RS*	*57*	*Rat*	*CA*	*1cm distal to caecum*	*End-end*	*5*	*8/0*	*Daflon*	X	√
12	Colon	*Alvarenga et. al. (Brazil)*	*2019*	*RS*	*65*	*Rat*	*CA / Colitis*	*4cm proximal to rectum*	*End-end / single layer*	*1*	*6/0*	*Polypropylene*	X	√

3.5.13 ARRIVE Guidelines Compliance

Median ARRIVE guideline compliance across all 12 studies was 56% (IQR 45-62). Colorectal anastomosis studies had a greater compliance (61%, IQR 61-63) compared to non-colorectal studies (45%, IQR 42-47) (Table 8) (Appendix 2). (227)

3.5.14 International Consensus Compliance

Of the seven colorectal anastomosis model studies, one studied scored 4/5, 4 studies scored 5/6 and 2 studies scored 6/6 for compliance to the Lower Gastrointestinal Consensus Criteria (Table 9) (Appendix 3). (210)

#	Anatomical Location	Authors	Date	Study Design	N	Population	Lower GI Consensus (6 Categories)	Median ARRIVE Guideline Criteria (38 point)
1	Oesophagus	*Xue et. al. (China)*	*2019*	*RS*	*21*	*Rabbit*	n/a	43.4
2	Gastric	*Komiyama et. al. (Japan)*	*2013*	*Cohort*	*40*	*Rat*	n/a	50.0
3	Bile Duct	*Zhang et. al. (USA)*	*2020*	*Cohort*	*9*	*Pig*	n/a	39.5
4	Bile Duct	*Hara et. al. (Japan)*	*2020*	*Cohort*	*11*	*Pig*	n/a	50.0
5	Small Bowel	*Maruya et. al. (Japan)*	*2017*	*RS*	*7*	*Micro Pig*	n/a	46.1
6	Colon	*Pascual et. al. (Spain)*	*2008*	*RS*	*40*	*Rat*	4/6	44.7
7	Colon	*Adas et. al. (Turkey)*	*2011*	*Cohort*	*40*	*Rat*	6/6	61.8
8	Colon	*Yoo et. al. (Korea)*	*2012*	*RS*	*60*	*Rat*	6/6	60.5
9	Colon	*Adas et. al. (Turkey)*	*2013*	*Cohort*	*40*	*Rat*	5/6	64.5
10	Colon	*Van De Putte et. al. (Belgium)*	*2017*	*Cohort*	*48*	*Rat*	5/6	60.5
11	Colon	*Sukho et. al. (Netherlands)*	*2017*	*RS*	*57*	*Rat*	5/6	69.7
12	Colon	*Alvarenga et. al. (Brazil)*	*2019*	*RS*	*65*	*Rat*	5/6	64.5

Table 9 | Study design and scores for compliance to ARRIVE guidelines and International Lower GI consensus.

3.6 Discussion

The prevention of AL remains an unmet clinical need and attempts to reduce, detect and treat its occurrence continue across the surgical and research communities. In humans, the basic principles of anastomosis formation centre around adequate blood supply, avoidance of tension at the anastomotic line, and submucosal suturing. (251) However, there is widespread variation in technique, (97,252) definition, measurement (253) and reporting of outcomes (254,255) with comparable results in both stapled and hand sewn anastomoses. (256) This is likely to have confounded previous investigation of prophylactic interventions and has limited the comparison of outcomes between trials as is also evident, perhaps, in this systematic review of animal models. Internationally there is a move to standardise anastomotic technique (257,258) definition of leak, (9) reporting of outcomes, (10,259) interventional study design and the translation of technologies to prevent AL in humans. (147)

Technological attempts to reduce the incidence of AL have failed to establish efficacy when translated to clinical practice despite some promising *in vivo* results. Interventions include devices to improve anastomosis formation through stapling and anastomotic line compression, (260) anastomotic buttressing, (261) intra-luminal stents (166) gels, (262) surgical sealants/adhesives (263,264), fibrin glue (265,266) and assessment of anastomotic blood supply (143). Unfortunately, none of these technological advances has made an impact on the incidence of AL.

If animal models are to be used effectively to test preclinical interventions in AL it is paramount to standardise both the model and outcome measures. (267,268) This will increase the reliability of findings, reduced heterogeneity between studies, aid the comparison of outcomes, reduce the number of animals required for testing and streamline the translation of novel interventions into clinical practice. (269) Of the 12 studies included in this review only Yoo *et. al.* tested the application of MSCs in an established model. (244) Fifty percent of studies attempted to replicate conditions unfavourable to anastomotic wound healing (Table 8) but no study used a model of AL with a known leak rate meaning that the efficacy of the intervention was reliant on surrogate measurements of wound healing. There is a need to strictly define outcome measures in animal studies of anastomotic leak with a clear difference between macroscopic efficacy (mortality, leak rate) and histological efficacy (surrogate markers of wound healing). Only half the studies reported mortality figures, which is contrary to the 3R principles and non-compliant with ARRIVE guidelines. (226)

The process of harvesting mesenchymal stem cells results in a multipotent mixed cell population (the stromal vascular cell fraction or SVCF) with the capacity to differentiate into osteoblasts, adipocytes and chondroblasts. There is wide variation in the source, isolation and characterisation of mesenchymal stem cells. In 2016 Dominici *et. al.* generated consensus on how to correctly define a mesenchymal stem cell population in humans (Appendix 1), but similar criteria do not exist for animals, where the cluster of differentiation proteins and cell markers vary from species to species. (202) Of the twelve studies included in this review there were some attempts to define animal mesenchymal stem cell populations. Where this occurred, most studies described experiments demonstrating differentiation capacity as proof of cell population harvest or utilised human cluster differentiation proteins as defined by Dominici *et. al*. All studies cultured mixed cell populations or isolated MSCs prior to delivery or application.

Eight of the included studies used subcutaneous adipose tissue and three used bone marrow aspirate as the source of MSCs. Current lipoaspiration procedures are less painful than harvesting bone marrow stem cells, with less ethical considerations. (201) However, they are not without morbidity. (214–217) Other sources may be more suitable and translatable for use in animal and human models of AL to allow delivery of a mixed cell population in a single operation, for example omentum during laparotomy. (218,219)

In humans, AL usually occurs between day 4-7 following surgery, depending on where the anastomosis is performed along the gastrointestinal tract. (270) Overall, the leak rate across all studies in this review was low in the models used. The quality and rate of wound healing varied between animal species (271) and was likely influenced by the chosen model of AL. In animal models it is therefore important to use an established model with a known leak rate and an accurate estimate on which day after the anastomosis that a leak is likely to occur. Cross sectional imaging or endoscopic examination of the anastomosis in animal models may streamline studies and avoid the unnecessary suffering of subclinical leaks and early termination of animals with 'missed leaks'. (272–274) Current attempts to standardise anastomosis formation in humans should be matched with standardisation in animal models of AL to allow accurate comparison of novel AL prevention or mitigation strategies in pre-clinical studies. (257) Studies may not require AL leak models where an intervention's effect on anastomotic healing or surrogate markers of wound healing are the primary outcome and model choice may be influenced by whether the intervention aims to prevent leak or mitigate the effect of post-leak pelvic sepsis.

It is unclear whether burst pressure is an accurate surrogate for anastomotic integrity, yet this continues to be routinely used in pre-clinical studies. (275) It is uncertain whether adhesions are a macroscopic sign of a small AL and whether this reflects a continuum of severity from adhesion to localised abscess formation though to feculent peritonitis. Where adhesions are measured, a standardised, validated scoring system should be used. (276,277) Inflammatory markers, neovascularisation and collagen formation are appropriate surrogate markers of normal wound healing and can be used to measure the effects of inflammation and fibrosis, which may be mediated through the paracrine action of MSCs. (199) These should be measured where possible using standardised or widely used scoring systems. (278) The type of collagen deposited varies in bowel injury compared to cutaneous wound healing. (71) More detailed conclusions may be drawn if collagen type is included during histological analysis of AL. Constituent analysis, such as hydroxyproline content, are commonly used as a direct measure of the amount of collagen present as per Maruya *et. al.* and could be considered. (279)

This review utilised two simple, non-validated scoring systems to quantify compliance to current best practice, which requires further validation. There was wide study heterogeneity, even amongst the rat colon models which made up seven of the twelve studies. This meant that quantitative comparison of outcome measures was not feasible.

Recommendations

1. Pre-clinical safety and efficacy studies should use an established animal model of AL with a known leak rate and known likely post-operative day of AL. Efficacy studies should perform an appropriate sample size calculation in line with ARRIVE guidelines and report both mortality and leak rates. (227)
2. Where wound healing, or a surrogate marker of wound healing, is the primary outcome measure, an animal model that does not leak is more suitable.
3. A method for correctly defining mesenchymal stem cell populations across animal species is required.
4. Alternatives to bone marrow and subcutaneous adipose tissue as sources of mesenchymal stem cells should be considered.
5. Surrogate outcome measures of wound healing should be reported using validated methods of assessment, including anastomotic line inflammation, neovascularisation and collagen deposition.
6. All studies should comply with ARRIVE guidelines. (226,227)
7. All studies involving the lower gastrointestinal tract should conform to current International consensus. (229)

4. Investigating the feasibility of omental derived regenerative cell harvest, stromal vascular fraction composition and delivery vehicle.

In Chapter 3, eleven different methods of ADRC delivery to the site of an anastomosis were highlighted. The most common method of harvest was from subcutaneous adipose tissue with a local method of delivery. All studies to date have cultured cells before implantation, which when translated to the human scenario would add an extra procedure and second surgery with additional risk of morbidity and mortality. An allogenic stem cell implant was the most common type used with most studies culturing cells under standard conditions without isolation or influence of subtypes. The feasibility of ADRC technology would be improved in human subjects if allogenic stem cells could be harvested from the omentum and implanted at the anastomotic line within the same operation. In this chapter, a method for harvest and culture of omental mesenchymal stem cells, characterisation of the omental stromal vascular fraction (OSVCF), and the identified delivery vehicle for application to an anastomosis are described.

4.1 Background

4.1.1 Harvest

In 1966, Rodbell *et. al.* pioneered the isolation of stromal vascular cell fraction (SVCF) from adipose tissue using collagenase digestion. (280) This process has been refined over the last half a century and now involves the following steps: (281)

1. Mincing of resected adipose tissue to increase surface area;
2. Collagenase digestion and incubation;
3. Filtering and Centrifugation;
4. Plate Seeding and Incubation.

The final step of centrifugation separates the SVCF into three fractions from the top of the liquid to bottom: 1. Mature Adipocytes, 2. Aqueous adipose fraction, and 3. Cellular SVCF Pellet. Erythrocytes often accompany the SVCF pellet. A red cell lysis buffer can be incorporated into the protocol to make a purer SVCF sample through removing any red cells harvested during tissue resection.

In humans, various positive and negative cell markers have been used for ADRC identification. CD45, a haemopoietic cell surface marker, which is low or undetectable in ADRCs compared to SVCF cells, is often used. Positive surface cell markers include CD73, CD90, CD44 (Cancer stem cell markers) and CD105 (Endoglin, an accessory receptor for transforming growth factor beta). ASCs can be distinguished from bone marrow aspirate MSCs through negative CD106 (Vascular cell adhesion protein) and positive CD36 (Human adipocyte progenitor protein). (282,283)

An ideal source of ADRCs for harvest during abdominal operations is the greater omentum. If omental derived regenerative cells (ODRCs) can be harvested through the modification of existing ADRC isolation methods they could be a valuable resource for gastrointestinal repair and other applications. (218–221) In addition to ADRC multipotency, they secrete paracrine factors (284) and multiple growth factors conducive to wound healing. (285)

Current lipoaspiration techniques (213) provide a superior yield of ADRCs as compared to bone marrow aspiration (201) however, both techniques are not without morbidity. (214–217) ADRCs are also found in the SVCF of white adipose tissue (212) which is present in the greater omentum. (218–221)

Delivery of MSCs to an anastomosis in an animal model has been shown to be feasible and safe with potential benefits in terms of reduced leak, mortality and associated complications, and surrogate histological outcomes. (287)

4.1.2 Selecting the Optimum Animal

International consensus supports the use of rat, mouse and pig as the most appropriate models of AL and the closest to the human scenario. (229) No model is currently described using the pig (288) and the majority of experimental colorectal AL models described have used rats. However, even under extreme conditions, the rat rarely develops anastomotic leakage with abscess formation or faecal peritonitis. The reasons for this remain unclear, but this limitation makes the rat model unsuitable for routinely investigating AL and treatments, leaving the mouse as the only practical option. The C57BL/6 (B6) mouse is the most widely used strain in biomedical research. (289,290)

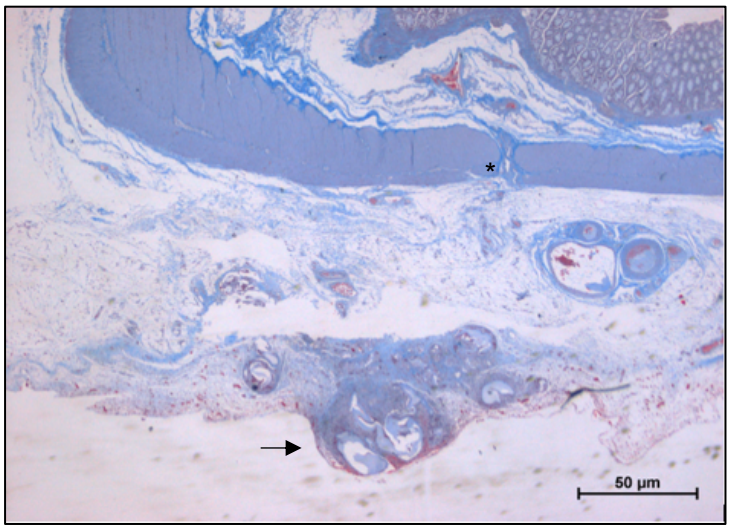
Murine omentum is structurally similar to human omentum but its cellular composition is poorly defined. (291) Wilkosz *et al.* utilised scanning electron and transmission electron microscopy to study the structure of human and murine greater omentum, (219) demonstrating that both had similar microscopic structures. Two distinct tissue types were identified: I. Adipose Rich and II. Translucent and Membranous. Omental regions rich in adipose tissue were well vascularised with a mesothelial cell layer covering except where ‘milky spots’ (areas with a high density of inflammatory cells) were present. In contrast, translucent areas were poorly vascularised with fenestrations. (219)

4.1.3 Selecting the optimum delivery vehicle for ODRC application to an anastomosis

Alginate hydrogels are used in a wide variety of biomedical applications, including tissue engineering, and cell and drug delivery. (292) They are widely available, biocompatible, non-immunogenic, and FDA approved for use in human trials, and can be formulated to rapidly set at physiological temperatures and pH. Importantly, they do not impair colonic anastomotic healing and are not known to cause complications. (177) Alginate hydrogels also protect the viability of contained cells from forces exerted during injectable delivery and may prevent post-surgical adhesion formation. (293)

A previous researcher, Anabelle Williams, University of Leeds, has optimised a 4% alginate and calcium chloride solution for gelation as a vehicle for application and characterised its physical properties. Alginate gels biodegrade over time *in vivo,* however my preliminary findings using an in-house pig anastomosis model has confirmed that alginate gel is still present at an anastomosis 7 days after application (Figure 5). Once this was confirmed the model was discontinued given the previously discussed international consensus.

Figure 5 | Application of alginate gel to Masson a 7-day old pig colon anastomosis with gel (Arrow) present at the join line (*) x20 Masson Trichrome.



4.2 Aims

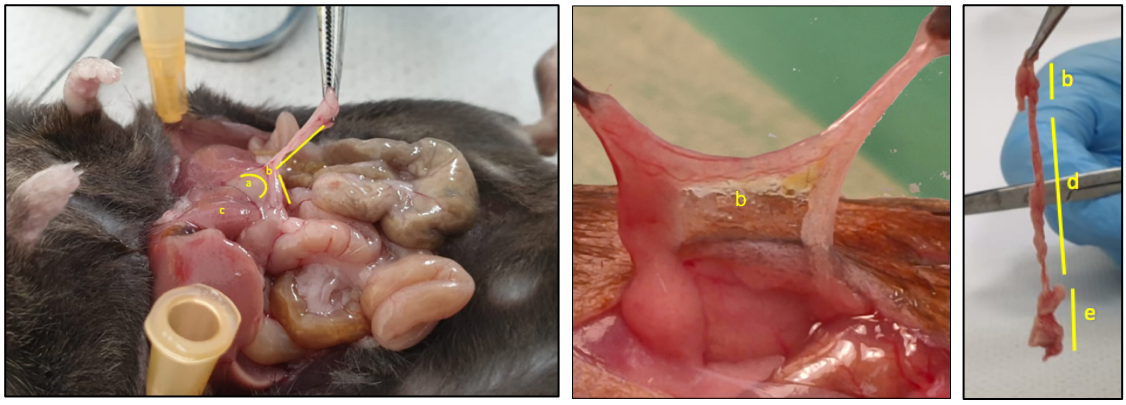
1. To assess whether the SVCF can be harvested and cultured from the greater omentum of Black C57bl-6 mice using protease extraction methods.
2. To characterise the SVCF of the C57bl-6 mouse omentum using flow cytometry.
3. Refine the formulation and micro delivery of a rapid setting, biologically compatible alginate gel to embed omental SVCF.

4.3 Method

4.3.1 Harvest of C57bl-6 omentum

The harvest protocol was optimised using male C57BL/6 mice (Appendix 4), aged 10-12 weeks weighing approximately 20g. Four black B6 mice (Charles-River, Canterbury, UK) were held under institutional licence (University of Leeds), approved by the local animal welfare committee (AWERB - University of Leeds, PP9886008), following UK Home Office Regulations. All animals were maintained in individually ventilated cages under pathogen-free conditions in the Animal Experiment Facility of the University of Leeds and provided with food and water *ad libitum*. Mice were subject to Schedule 1 killing. A midline laparotomy was performed and the greater curvature of the stomach and the attached greater omentum was identified (Figure 6). The omentum was dissected out and placed into 4% PFA (Sigma-Aldrich, Dorset, UK). Specimens were placed into fixing cassettes and submerged in 70% ethanol (Sigma-Aldrich, Dorset, UK) overnight. The specimens were then embedded in wax, sectioned at 5μm using a microtome (Epredia, Runcorn, UK), and stained using H&E (Sigma-Aldrich, Dorset, UK) to confirm harvest of mouse omentum.

Figure 6 | Macroscopic appearance of murine omentum (b) and its anatomical relationship with the greater curvature of the stomach (a) the liver (c), the stomach-spleen connective tissue complex (d) and the pancreas (e).



4.3.2 Isolation of C57bl-6 ODRCs

The isolation protocol was optimised using the four additional male C57BL/6 mice treated as described in 4.3.1 (Appendix 4) (Charles River, Canterbury UK). Following schedule 1, the abdomen was shaved and the skin prepared using chlorhexidine 0.5% / ethanol 70% spray (Sigma Aldrich, Dorset UK). A 1.5cm laparotomy was made from xiphisternum to mid abdomen. The omentum was resected and washed with 5mls of HBBS containing 5% Amphotericin (Sigma Aldrich, Dorset UK). The specimen was next placed in 5mls of 0.25% EDTA/Trypsin (Sigma Aldrich, Dorset UK) for 20 minutes at 37℃. Following neutralisation with 1ml FCS, the specimen was mashed though a 100µL cell sieve. The resulting SVCF solution was combined with 5mls of complete 10% DMEM (450mls DMEM, 50mls FBS, 5mls L-glutamine and 5mls of Penicillin / Streptomycin) and centrifuged at 2000rpm (440RCF) at 37℃ for 5 minutes. Media was removed and the cell pellet re-suspended in 1ml of complete DMEM. Live/dead cell counts were performed following trypan blue (Sigma-Aldrich, Dorset, UK) staining using a Countess™ II Automated Cell Counter (Thermofisher, UK). Cells were seeded

in 6 well plates at $5x10^5$/ml. Media was changed at 72 hours and then every 48 hours aiming for 70% cell confluence.

4.3.3 Flow Cytometry of C57bl-6 ODRCs

Whilst C57BL/6 mice remain the most common strain in scientific research some of the models identified in Chapter 1 also utilised the BABL/c mouse. Another genetically identical mouse strain. To ensure results are as comparable as possible to current literature O-SVCF was compared across two different mice lineages using splenic tissue as a control. Six C57BL/6 (3F:3M) and six BALB/c (3F:3F), 10-12 weeks of age and an approximate weight of 20g, were purchased from a commercial supplier (Envigo, Hillcrest, UK) and were treated in the same manner as described in section 4.3.2. All animals were maintained in individually ventilated cages under pathogen-free conditions in the Biological Services Facility (BSF) of the University of Manchester and provided with food and water *ad libitum*.

An aliquot of total omental derived stromal vascular cell fraction containing $1x10^6$ cells, was stained using reagents in a viability assay kit according to manufacturer's instructions (Zombie UV, Biolegend, London, UK) and blocked with 5 µg/mL anti-CD16/32 (clone 93; Biolegend, London, UK) and heat inactivated normal mouse serum (1:10, Sigma-Aldrich) in flow cytometry buffer (0.5% BSA and 2 mM EDTA in Dulbecco's PBS) before addition of antibodies whilst keeping the solution on ice.

4.3.4 Flow Cytometry Antibody Panel

There is a lack of consensus on the criteria for defining animal mesenchymal stem cells and there is huge variability within the published literature in the mouse population. The minimum cell expression profiles seen in humans (Appendix 1) are

generally accepted in other animal species, with some differences in surface cell markers accounting for inter-species variation. (294,295)

Following a review of the literature, and in collaboration with Dr Rinal Sahputra, Post-doctoral Researcher, University of Manchester, a bespoke antibody panel for murine SVCF characterisation was selected (Table 10). There is general acceptance that mouse MSCs positively express Sca-1, CD105, and CD29 and are negative for CD45, CD31, Lyve, CD90.2, WT1, CD116, PDPN, PDGFRa.

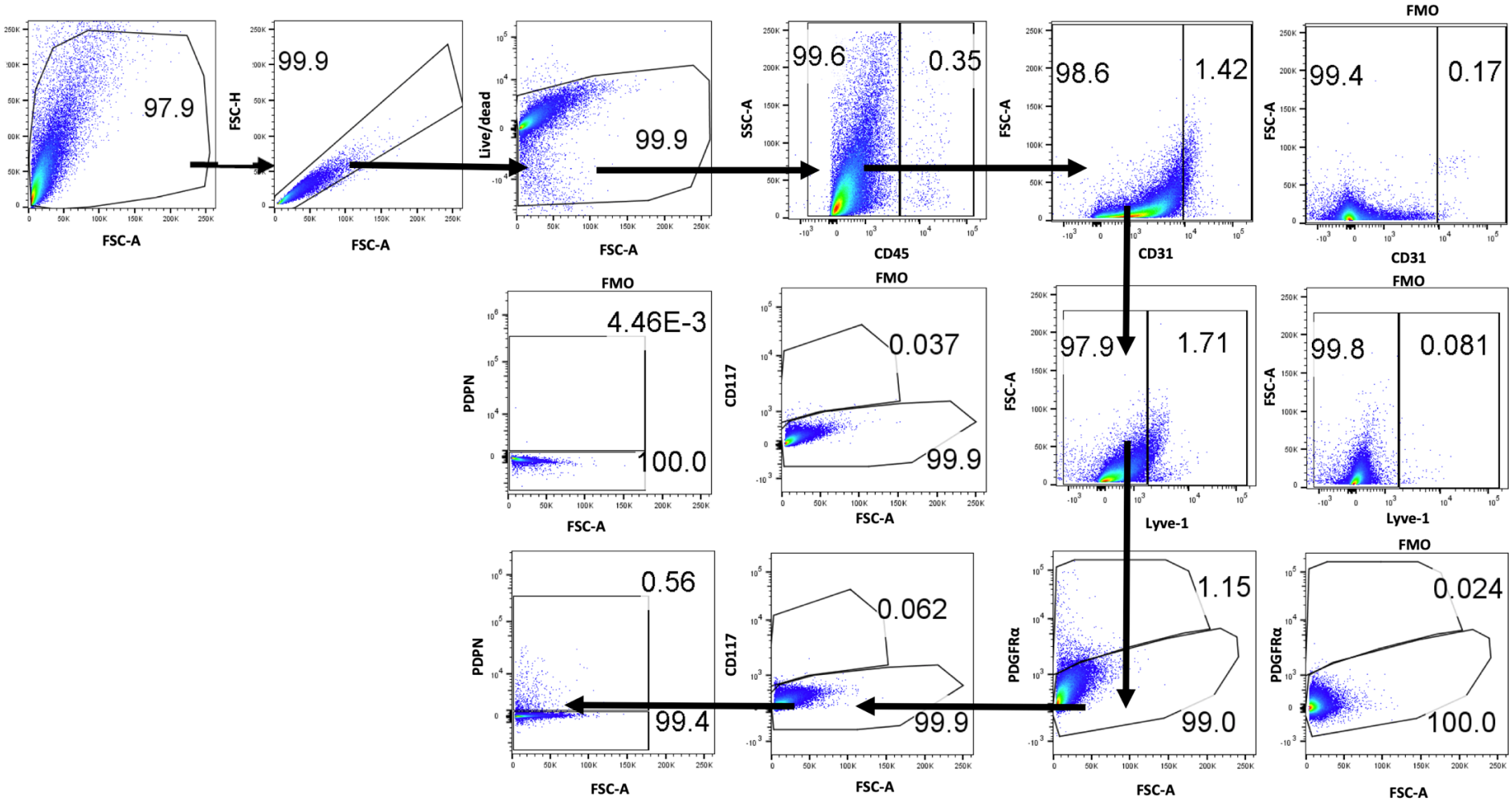
All antibodies were purchased from Biolegend, unless stated otherwise. Flow cytometry was carried out with the assistance of Dr Rinal Sahputra, post-doctoral researcher, University of Manchester as described by Sahputra *et. al.* in 2022. (296) PECs were analysed on a BD FACSymphony machine using BD FACSDiva software (BD Biosciences). Post-acquisition analysis and population proportions of single live cells (such as MSCs) was performed using FlowJo v10 software (BD Biosciences, London, UK). The gating strategy for flow cytometry is shown in Figure 7.

Filter	Fluoro-chrome	Antigen	Clone	Dilution	Supplier	Endo-thelial	Meso-thelial	Fibro-blast	MSC	1' Ref
355 450_50	BUV440	Live/Dead	Zombie UV	1:1000	Biolegend	N/A	N/A	N/A	N/A	
561 670_30	PE-Cy5	CD45	30-F11	1:400	Biolegend	-	-	-	-	(297)
637 670_30	APC	CD31	MEC13.3	1:100	Biolegend	+				(298)
488 530_30	FITC	Lyve1	ALY7	1:100	Invitrogen	+				(299)
405 450_50	PB	CD90.2	30-H12	1:100	Biolegend	+	+			(300,301)
637 730_45	AF700	WT1	30-F11	1:100	Biolegend	+	+*			(302,303)
405 525_50	BV510	CD117	6D5	1:100	Biolegend	+		+	-	(304)
561 586_15	PE	PDPN	8.1.1	1:100	Biolegend		+	+/-	+/-	(305)
561 780_60	PE-Cy7	PDGFRa	IAFS98	1:100	Biolegend		-	+	-	(306)
56b 610_20	PE-CF594	CD29	HMβ1-1	1:100	Biolegend				+	(307,308)
637 780_60	APC-cy7	CD105	1A8	1:100	Biolegend				+	(294,295)

405 710_50	BV711	Sca1	D7	1:180	Biolegend				+	(294,295)

Table 10 | Flow Cytometry Panel for C57bl-6 ODRCs. “+” = Positive cell marker. “-“ = negative cell marker. “*” = weak signal due to two-hour staining protocol.

Figure 7 | Gating Strategy for Flow Cytometry Panel analysis and gating of lineage+ or lineage- cells identified as described in Table 10 for omental stromal vascular cell fraction for 6 (3M:3F) C57BL/6 mice.



4.3.5 Development of Alginate Gel Delivery Vehicle

100mls of 10% complete DMEM was placed in a 500ml Duran bottle. 4g of Sodium Alginate (Sigma, UK) was added slowly and the solution stirred with a magnetic stirrer for 5 minutes. The solution was then sterilised in an autoclave. Omentum was processed as per the harvest and isolation protocol to extract MSCs (section 4.3.1) (Appendix 4).

4.3.6 Statistical Analyses

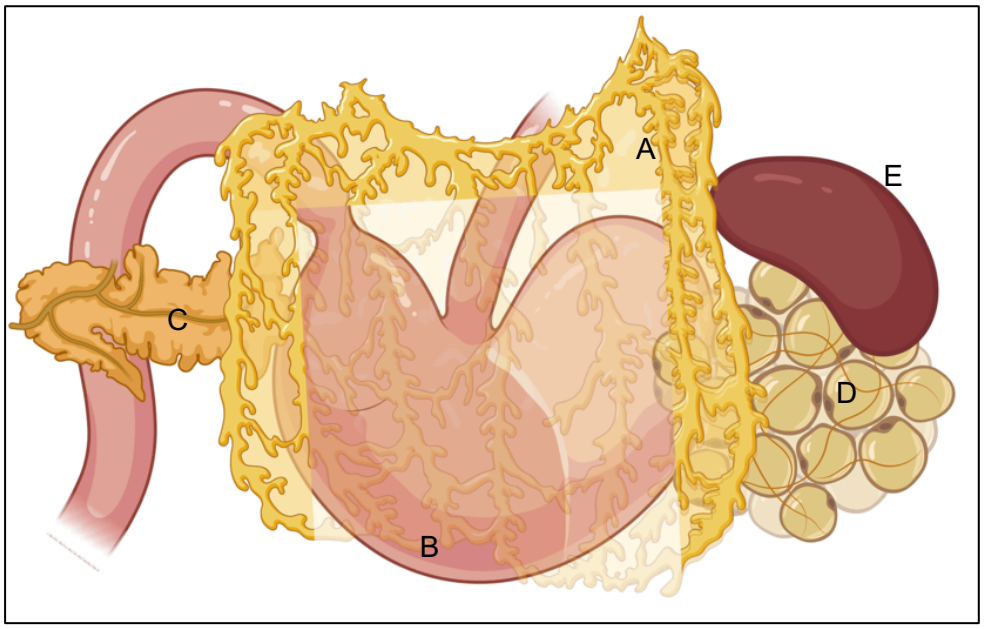
Statistical analyses were performed using Prism 10 (Graph-Pad software Inc., La Jolla, CA, USA). Data were tested for normal distribution using a Shapiro-Wilk test prior to analysis of significant differences between groups. Data deemed to follow Gaussian distribution were subsequently analysed using the Student's t-test for two groups, or ANOVA followed by Tukey or Sidak posthoc test for experiments with more than 2 groups. Data with non-Gaussian distribution were analysed using the Mann-Whitney test (2 groups) or Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test for experiments with more than 2 groups. Differences with a p-value of less than 0.05 were considered statistically significant.

4.4 Results

4.4.1 Omental Harvest

Optimisation of the technique required removal of the omentum without incorporation of splenic and pancreatic tissue (Figure 8).

Figure 8 | Anatomical relations of the murine greater omentum (A), the stomach posteriorly (B), pancreas medially (C), splenic adipose tissue laterally (D) and spleen postero-laterally (E). Created with Biorender.com.



In optimisation experiments, the splenic adipose tissue was inadvertently harvested as part of the omental sample which was undesirable and could have skewed the results of the O-SCVF isolation and characterisation. Refinement of the technique through practice and repeated histological confirmation ensured that only omental tissue was included in the O-SVCF characterisation studies.

4.4.2 Confirmation of C57bl-6 omental harvest

All specimens from the first three mice contained pancreatic tissue (Figure 9). This process was repeated with one further C-57bl6 (B1) mouse where the omentum and pancreas were harvested separately to ensure the correct tissue was bring isolated (Figure 10).

Figure 9 | Microscopic appearance (x20 magnification) of murine pancreas (a) and omentum (b) harvested from a black C-57bl6 mouse.

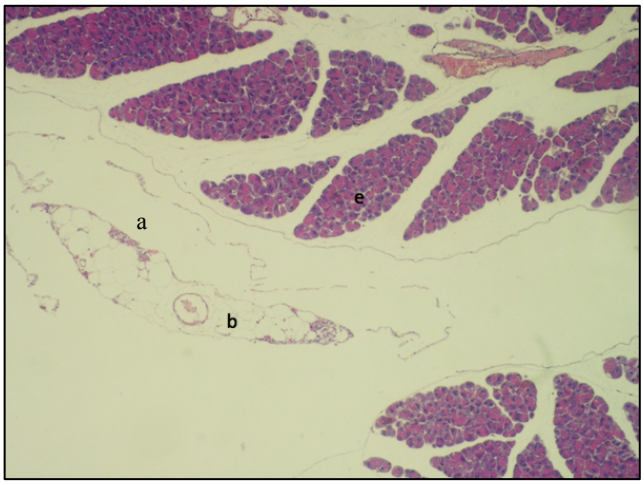
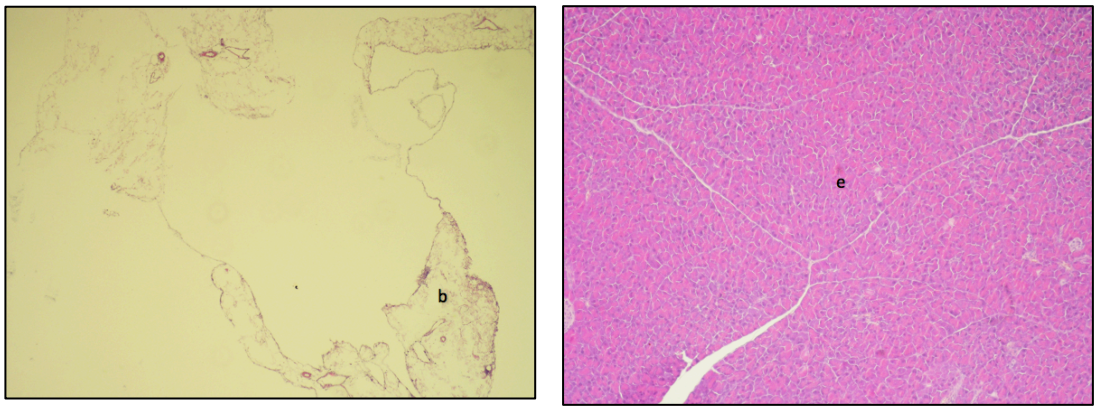


Figure 10 | Microscopic appearance (x20 magnification) of murine pancreas (e) and omentum (b) harvested from a black C-57bl6 mouse confirming that the omentum could be isolated without including pancreatic tissue.



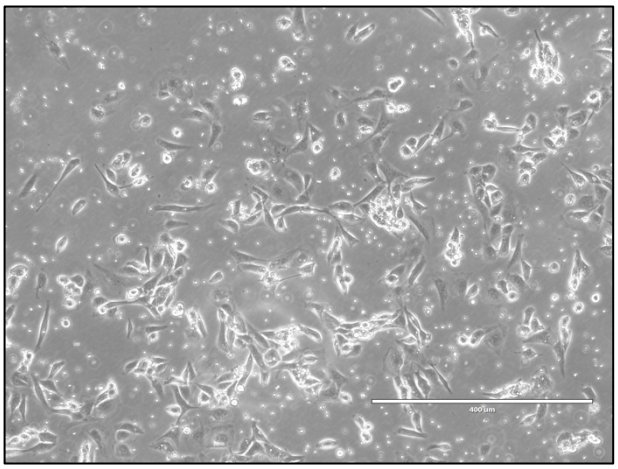
4.4.3 Culture of C57bl-6 omental harvest

Omentum was successfully harvested from three mice (Table 11) and MSCs cultured to day 7 (Figure 11).

Table 11 | Cell counts and Live/Dead proportions at time of harvest for 3 C57bl-6 mice

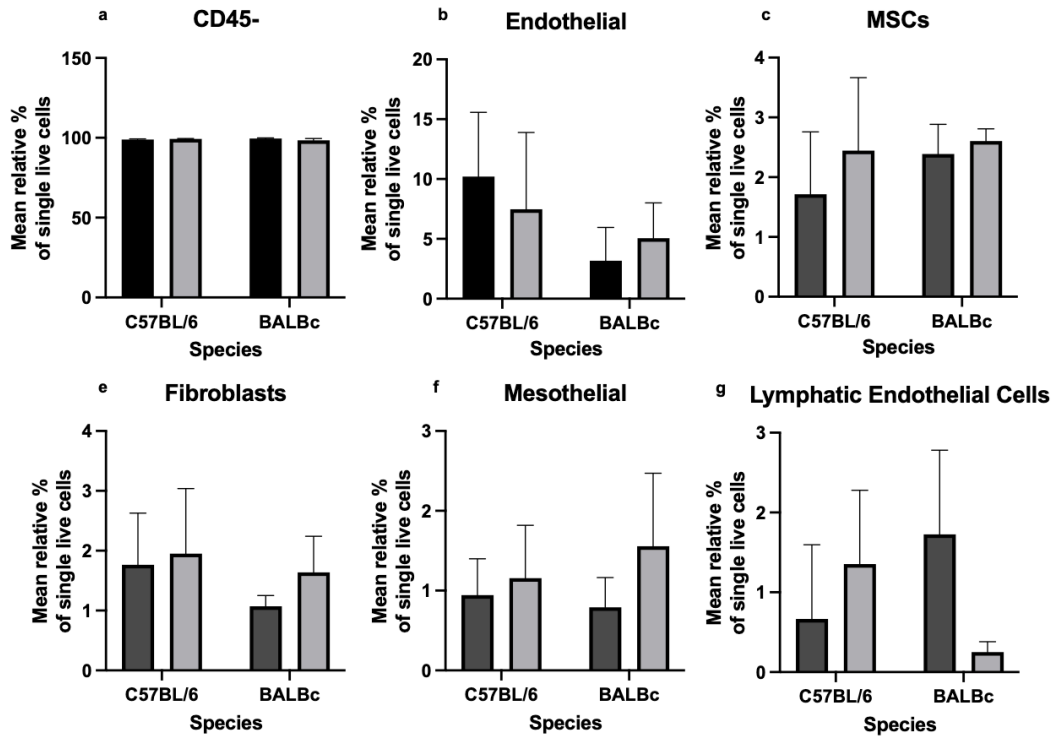
Mouse	Sample	Full Count	Live (%)	Dead (%)
12W-M	Omentum 1	9.56x10^5	97	3
12W-M	Omentum 2	5.4x10^5	97	3
12W-F	Omentum 3	4.46x10^5	95	5

Figure 11 | C57bl-6 ODRCs culture day 7.



4.4.4 Flow Cytometry of C57bl-6 ODRCs

Around 99% of O-SVCF cells were CD45 negative (non-haemopoietic). There was no significant difference between mean relative percentage of single live cells between either strain of mice or sex. (Figure 12). Endothelial Cells were the most common cell type, accounting for 2.1 – 2.5% of cells positive in the selected panel of mesenchymal stem cell markers (Figure 12).



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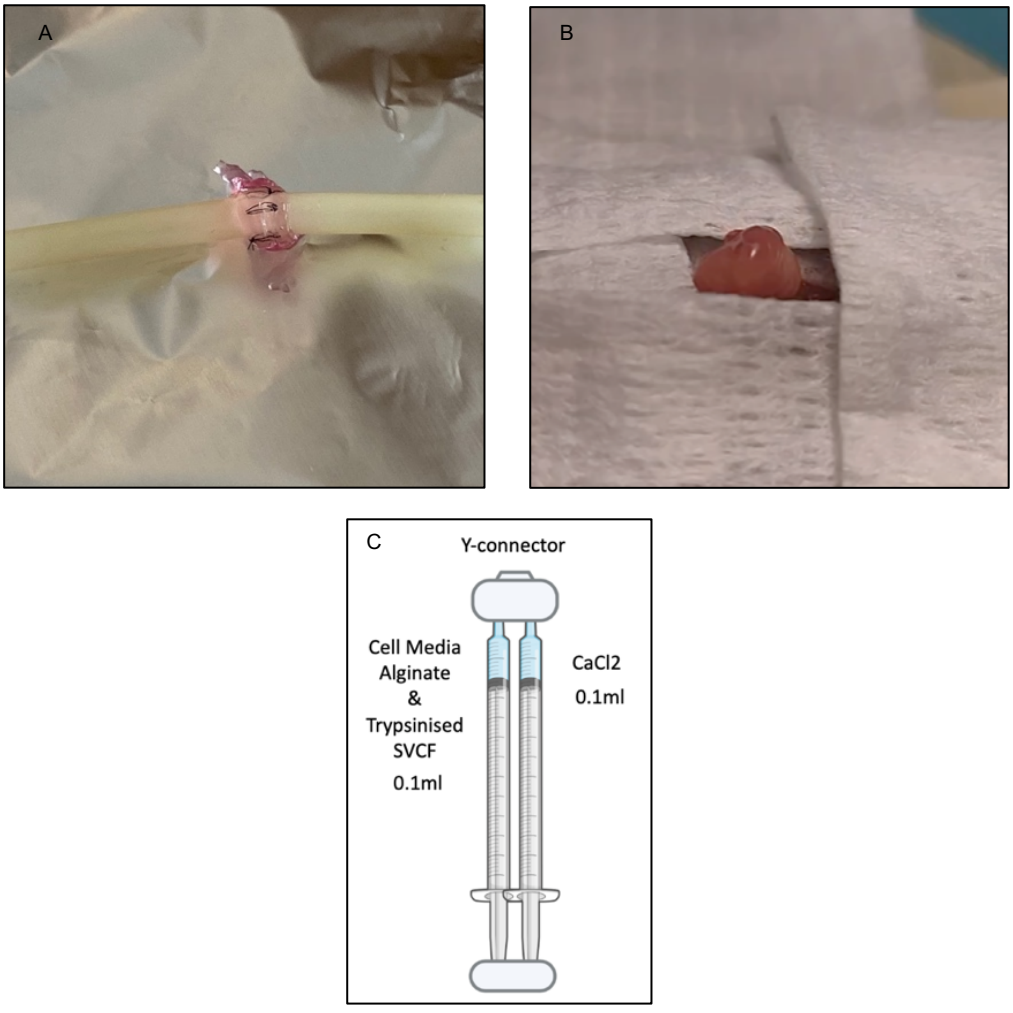
Stromal Marker											Cell Type	Mean Relative % Single Live Cells		*p*
CD45	CD31	LYVE1	CD90.2	WT	CD117	PDPN	PDGFRa	CD29	CD105	Sca1		C57BL/6 Pooled	BALBc Pooled	
-	-	-	-	-	-	-	-	-	-	-	CD45 –ve	99.15 (±0.34)	98.88 (±0.99)	0.70
-	+	+	+	+	+	-	-	-	+	-	Endothelial	6.70 (±5.50)	6.30 (±2.78)	0.12
-	-	-	-	-	-	-	-	+	+	+	MSCs	2.10 (±1.10)	2.50 (±0.36)	0.41
-	-	-	-	-	-	-	+	-	-	-	Fibroblasts	1.90 (±0.89)	1.40 (±0.50)	0.28
-	-	-	-	-	+	+	+	-	-	-	Mesothelial	1.10 (±0.52)	1.20 (±0.73)	0.75
-	-	+	-	-	-	-	-	-	-	-	LECs	1.20 (±0.91)	0.80 (±1.05)	0.96

Figure 12 | Flow cytometry data showing cellular composition of omental stromal vascular cell fraction. (Black Bars: Male, Grey Bars: Female) Mean relative percentage of single live cells with (a) CD45 negative non-haemopoietic cells (b) Endothelial cells (c) Mesenchymal stem cells (d) Fibroblasts (e) Mesothelial cells (f) Lymphatic endothelial cells and (h) pooled species mean relative percentage of single live cells. Statistical analysis using ANOVA.

4.4.5 Gel Production

0.10 ml of media containing cells was mixed with a 0.15mls of 4% DMEM / Gel and placed through a y-connector containing 0.250mls of 150mM calcium chloride to produce a cell/gel ribbon (Figure 13). This was trialled on the bowel of a Schedule 1 mouse in triplicate for each concentration showing good gel adherence, which remained in place through closure and re-laparotomy (Figure 13). The use of complete DMEM did not alter the desired gelation time using the scaled down micro-gel applicator (Figure 13).

Figure 13 | Cell/Gel ribbon adherence to bowel wall simulated (A) & live (B) after using a using a dual syringe system with 3d-printed Y-connector.



4.5 Discussion

O-SVCF can be harvested from mouse omentum and incorporated within a rapidly setting alginate gel applied to murine bowel wall. Multiple techniques for O-SVCF harvest have been described in the literature, including omental explant (309) and peritoneal lavage. (296) However, neither are easily translatable to humans because an extra procedure would be required or enzymes would need to be infiltrated into the peritoneal cavity. Alternative sources of adipose tissue were also considered, such as the lipotransfer techniques which have been described previously in human breast augmentation. (310) These would still require an additional procedure. Controversy remains over the ideal panel for characterisation of mouse MSCs. The described panel of cell markers included a broad representation of cell phenotypes that were positive and negative for markers of mouse MSCs previously described in the literature.

Whilst several delivery vehicles were considered, alginate gel was deemed the best for delivery of O-SVCF to an anastomosis. Alginate is FDA approved, is cheap, and can be scaled down for micro-applications. The design and production of the 3d micro Y-connector device was done so with the help of Dr Benjamin Johnson (Experimental Officer, School of Physics and Astronomy, University of Leeds) which can deliver a reproducible cell/gel ribbon that adheres to bowel wall in a mouse model.

In this chapter, I have demonstrated the presence of Omental MSCs in samples of mouse omentum. There were no significant differences between sex or between two mouse strains. Given that Pommergaard *et. al.* utilised a C57BL/6 mouse and there is no apparent difference in their characterisation profile of the omentum the decision was made to proceed with experiments using this strain of mice. This was to allow for comparison of results between the identified studies also by Komen *et. al*. and Pantalis *et. al.* (289,327,328)

5. Investigating the Biology of Anastomotic Wound Healing in a Temporal Mouse Model

To better understand why some anastomoses fail to heal, an increased understanding of the normal gut healing process is required. The literature is replete of information on how human bowel heals because of the ethical considerations involved in harvesting a healed anastomosis for histological analysis. Previous research is limited to a single retrospective study. To further investigate anastomotic wound healing, an animal study investigating a 50% anastomosis in C57BL/6 mice was conducted. These anastomoses allow comparison of traumatised but healing bowel with un-touched bowel wall within the same animals. This has generated a histology tissue bank for future detailed analysis. The development, techniques and optimisation of the model, and a narrative of the wound healing process at the anastomotic line, are presented in this thesis.

5.1 Background

In 2003 Shamafi *et. al.* examined 30 human bowel anastomoses at post-mortem in an attempt to characterise colonic healing. Subjects were heterogenous in terms of the bowel pathology dictating resection, all-cause mortality, period between anastomosis, death and resection. Therefore any meaningful conclusions are limited. The histopathological examination of the anastomoses that leaked (n=5) showed full thickness necrosis of the mucosa with the formation of a surface membrane of fibrin, acute inflammatory cells and pus. There was discontinuity of the muscle layer with focal areas of necrosis and neutrophilic infiltrate with early features of granulation tissue formation, but no foreign body giant cell reaction. To date, there have been no further attempts to characterise anastomotic wound healing in humans or animal models.

The development of murine small intestine is markedly similar to humans (311) but differs in that mice have 50% less large bowel per Kg. The understanding of the development of murine large bowel is minimal (312) There is some variation in intraluminal content with a higher water component, lower pH, and a greater amount of lymphoid tissue in the mouse colon compared to humans (313) The intestinal immune system is highly conserved between mice and humans with similar micro-anatomical features (e.g. immunoglobulins) and the outcome of intestinal immune response is the same (e.g. tolerance of food, commensal flora, maintenance of the integrity of the epithelial barrier with some differences in the pathways that achieve the immune response). (314) As previously described, there is international consensus that a mouse model is a favourable animal for conducting anastomotic experiments with the additional benefit of potential knockout models.(229) The suitability of a mouse model of anastomotic healing is also discussed in section 4.1.2.

5.2 Aim

1. To develop a mouse model of anastomotic leak with a 0% leak rate that provides within-subject control.
2. To provide a narrative of the histological wound healing processes at the anastomotic line.

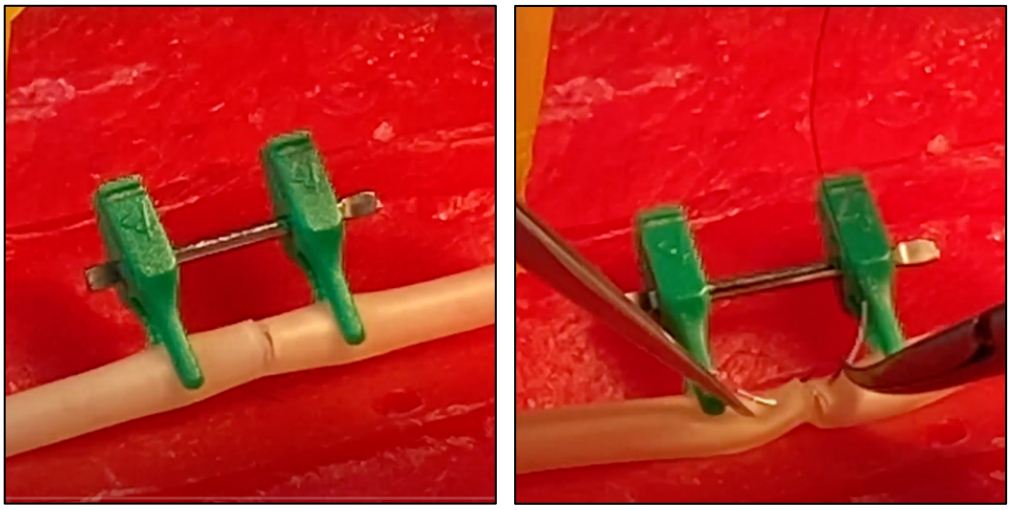
5.3 Methods

5.3.1 Simulation (Plastic Vessel)

The technical ability to perform small luminal anastomoses requires practice, specialised instruments, and a high-quality microscope. To ensure methodological

rigour through standardisation of technique and to ensure that the researcher was safe and able to perform surgery on live animals, a series of simulated experiments were performed under the guidance of Mr Jason Wong, Consultant Plastic Surgeon, an expert in microvascular anastomoses. (315,316) The surgical literature suggests that it takes a minimum of 50 cases to reach the plateau or “turning point” of the learning curve for a new surgical technique. (317–320) Therefore, 50 anastomoses on 3mm artificial vessels (Limbs & Things, UK) (321) (which represents the diameter of the mouse colon at 1cm from the caecum) (322,323) as per Pommegard *et. al.* (289) were performed (Figure 14). A microvascular clamp was used to stabilise the vessel. In subsequent animal experiments (section 5.3.2) the anastomoses were performed without the clamps to avoid inadvertent injury to the bowel. (324,325)

Figure 14 | To overcome the learning curve associated with micro anastomoses simulation experiments were performed on artificial vessels to allow for a standardised anastomosis.

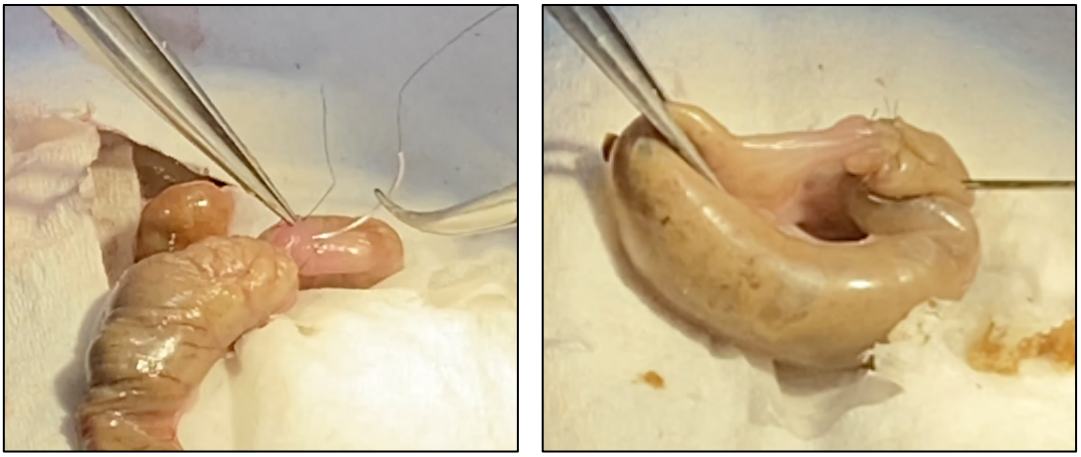


5.3.2 Simulation (Schedule 1 Black C57BL/6 Mice)

Following rehearsal on the simulated dry lab models, 3 anastomoses on Schedule 1 C57BL/6 mice (Figure 15) were performed. A modified leak test was conducted after

each anastomosis to check for anastomotic integrity by injecting the bowel lumen with sterile water (Figure 15). If there was any air or water leak, the sutures were taken down and the 50% anastomosis refashioned.

Figure 15 | To ensure the anastomotic technique learned on artificial vessels was transferable to animal subjects the simulation experiment was repeated on schedule 1 mouse colon of the same diameter.



5.3.3 Temporal Study (Black C57BL/6 Mice)

The previously described husbandry conditions described in chapter 4 were utilised using male C57BL/6 mice, aged 10-12 weeks and an approximate weight of 20g for the temporal study. Animals were purchased from a commercial supplier (Charles River, UK).

Mice were anaesthetised and a midline laparotomy performed as per section 4.3.2. Following midline incision, the caecum was delivered and a 50% transection performed across the antimesenteric border to mesentery using microscopic scissors. Two stay sutures were placed at 0 and 180 degrees to ensure even spacing between the additional sutures on either wall. Five Interrupted submucosal sutures were placed on the opposing bowel wall ends, and knots were tied conventionally with an initial double throw, followed by an additional two single throws using 8.0 vicryl submucosal sutures

(BD, London, UK) (Figure 15). An air leak test was performed by submerging the join line under sterile water and gently forcing intraluminal contents through the lumen passing the join line. If there was leakage of air or content, the sutures were taken down and the 50% anastomosis refashioned.

Healing of the mouse colon is rapid under normal conditions, and might be faster in some C57BL/6 models of severe colitis when re-epithelisation of the mucosa occurs within 2 days. (326) To capture early histological changes at the anastomotic line, six (3 male and 3 female) C57BL/6 mice were sacrificed at day 1,3,5,7 and 14 days (n=30).

5.3.4 Histological Analysis

5.3.4.1 Sectioning

The processing of mouse colon for histology is difficult due to its friable nature and tendency for the bowel to collapse. There is also the decision of whether to section the colon in either the longitudinal or transverse plane. Following repeated trial histology experiments the decision to apply a pink 16-gauge Venflon cannula (1.1mm in diameter) (BD, London, UK) through the lumen of the bowel prior to fixing with 4% paraformaldehyde (Sigma-Aldrich, Dorset, UK) overnight with removal prior to wax embedding was made. This held the lumen patent, helped stop the distortion of tissue and aided in alignment of samples (Table 12). 5-micron sections of the bowel, including the anastomosis, were taken 100 microns apart. The longitudinal sections had the advantage of being able to visualise the anastomosis in any section, so this option was preferred. Eight sections were taken in 3 areas of the bowel (low, middle and high) (Figure 16).

Table 12 | Optimisation of bowel sectioning to demonstrate histological changes at the anastomotic line.

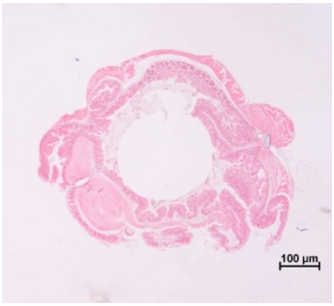
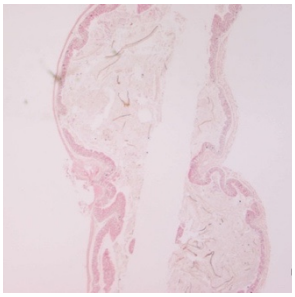
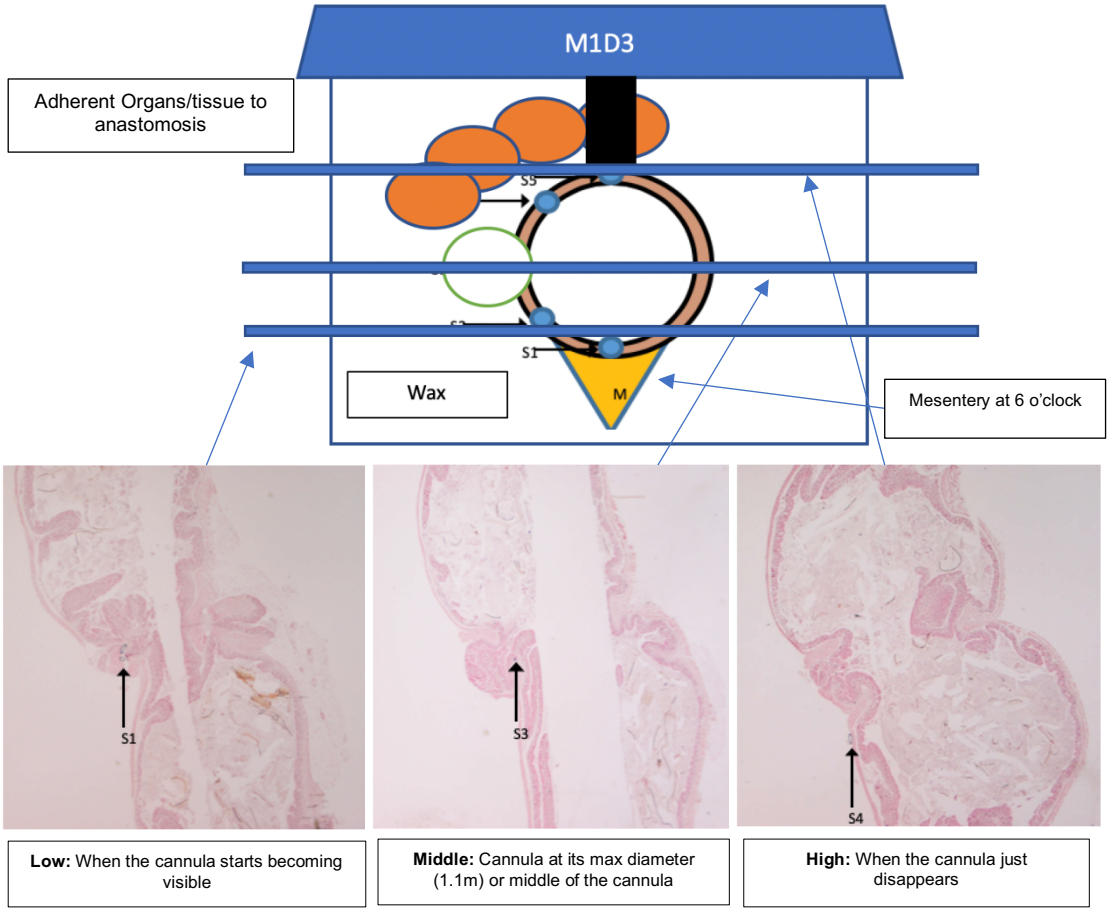
Section	Transverse	Longitudinal
Example		
Advantages	▪ Visualise entire circumference of bowel ▪ Allows comparison of injured to non-injured sides	▪ Can visualise the anastomosis no matter where the section is taken. ▪ Allows for comparison of injured to non-injured sides
Disadvantages	▪ Difficult to capture anastomotic line when sectioning	▪ More difficult to visualise sutures

Figure 16 | Optimisation of bowel sectioning in the longitudinal plane to capture the anastomosis. The cannula is removed prior to sectioning.



5.3.4.2 Narrative of Anastomotic Healing

There is no consensus on the best histological markers to assess bowel wall healing. Markers were selected based on section 1.1.4.1 (Response to tissue injury in the skin), section 1.1.4.2 (Physiology of Anastomotic Healing) and following the findings of the systematic review presented in Chapter 2.

5.3.4.3 Cytochemistry and Immunohistochemistry of sectioned tissue specimens

Tissue sections were subject to histological analysis (Table 13). All antibodies and solutions were prepared according to predefined protocols (Appendix 5). In brief, sections were mounted on glass slides, dewaxed and rehydrated, and subjected to antigen retrieval according to Table 13. Hydrogen peroxide was then applied to the sections (200mls of methanol (Sigma-Aldrich, UK) in 2mls of H_20_2 for 10 minutes). Following rinsing with water and Tris-Buffered Saline (TBS), a pre-block was performed in antibody diluent with subsequent administration of the primary antibody for one hour at room temperature. Following rinses with Tris-Buffered Saline with 1% Tween (TBST) and TBS, the secondary antibody (Goat anti-rabbit; Abcam, UK) was applied and incubated for 30 minutes at room temperature. Following rinses with TBST and TBS, Diaminobenzidine (DAB) (Sigma-Aldrich, UK) substrate was applied and incubated for 10 minutes. Following water rinse the sections were counterstained with haematoxylin and eosin as per 5.3.4.3. Slides were then mounted using DePex (Sigma-Aldrich, UK).

Table 13 | Details of cytochemical and immunohistochemical agents used to investigate anastomotic healing.

N	Stain	Product	Cat No	Antigen Retrieval	Dilution	Supplier	Features Investigating
1	Haematoxylin	Haematoxylin Solution	100 470 05	-	-	VWR International	Epithelialisation, Muscle Distribution, Necrosis, Fibroblast activity, inflammation, tissue restructuring and cell organisation
1	Eosin	Eosin 1% Aqueous Solution	625 025	-	1:100	BioServUK	
2	Masson Trichrome	Trichrome Stain (Masson) Kit	HT1 5-1KT	10mM Citric Acid buffer pH 6.0	-	Merck Life Science UK Limited	Fibrosis
3	CD31	Rabbit anti-mouse CD31	Ab1 829 81	10mM Tris-EDTA pH 9.0 (no Methanol Block)	1:1000	Abcam	Neovascularisation
4	F4/80	Rabbit anti-mouse F4/80	MC A49 7GA	10mM Citric Acid buffer pH 6.0	1:750	BioRad	Macrophage Activity
5	α-SMA	Rabbit anti-mouse α-SMA	Ab5 694	10mM Citric Acid buffer pH 6.0	1:1000	Abcam	Smooth muscle cells
6	Secondary	Goat Anti Rabbit	Ab6 721	-	1:1000	Abcam	-

5.3.4.4 H&E staining of sectioned tissue specimens

Sections were mounted on glass slides and treated with Mayer's Haematoxylin solution before rinsing in cold running water for 1 minute. The slides were washed in Scott's Tap Water for 1 minute before rinsing in running water for a further 1 minute. Slides were counterstained with Eosin for 1 minute before being rinsed in running water for 1 minute. Slides were then dehydrated in ethanol, rehydrated in xylene, and mounted on to coverslips using DePeX Mounting Medium (Sigma Aldrich, UK).

Slides were qualitatively assessed to characterise anastomotic line healing. One C57BL/6 mouse from each day of harvest (1,3,5,7 and 14) was selected and analysed. A photomicrograph was taken and the distribution of cytochemical and

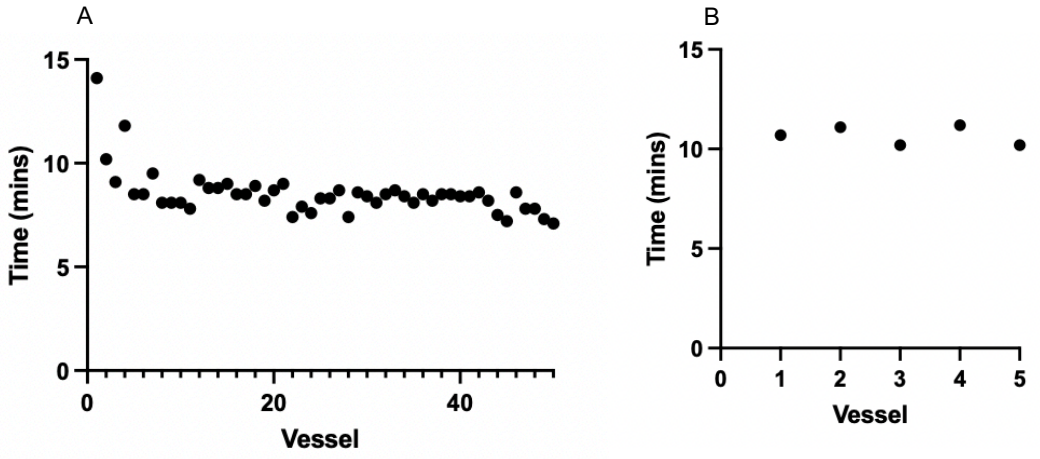
immunochemical reactivity was subjectively assessed at X5, X10, x20 and x40 magnification.

5.4 Results

5.4.1 Simulation (Plastic Vessel)

Fifty anastomoses were performed using the simulated vessels. The average time to perform an anastomosis was 8.51 [±1.10] minutes. The time for anastomosis decreased with increasing experience (Figure 17).

Figure 17 | A: time to completion of anastomosis in simulated vessels; B: time to complete anastomosis in Schedule 1 C67BL/6 mice. Time to completion gradually decreased and plateaued in simulation models with the consistency demonstrated in subsequent Schedule1 experiments. This suggested a learning curve of around 7 cases. There was no difference in anastomotic time in Schedule 1 experiments after completing the 50 simulated anastomoses.



5.4.2 Simulation (Schedule 1 Black C57BL/6 Mice)

Five anastomoses were performed using Schedule 1 Black C57BL/6 mice. The average time taken to perform an anastomosis was 10.67 [±0.43] minutes. The time to complete an anastomosis remained constant across the 5 experiments (Figure 17).

5.4.3 Temporal Study

5.4.3.1 Proving the model

Six (3 male and 3 female) C57BL/6 mice with a median age of 11 weeks [9-15] were anastomosed at day 1,3,5,7 and 14 days (n=30). Mean operation time was 25.3 minutes [±2.1]. Anastomotic leak rate was 0%, abscess formation rate was 0%, and feculent peritonitis rate was 0%. There was one Schedule 18 for a bleed at the anastomotic line that was reported to the UK Home Office. This experiment was repeated. 87% (n=26) of the anastomoses had other organs adherent to at least part of the anastomotic line.

5.4.3.2 Histology

Histology was completed successfully for all 5 cytochemical and immunochemical markers based on 3 sections from 6 mice (3M:3F) across 5 time points with day 0 as the control. This resulted in 540 slides for future quantitative analysis.

5.4.3.3 The Temporal Biology

Slides from five male mice were taken from each of the time points (Days 1,3,5,7 and 14) for microscopic analysis. The photographs shown in Figure 18-35 were all taken using either a x5 or x10 microscope objective lens. However, all images were reviewed using up x40 objective for more detailed analysis.

Figure 18 | Control colonic wall in C57BL/6 male mouse subjected to Haematoxylin and Eosin staining (Objective magnification x2 (top) and x20 (bottom).

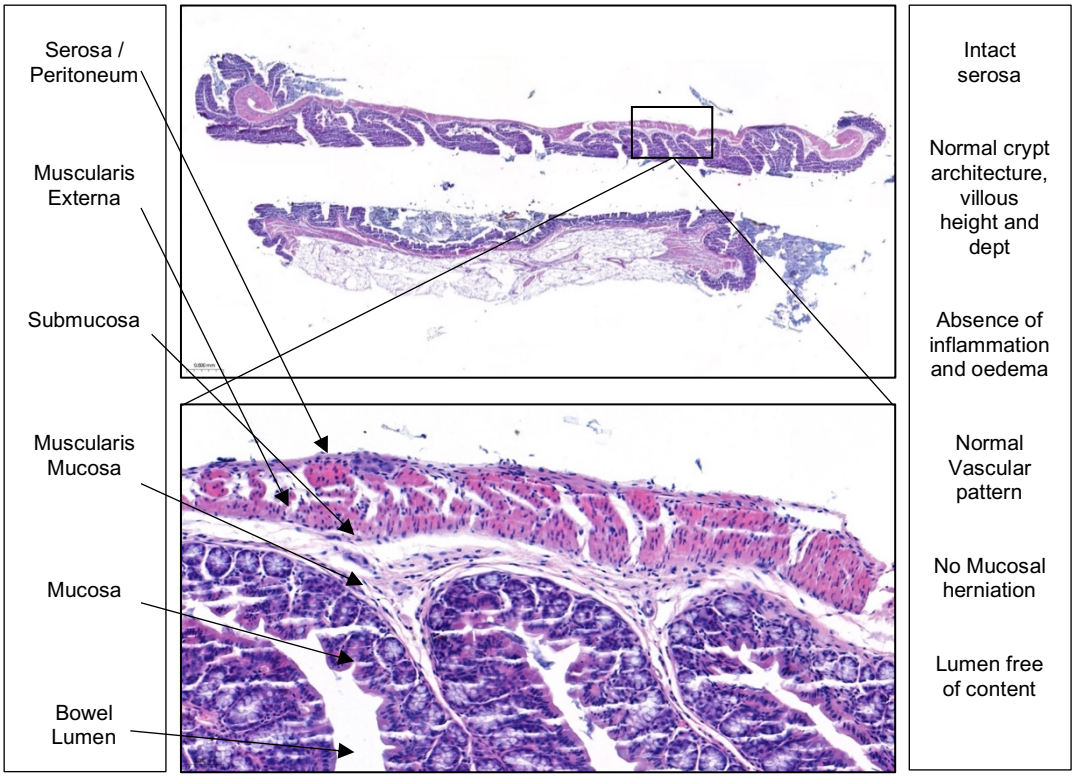


Figure 19 | Day 0 (Control) C57BL/6 male mouse - Haematoxylin and Eosin (x5). All layers of the bowel wall are intact with no inflammatory cells present.

Importantly there has been no tissue trauma caused using a Venflon to maintain the patency of the bowel lumen for sectioning.

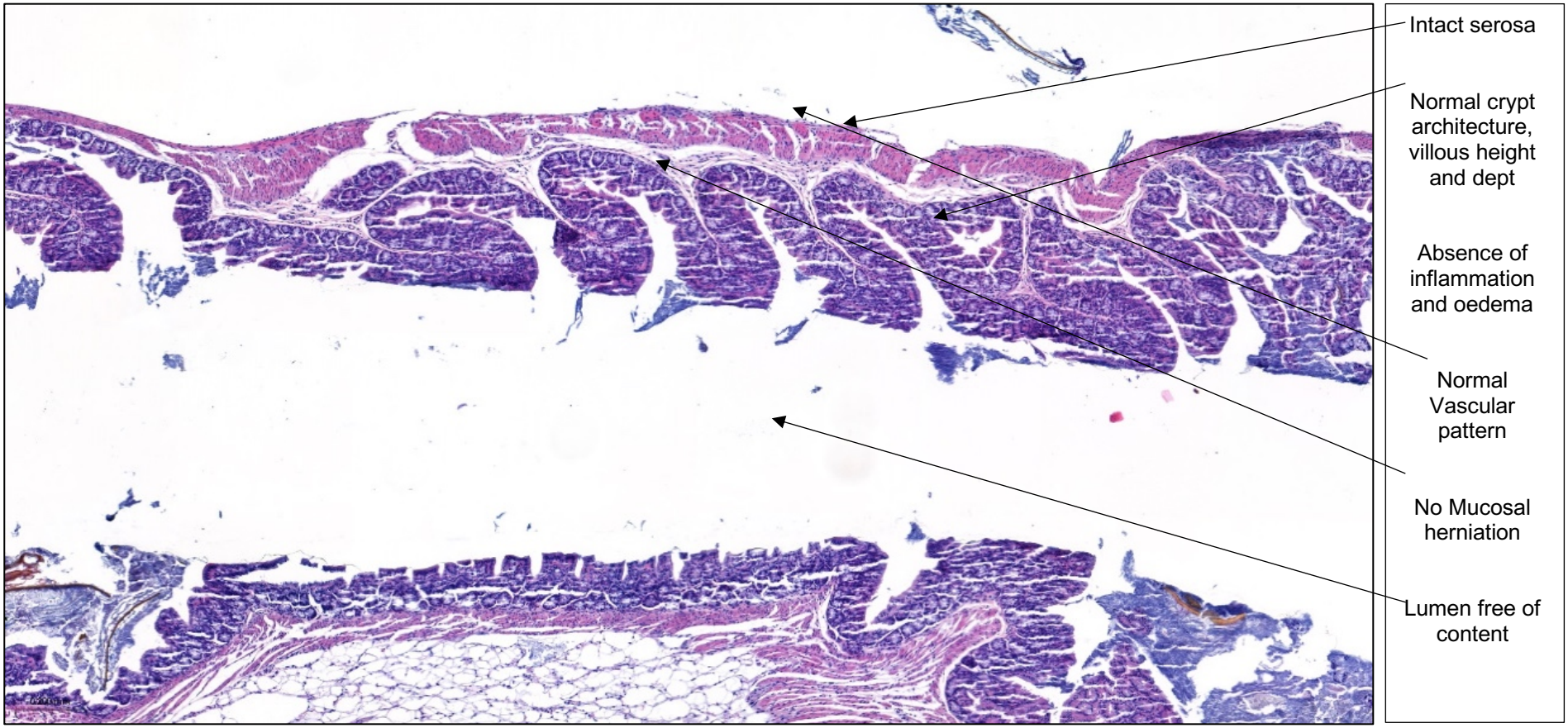


Figure 20 | Day 1 - Haematoxylin and Eosin C57BL/6 male mouse (x5). There is complete transection of all layers of the bowel wall with a concentration of inflammatory cells (neutrophils), platelet and a clot formation, and an early fibrin plug. There is no continuity of any layers of the colon with mucosal herniation, no layer bridging, no restoration of villous epithelium and no restoration of vascular pattern.

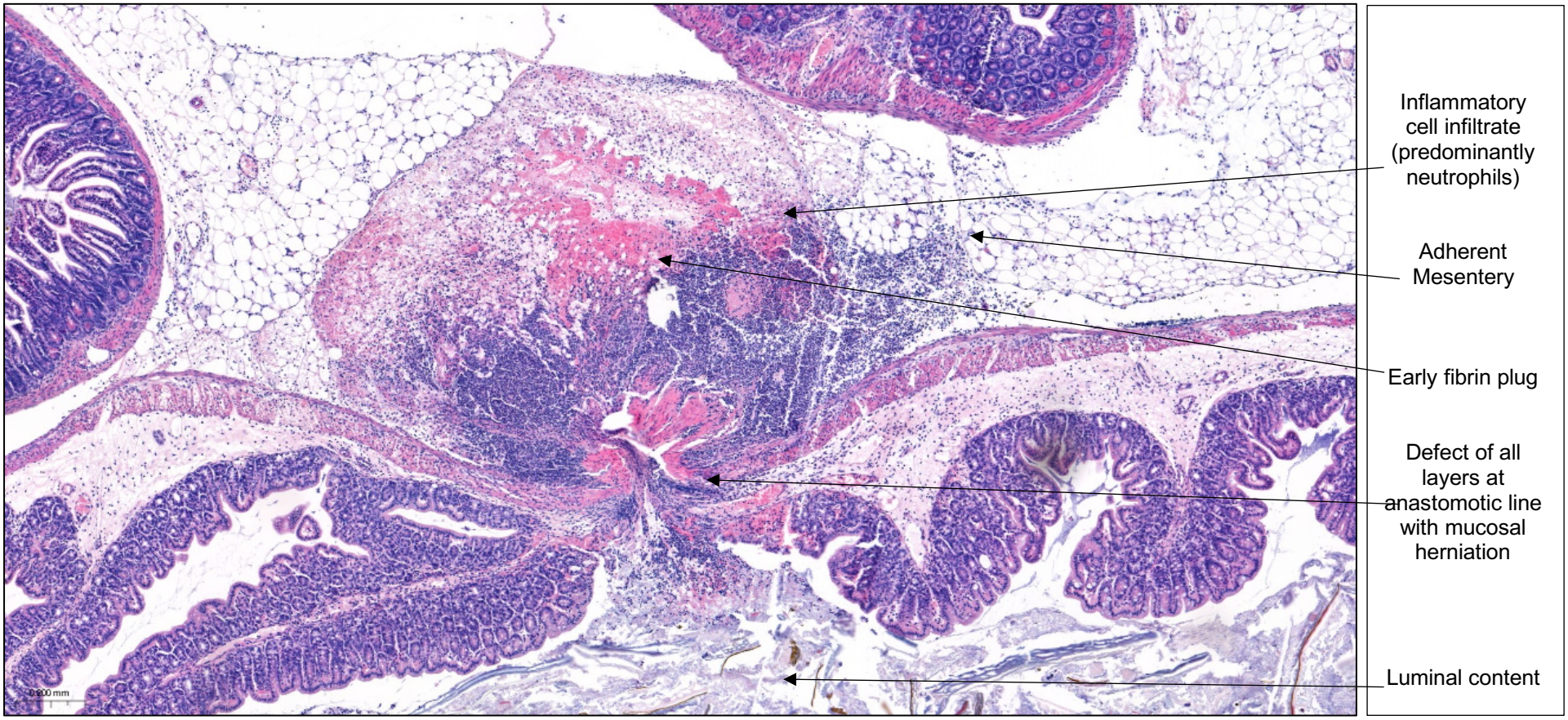


Figure 21 | Day 3 - Haematoxylin and Eosin x5. The serosa appears to be intact with continuing, Inflammatory cells are still present but in lower abundance and appear to be more organised. There is a suggestion of reconstitution of the basement membrane as it is starting to be deposited along the fibrin plug (confirmed on Masson Trichrome stain Figure 22) and some new blood vessels but no continuity of submucosa or muscular layers. There is minimal smooth muscle cell deposition (α-SMA Figure 23).

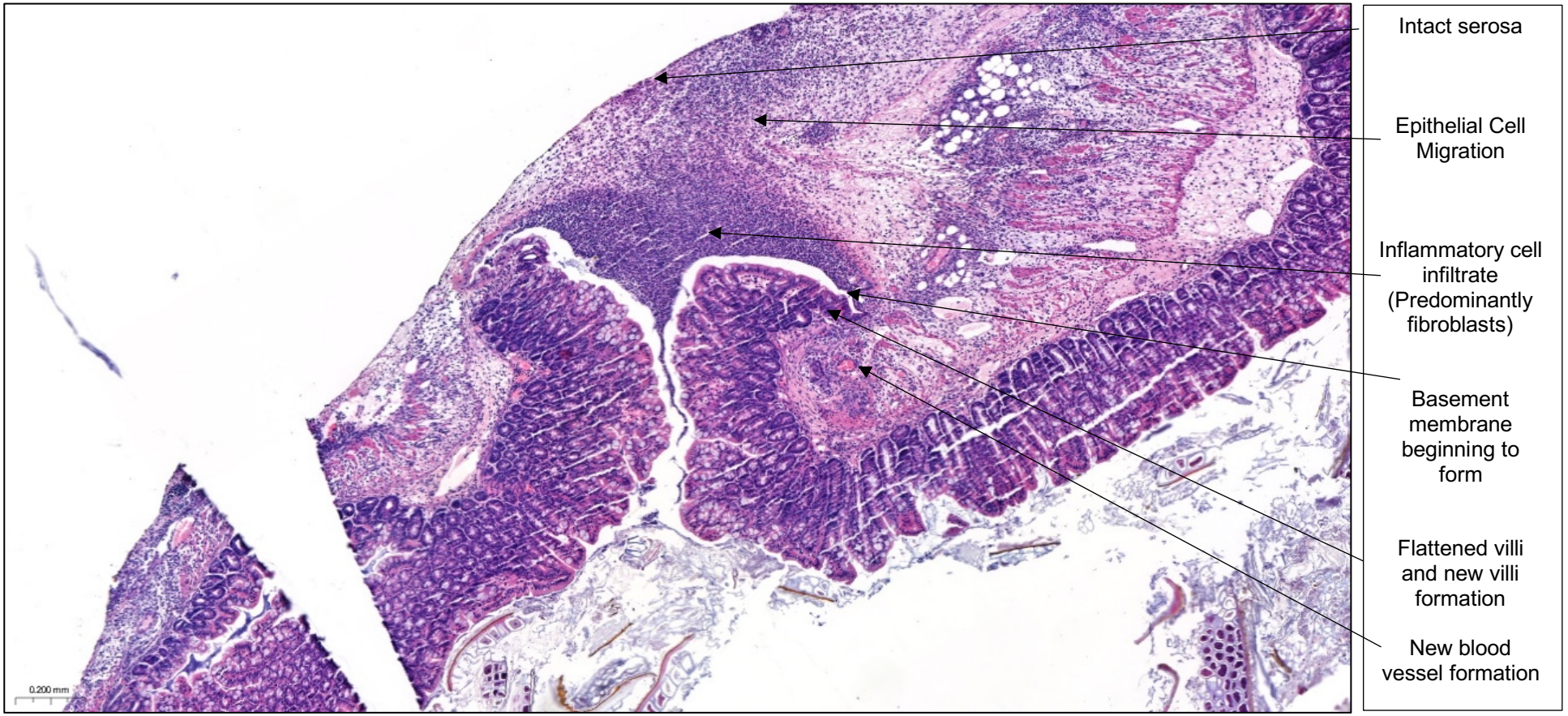


Figure 22 | Day 3 – Masson Trichrome x10. There is suggestion of reconstitution of the basement membrane stained blue as it is starting to make its way along the fibrin plug.

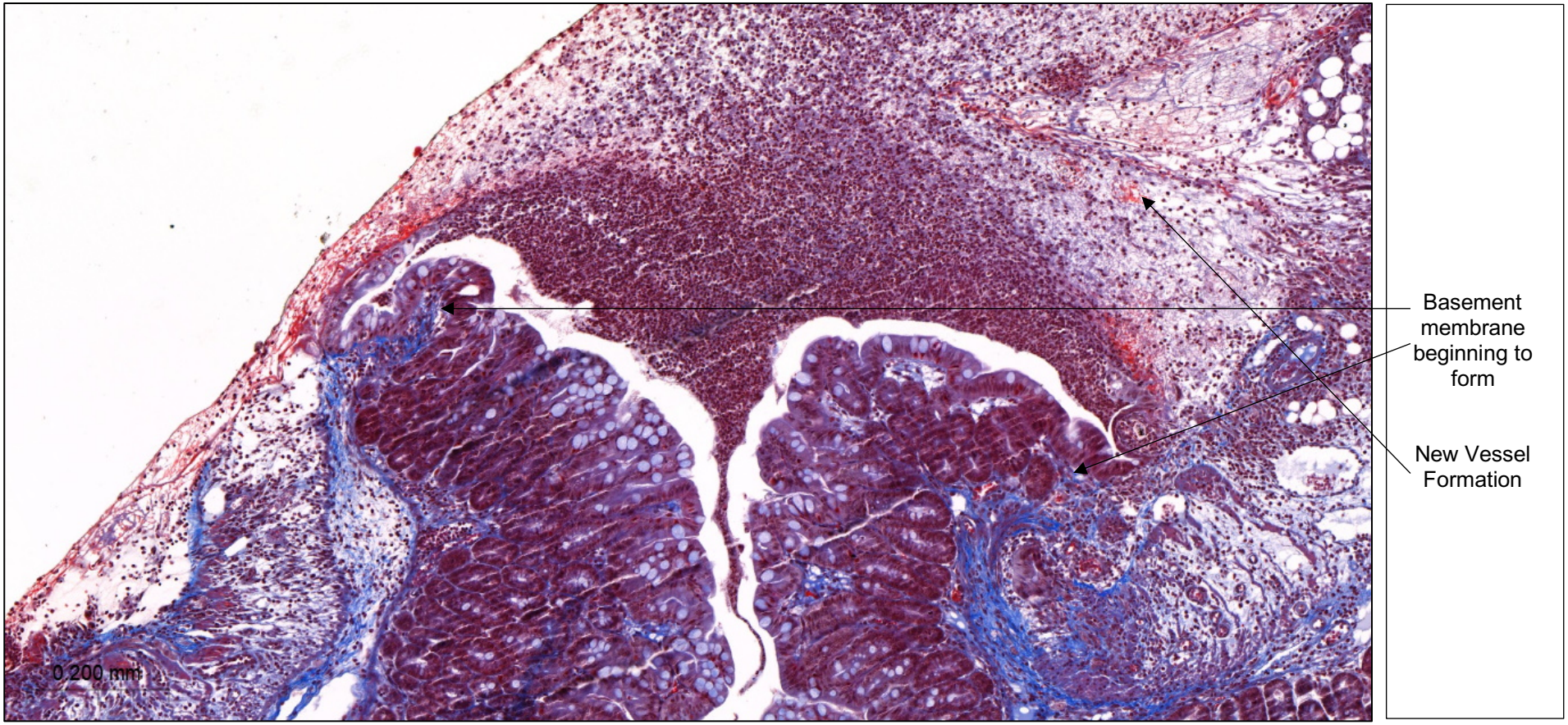


Figure 23 | Day 3 – α-SMA x10. Limited smooth muscle cells stained brown over the epithelial defect.

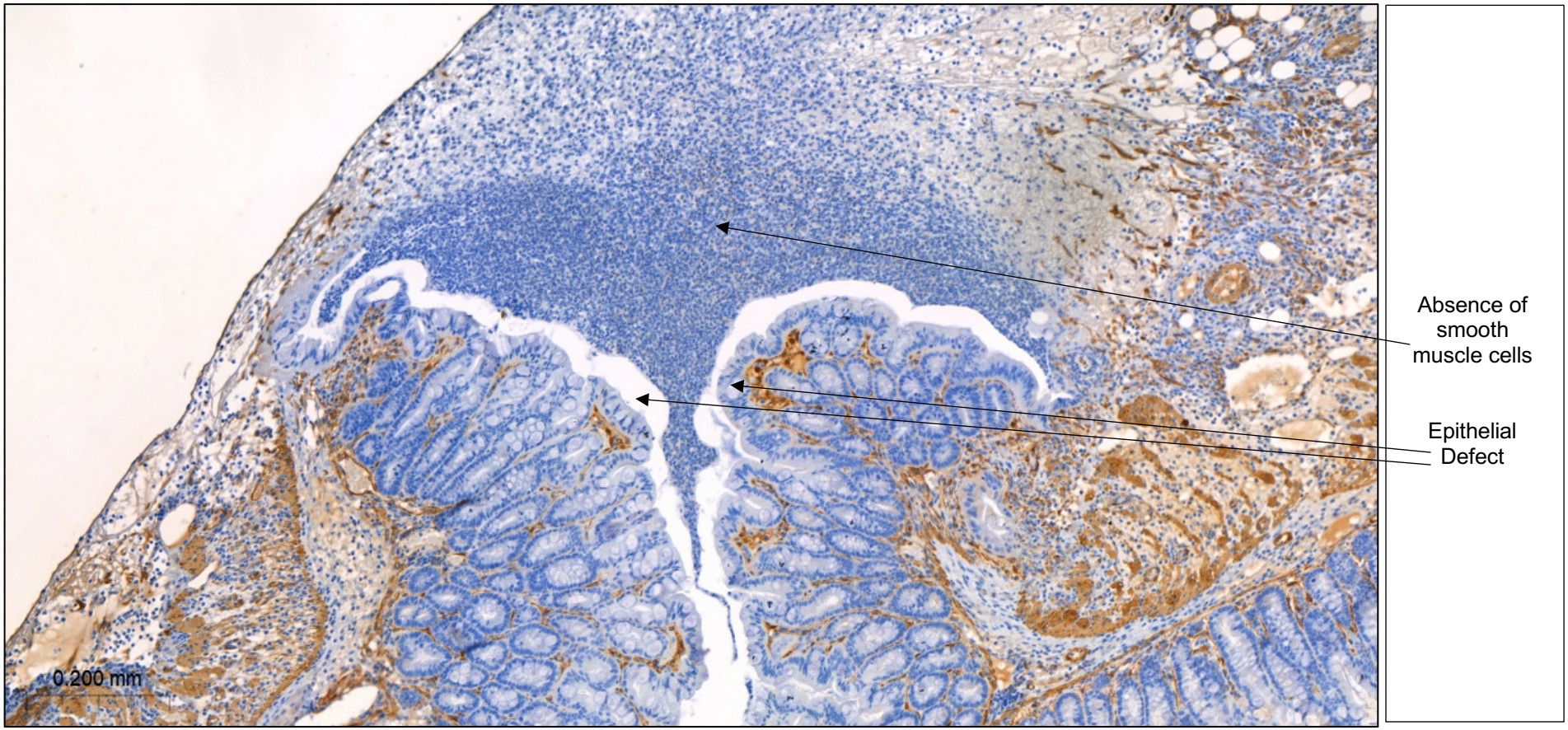


Figure 24 | Day 5 - Haematoxylin and Eosin x5. Basement membrane continues to develop, but is yet to be fully intact (Masson Trichrome Figure 25), with formation of multiple new blood vessels (CD31 Figure 26). Deposition of smooth muscle cells has begun within the extracellular matrix with apparent early bridging across the defect (α-SMA Figure 27) There remains a persistent epithelial defect with less inflammatory cell infiltrate and oedema than previous days. No continuity of submucosa or muscular layers.

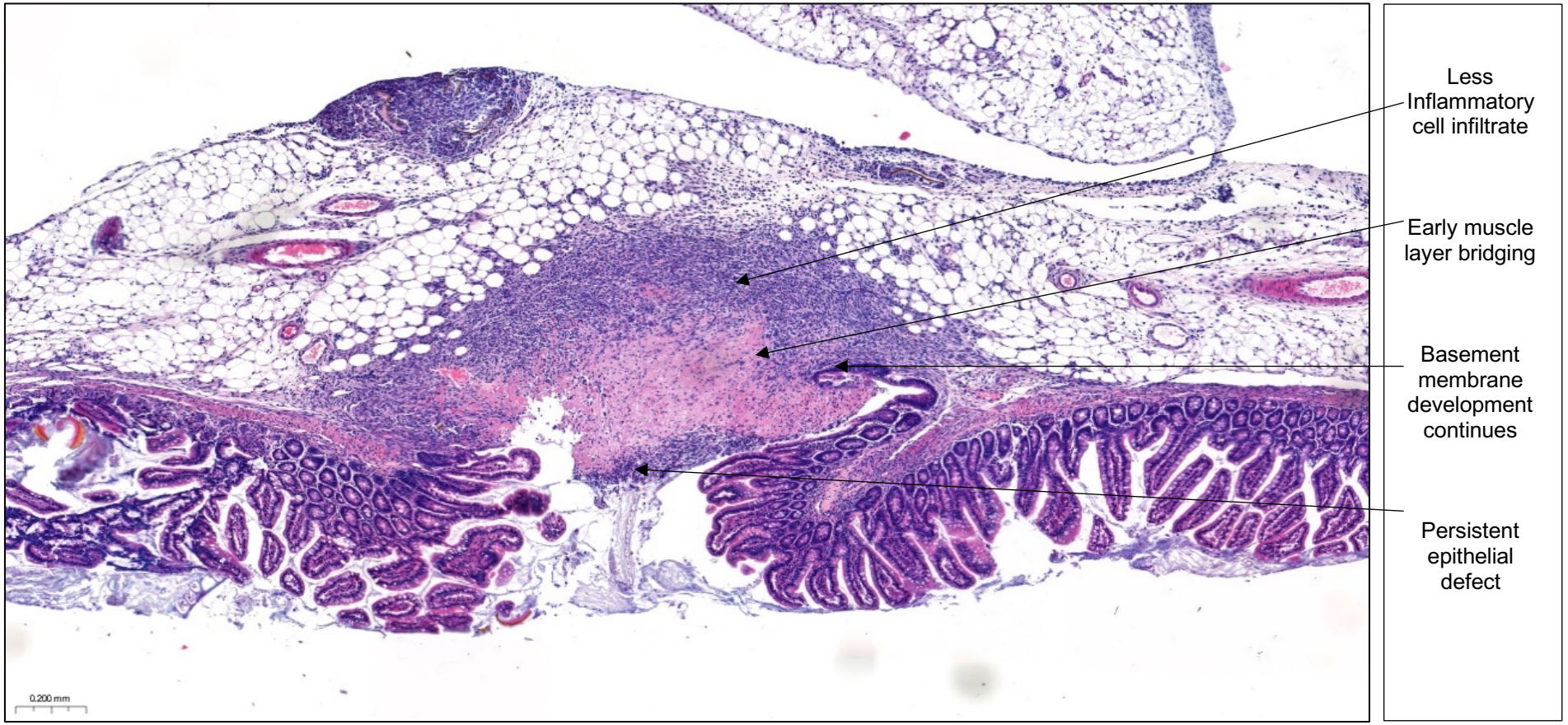


Figure 25 | Day 5 – Masson Trichrome x10. There is suggestion of continued reformation of the basement membrane stained blue as it continues to make its way along the fibrin plug.

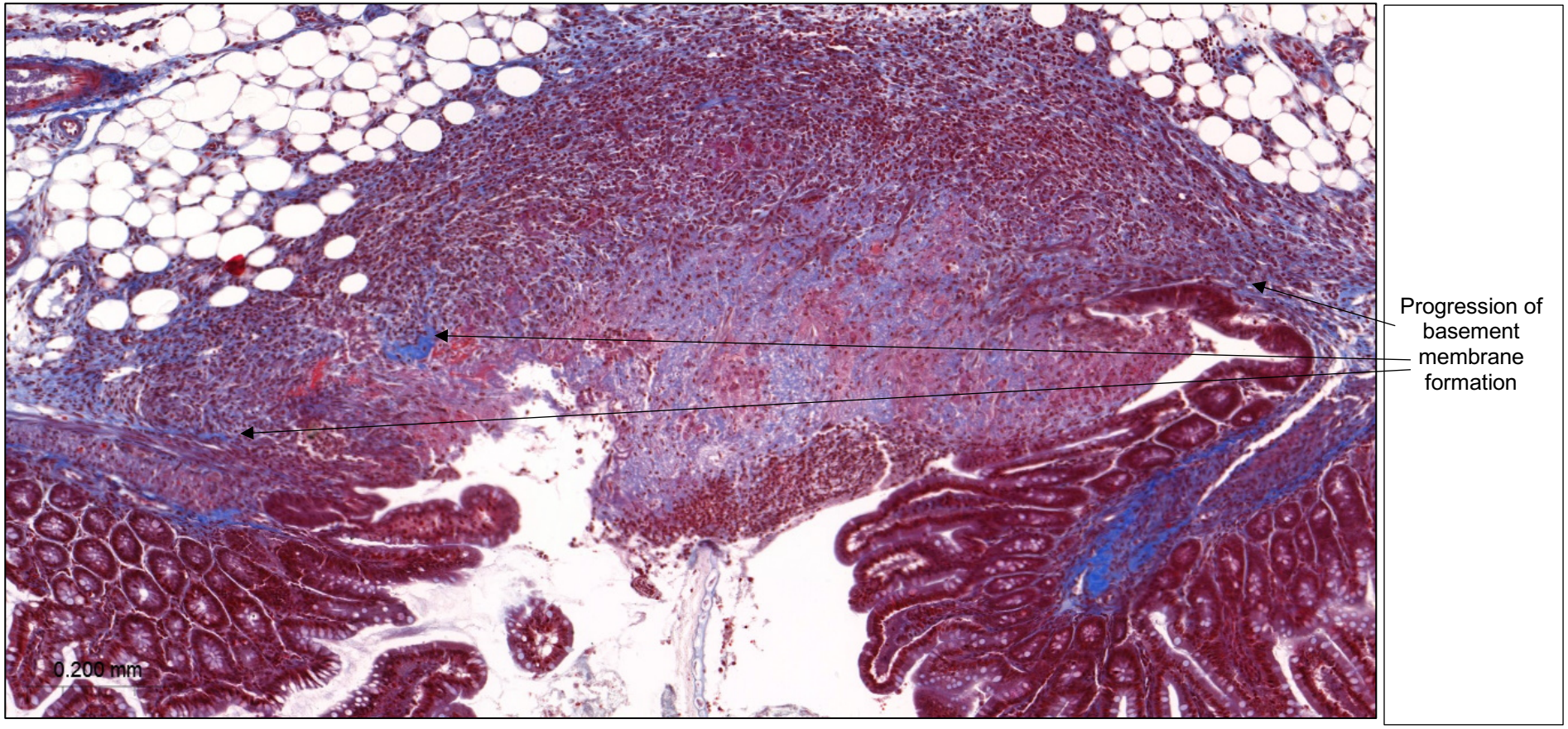


Figure 26 | Day 5 – CD31 x10. There is suggestion of multiple new vessels stained brown forming around the periphery of the extracellular matrix.

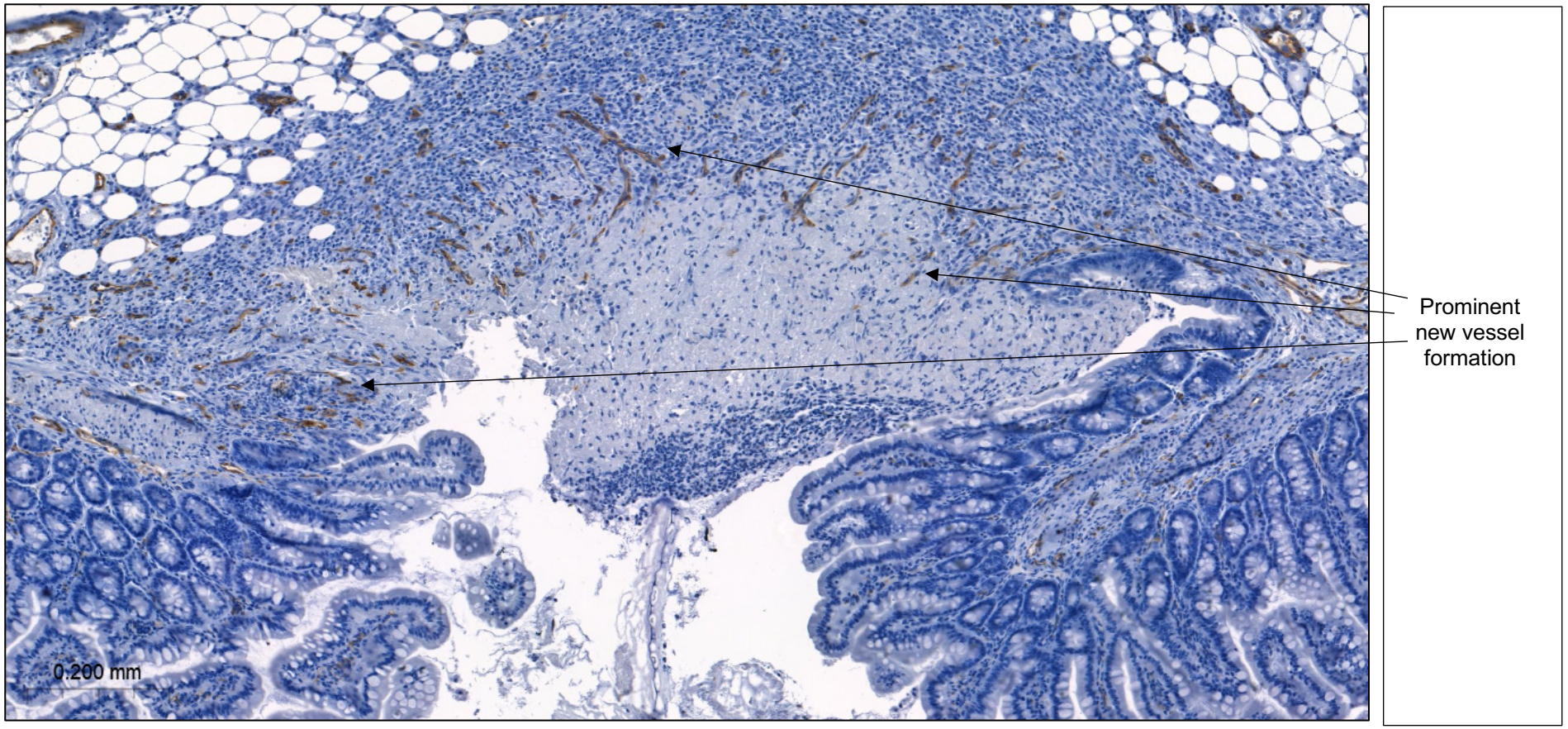


Figure 27 | Day 5 – α-SMA x10. An increased abundance of smooth muscle cells stained brown as the muscle layer begins to organise over the epithelial defect.

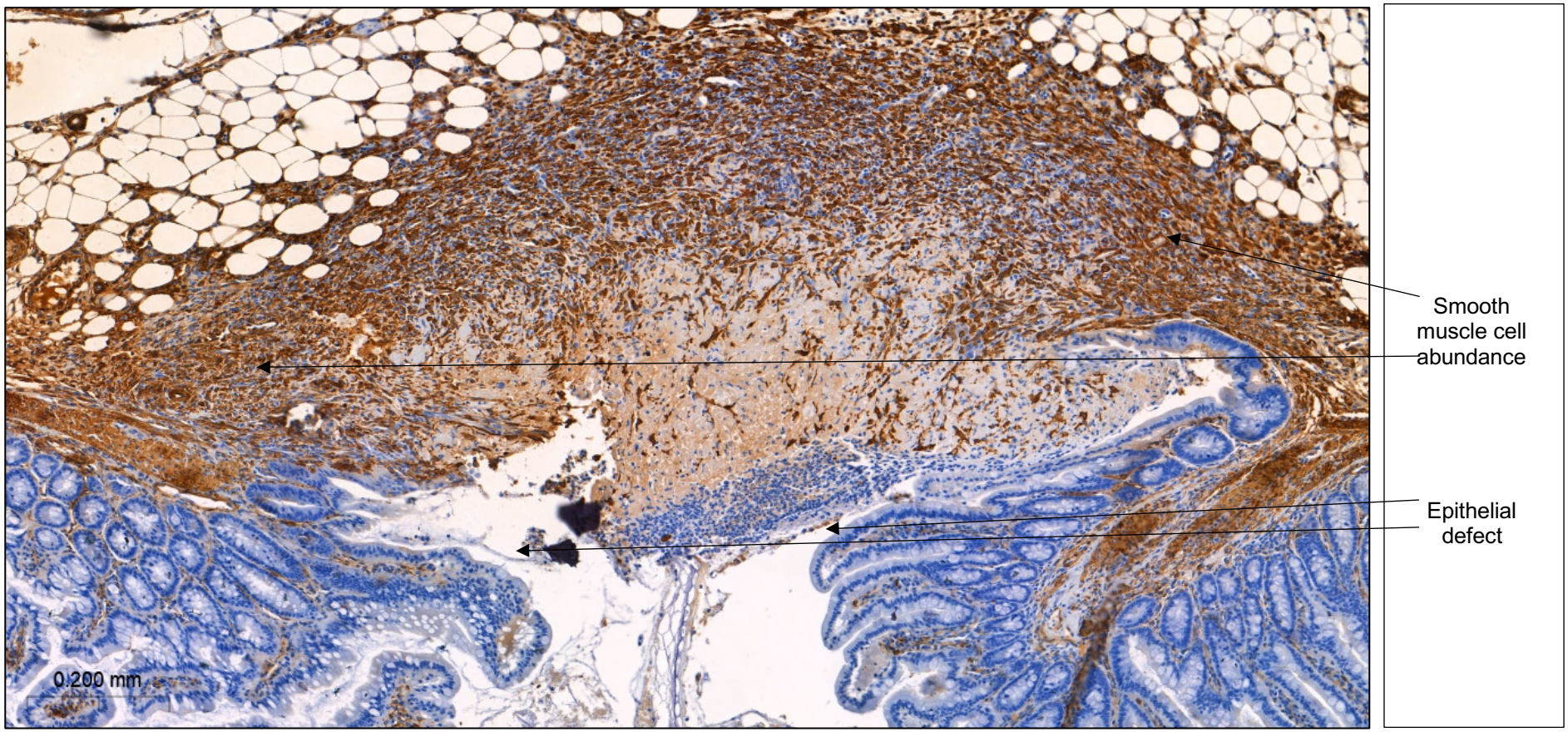


Figure 28 | Day 5 – F4/80 x10. There are some macrophages stained brown around the adherent adipose tissue but non-within the Extracellular matrix.

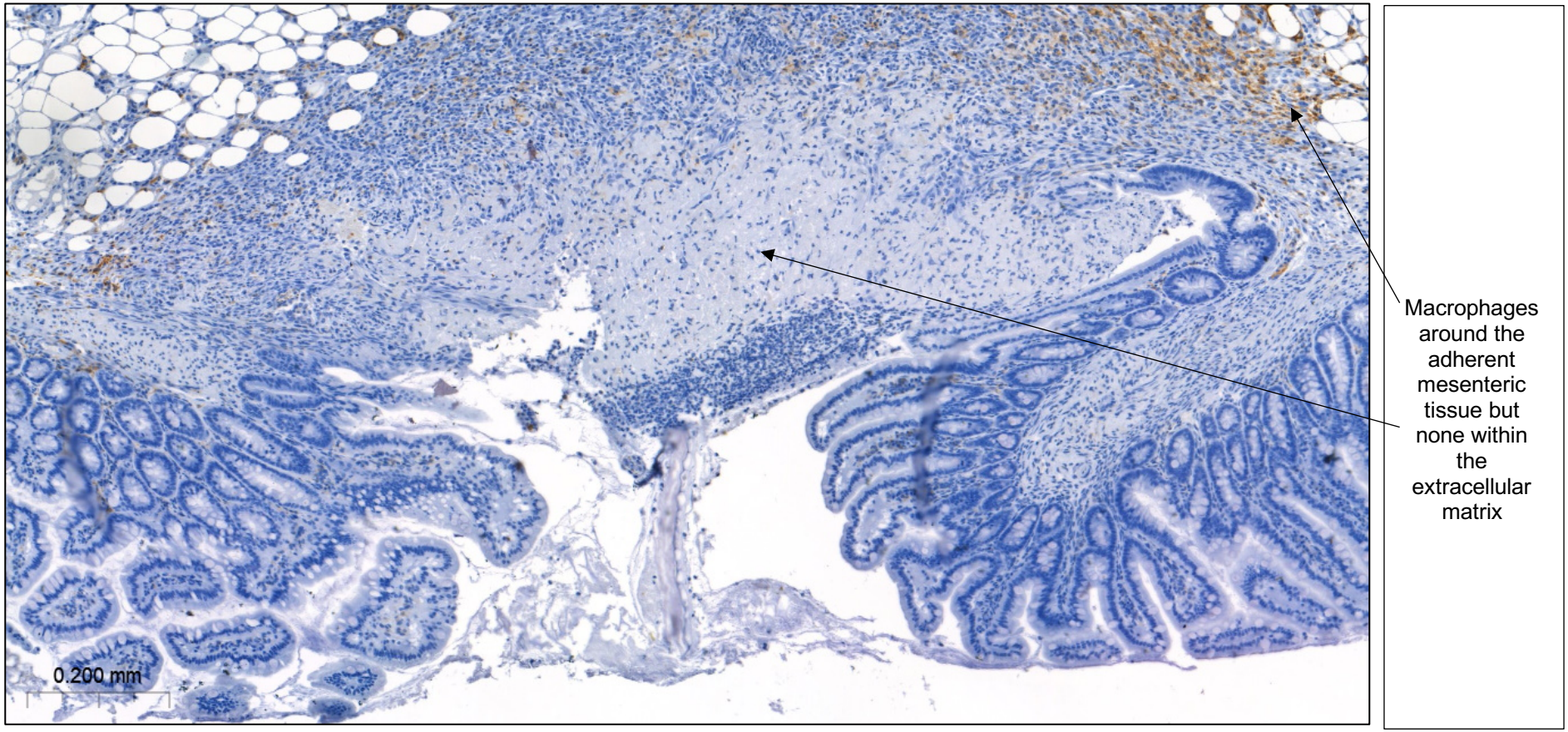


Figure 29 | Day 7 - Haematoxylin and Eosin x5. Basement membrane is now intact (Masson Trichrome Figure 30). The muscle layer is more organised and in continuity and there is submucosal apposition (α-SMA Figure 31). The epithelial defect has healed and there is minimal inflammatory cell infiltrate.

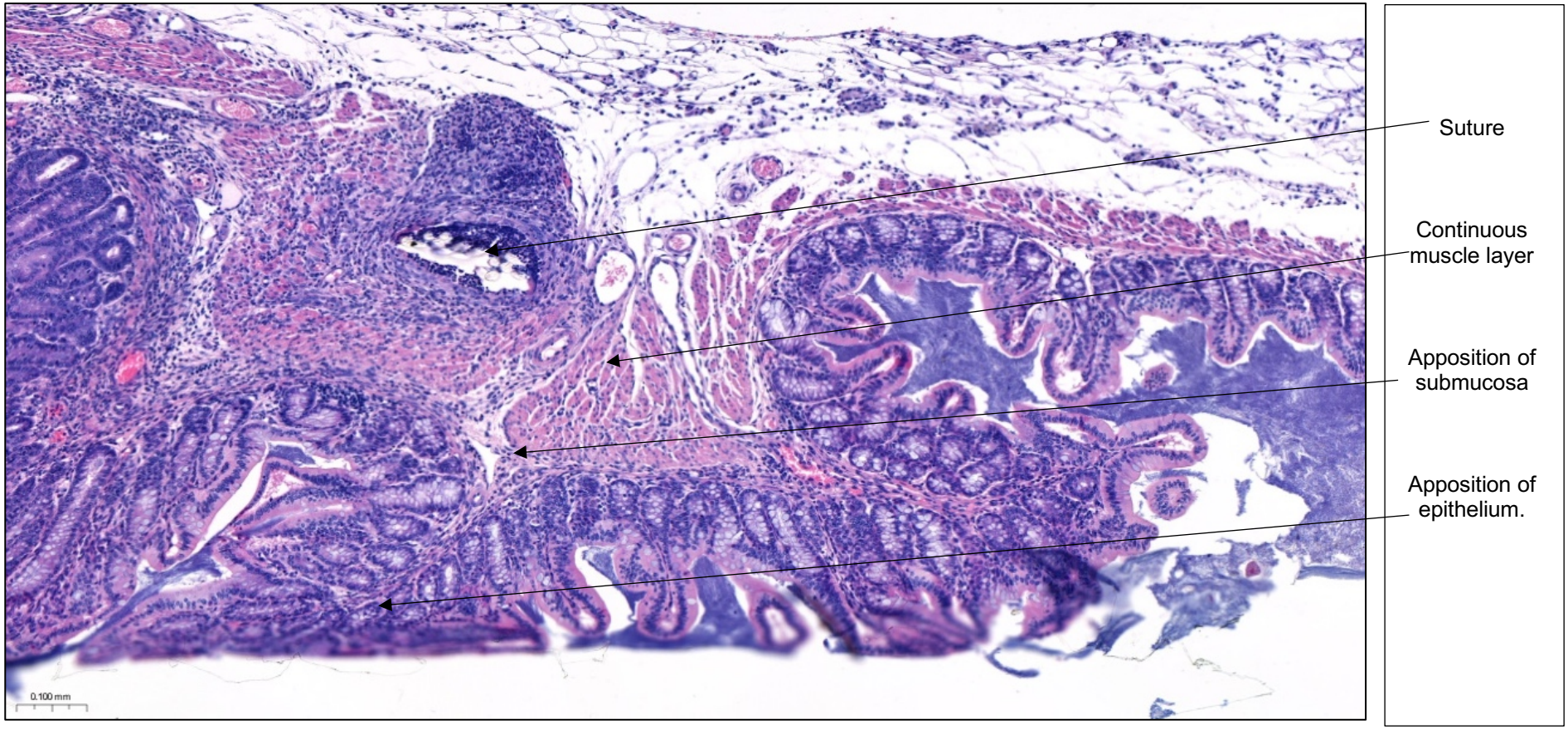


Figure 30 | Day 7 – Masson Trichrome x10. The basement membrane stained blue is completely intact.

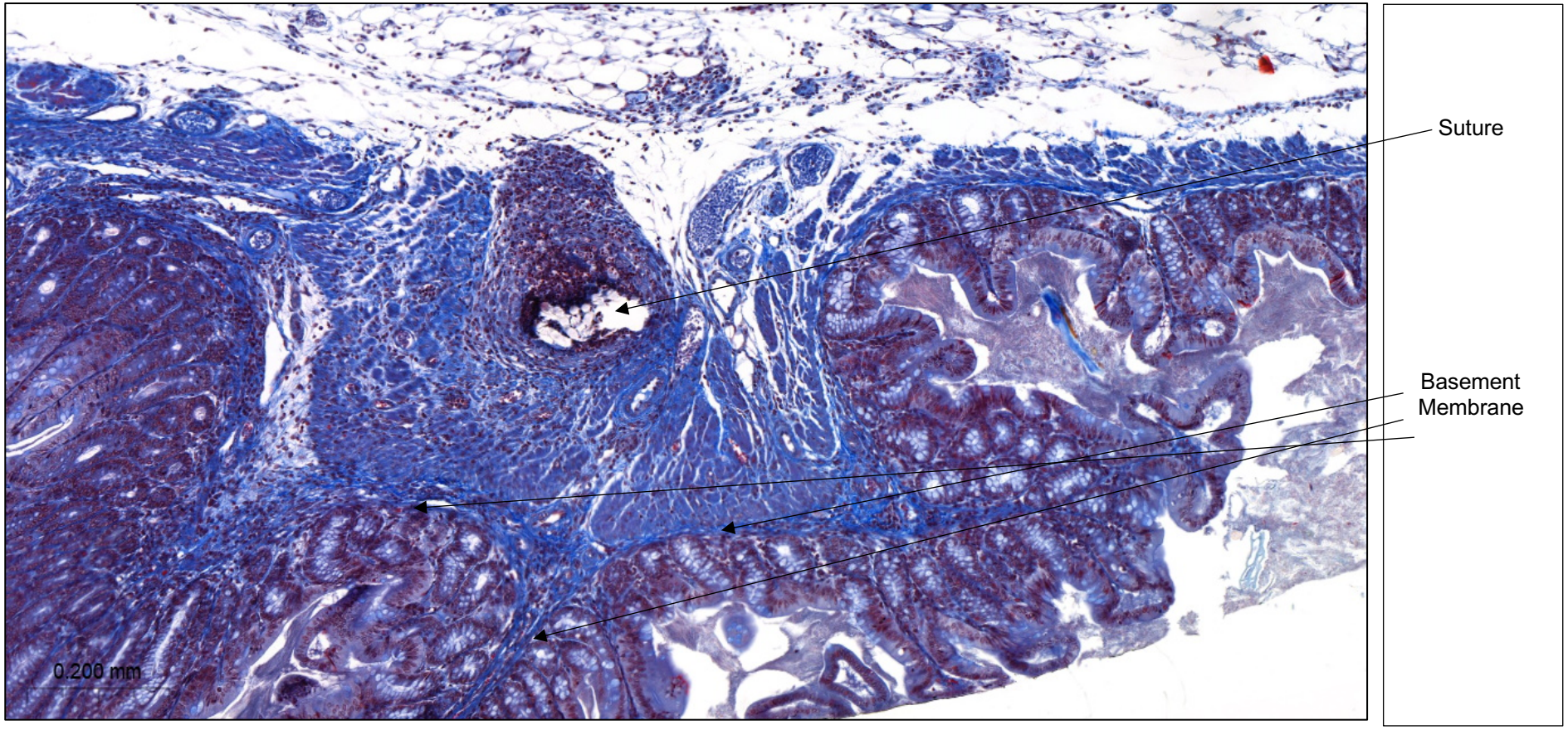


Figure 31 | Day 7 – α-SMA x10. Smooth muscle cells stained brown as the muscle layer is completely intact to organise over the continual epithelial layer.

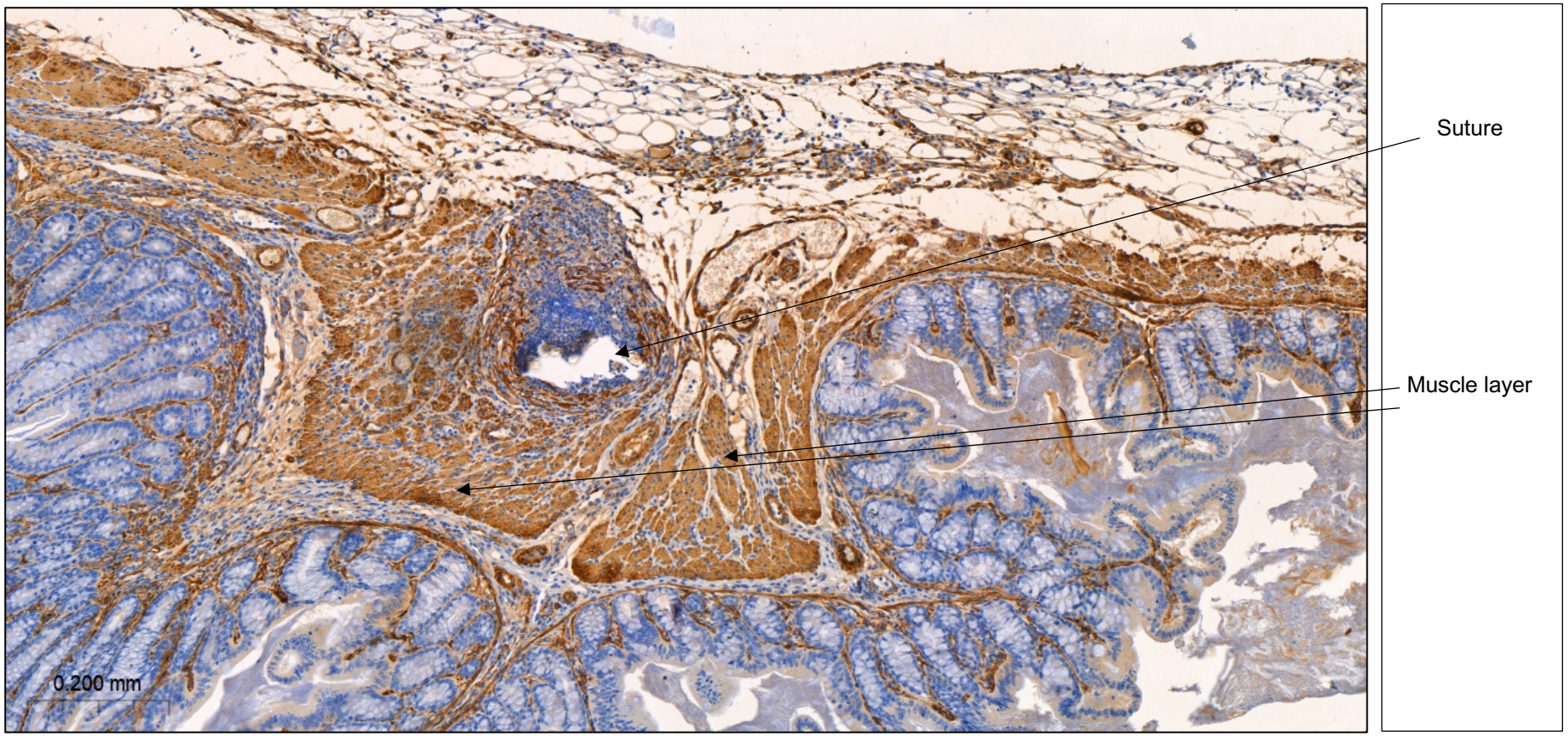


Figure 32 | Day 7 – F4/80 x10. There is an abundance of macrophages stained brown within the remaining extracellular matrix.

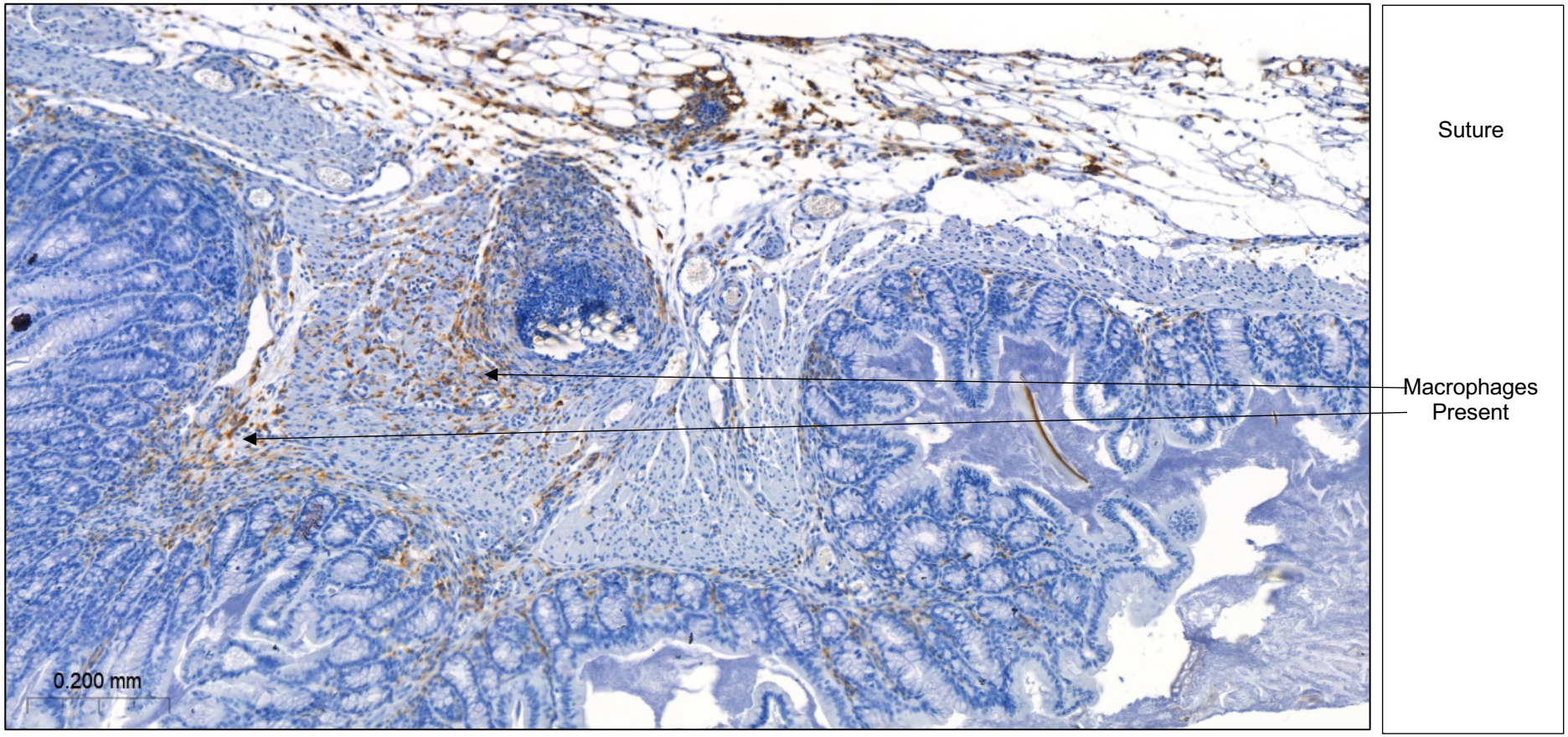


Figure 33 | Day 14 - Haematoxylin and Eosin x5. There is muscle thickening either side of the anastomotic line with “pinching” or contracture over the defect with some early fibrosis.

All layers of the bowel wall are intact demonstrated on Masson Trichrome (Figure 34). Macrophage concentration has reduced after the spike at day 7 (F4/80 Figure 35).

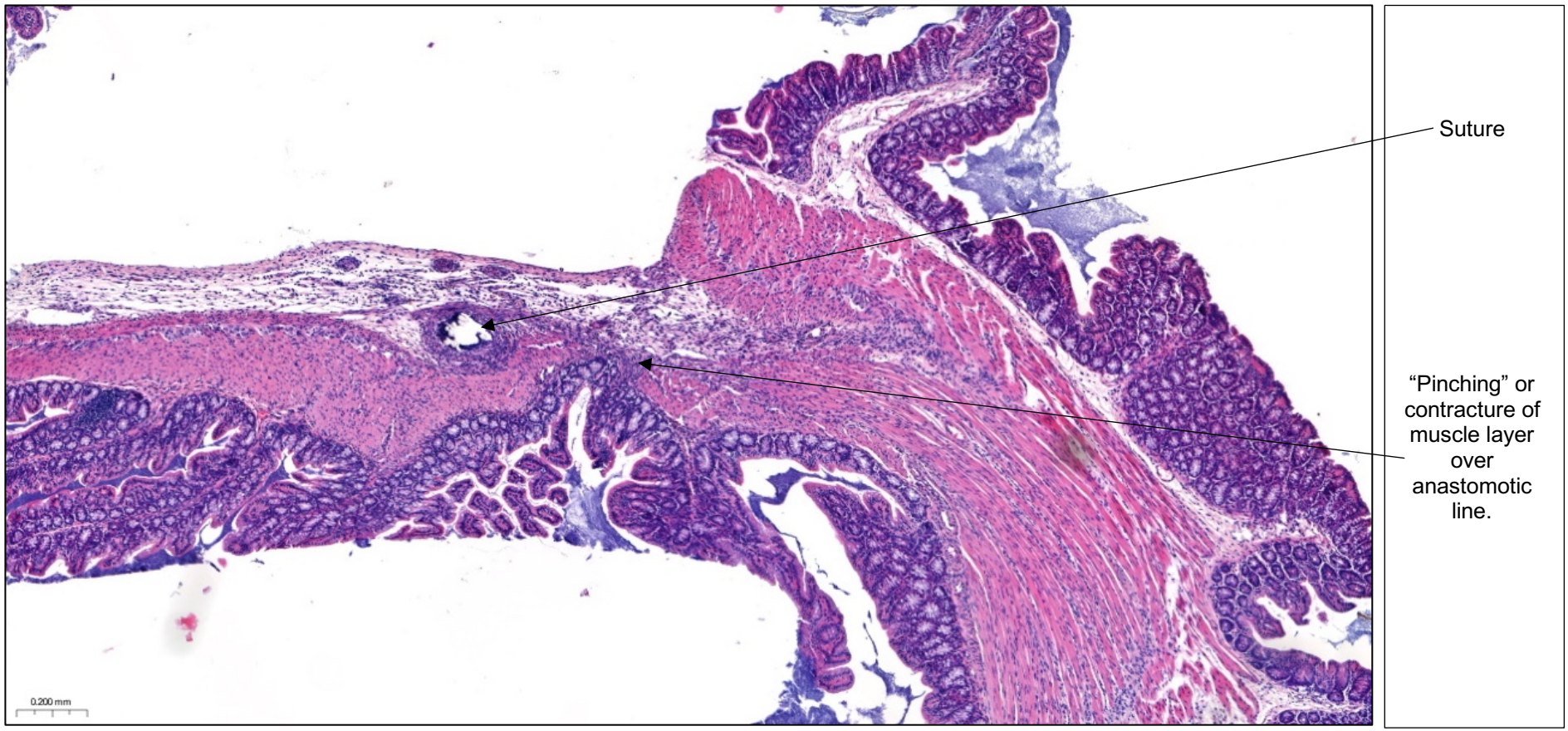


Figure 34 | Day 14 – Masson Trichrome x10. All bowel wall layers are complete intact with pinching of the serosa and sub-serosa at the anastomotic line.

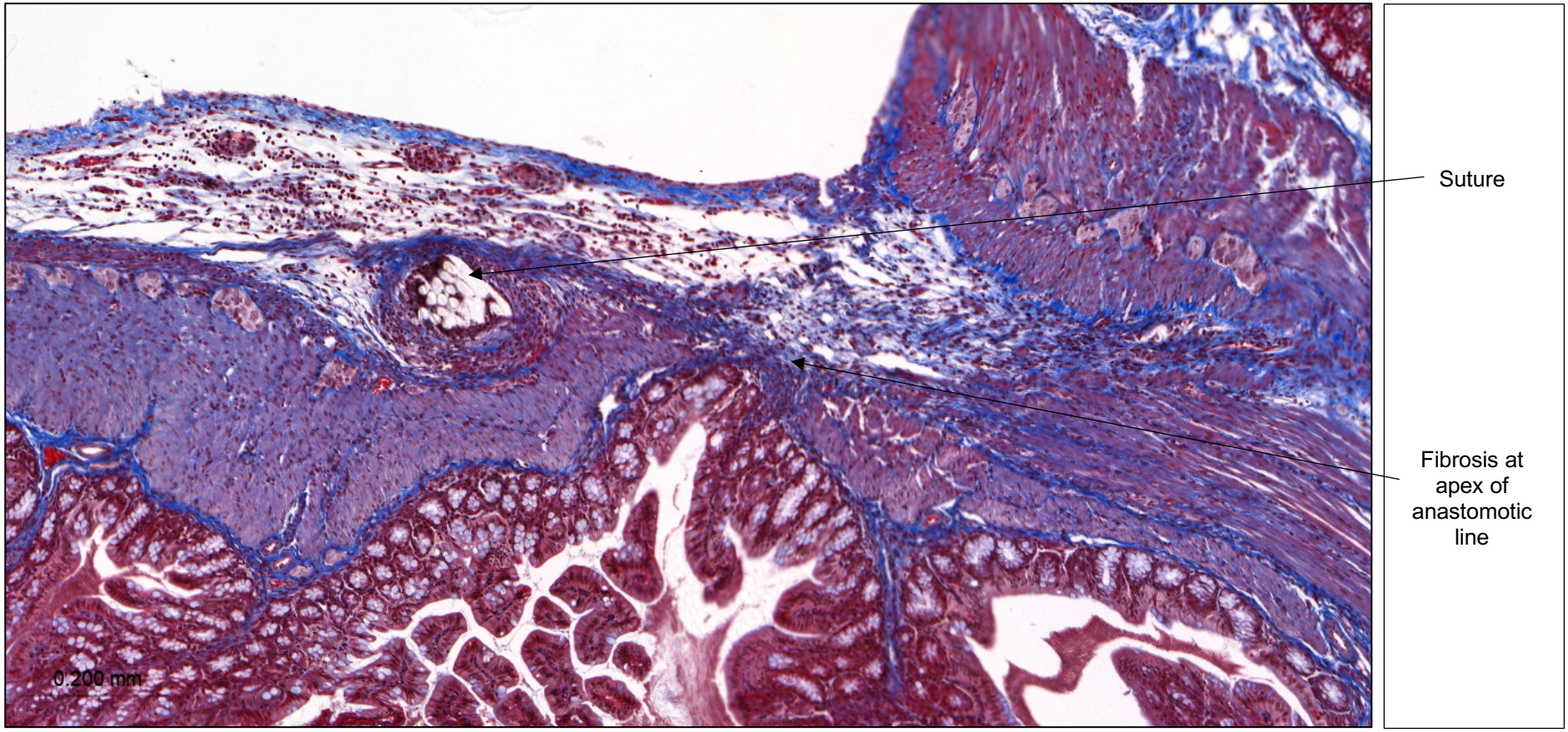
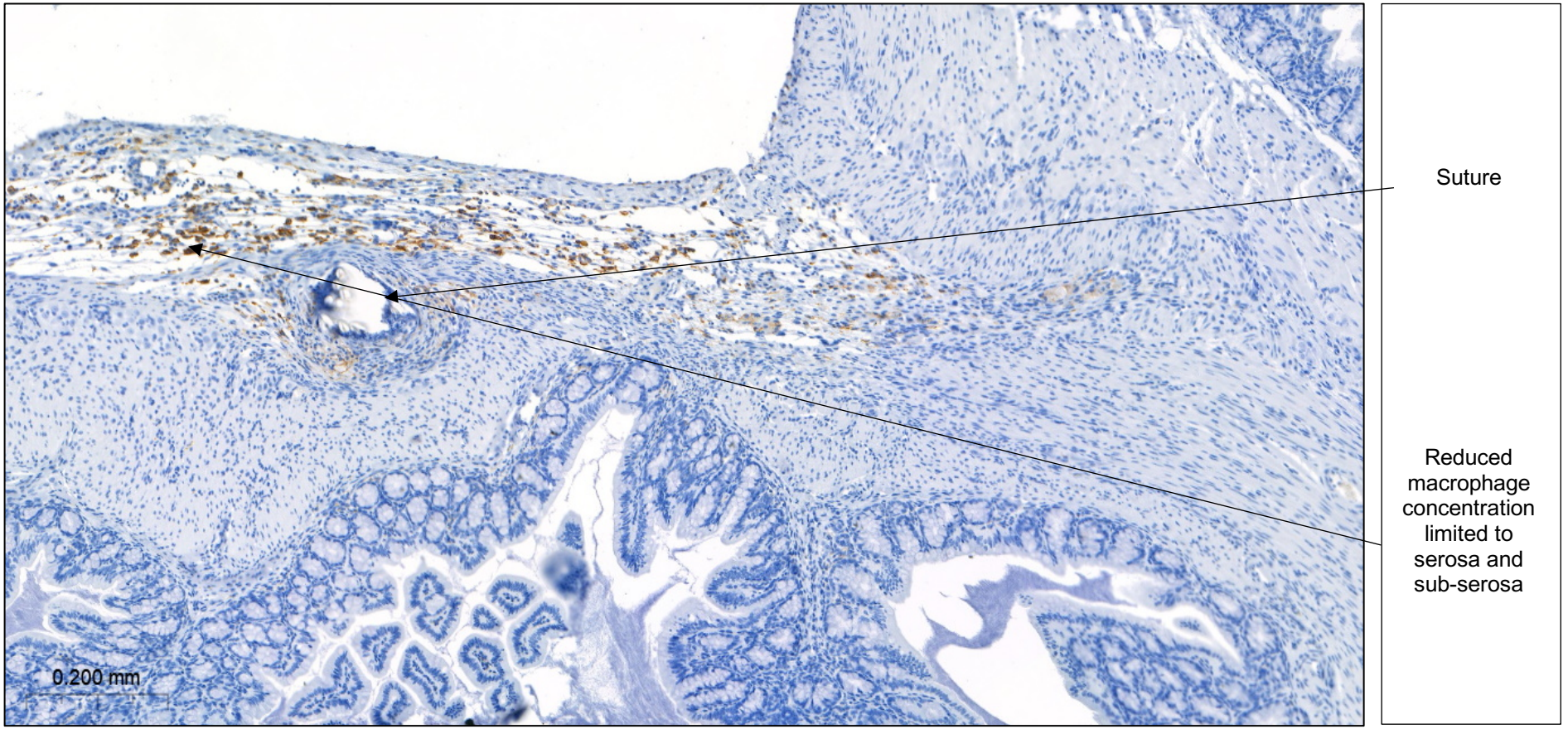


Figure 35 | Day 14 – F4/80 x10. Macrophage concentration stained brown has reduced after the spike at day



5.6 Discussion

Based on the qualitative assessment of this single mouse series murine anastomotic healing appears to broadly follow the steps as human skin healing as described in section 1.1.4.1 to restore normal structure and function to the injured tissue.

At day 1 (Figure 20) neutrophils are the predominant cell type within inflammatory infiltrate external to the anastomotic line with early fibrin-based clot evident to secure haemostasis as observed in human skin healing. (84) The submucosal sutures have resulted in apposition and external outpouching of the two ends of the anastomosis and whilst there is a breach in the layers of the colonic wall there is good opposition with no exteriorised luminal content. Mesentery is adherent to the fibrin plug at the anastomosis which would confer a protective effect.

At day 3 (Figures 21 - 23) the fibrin plug is more organised. The previously exteriorised layers of colon wall are no longer present and fibroblasts predominate with evidence of granulation tissue forming along with new villi at the edge of the mucosa where it meets the fibrin plug either side of the anastomotic line. There is an absence of smooth muscles cells in the fibrin plug however there is high expression laterally suggesting contraction around the anastomotic line; similar to that seen in human skin on day 3 or 4 of healing. (84-86)

At day 5 (Figures 24-28) there is a suggestion that the basement membrane is being deposited along the inflammatory infiltrate with marked new vessel formation and an increased abundance of smooth muscle cells. This indicates that the proliferative phase has started as we would expect in human skin. (87) The new villi formed at the cut ends of the mucosa appear to ‘curve’ towards the anastomotic line. Macrophages

are present outside the ECM close to mesenteric tissue, and have not yet started to break down the ECM.

At day 7 (Figures 29-32) the basement membrane is now intact with a more organised and continuous muscle layer and submucosal apposition. The epithelial defect has healed and there is minimal inflammatory cell infiltrate except around the suture hole. There is an abundance of macrophages within the ECM facilitating remodelling. It is possible that the peak of macrophage activity has been missed between the day 5 and day 7 samples. By day 14, the anastomosis has completely healed with muscle either side of the anastomosis remaining thickened and a 'pinching' or contraction of muscle at the anastomotic line with some early fibrosis. Macrophage activity is not limited to serosa and sub serosa.

It is predicted that following qualitative analysis, this model will provide baseline histological characteristics to then compare interventions. The model represents the 'perfect' anastomosis where it always heals without a deficit in the Halstead principles (e.g. poor tissue approximation). Future quantitative analysis will include the electronic counting of cell phenotypes and measurement of chronological changes at the anastomotic line using the non-affected side of the anastomosis as a control. 3D reconstruction will be used to described the "movement" of cell phenotypes and increase the accuracy of any conclusions. These methods are well described in the arterial wall with methodology well established at The University of Manchester (315,316)

To date there have only been eight studies of knockout models in bowel anastomoses. Most of these studies utilised an end to end anastomosis. The silenced genes include interleukin-10, the four-and-one-half LIM domain-containing protein 2 (FHL2), cyclooxygenase-2 (COX-2), annexin A1 (ANXA-1), thrombin-activatable fibrinolysis

inhibitor (TAFI), and heparin-binding epidermal growth factor (HB-EGF) gene. Following the completion of this research it would be interesting to investigate a knockout of the different collagen phenotype and of the MMP-9 family given the implications of poor anastomotic healing when this pathway fail or is altered as described in section 1.1.4.2.

5.7 Summary

In this chapter, the development of a temporal model of anastomotic healing on which to test future interventions to glean histological outcomes has been described. Through this I have demonstrated compliance to 3Rs guidelines using simulation prior to testing on animal subjects. The optimisation of the anastomotic technique, model, histology and immunohistochemistry is novel and reproducible. The qualitative synthesis in a single mouse anastomosis suggests that the wound repair process broadly mirrors that of the known human skin healing literature. The histological data acquired across the 30 mice is a rich source of information for future quantitative analysis to confirm the results presented.

6. Investigating the efficacy of mesenchymal stem cell / alginate gel composite on anastomotic healing – a large scale *in vivo* study.

This chapter described a study to evaluate the efficacy of a new technique to reduce anastomotic leak in a murine model translatable to human application. Applying omental-derived stromal vascular cell-fraction / alginate gel composite to the anastomosis resulted in a 9.1% reduction in leak rate compared to control groups. Applying the gel alone showed a similar reduction but also lowered the severity of the leak, favouring abscess formation rather than faecal peritonitis. This technique shows promise in reducing colorectal anastomotic leak rates. It is a low-cost intervention and an attractive means of modulating bowel healing and a promising application in human subjects.

6.1 Background – selecting the optimum model

The discussion surrounding international consensus of animal models for colorectal anastomotic leak and the process for selecting the optimum animal model described in section 4.1.2 also apply to this experiment and need not be repeated.

There are currently seven unique anastomotic leak models have been developed in the C57BL/6 mouse (Table 14) with varying suture types and suture frequencies. The model described by Pommergaard *et. al.* (2004) is advantageous to test an intervention with an anastomotic leak rate of ~ 40%. This is high enough to allow a demonstrable change to any intervention and keeps the sample size manageable. Pommergaard performed anastomoses in 40 mice, 20 with 5 sutures in the intervention group and 20 in the 12-suture control group. In the intervention group, 40% leaked with 4 experiencing fecal peritonitis and all four were subjected to Schedule 1 killing approximately 24 hours after the procedure. No animals died outside of the end points and Schedule 1 protocol (Table 14).

Table 14 | Current studies pertaining to colonic anastomotic leak in the mouse.

Author & Year	Anatomical Location	Suture Type	C57BL/6 Mice	Suture Number	Leak N	Leak Rate
Komen 2009 (327)	1cm Distal to Caecum	8-0 Daflon	9	12	0	0%
Komen 2009 (327)	1cm Distal to Caecum	8-0 Daflon	9	12	0	0%
Pommergaard 2014 (289)	1cm Distal to Caecum	8-0 Vicryl	24	12	6	25%
Pommergaard 2014 (289)	1cm Distal to Caecum	8-0 Vicryl	20	8	0	0%
Pantelis 2010 (328)	Ascending Colon	8-0 Vicryl	39	8	0	0%
Pantelis 2010 (328)	Ascending Colon	8-0 Vicryl	30	8	11	36.7%
Komen 2009 (327)	1cm Distal to Caecum	8-0 Daflon	9	6	1	11.1%
Komen 2009 (327)	1cm Distal to Caecum	8-0 Daflon	9	5	4	44%
Pommergaard 2014 (289)	1cm Distal to Caecum	8-0 Vicryl	24	5	16	67%
Pommergaard 2014 (289)	1cm Distal to Caecum	8-0 Vicryl	20	4	8	40%

6.2 Aim

This study aimed to evaluate the effects of a new strategy of omental-derived stromal vascular cell-fraction (O-SVCF) / alginate gel composite application on anastomotic leak using a murine model.

6.3 Methods

This is a prospective, partially blinded, randomised controlled study comparing the use of O-SVCF Gel Composite against controls in colonic anastomoses in C57BL-6 mice. All experiments were performed under Home Office Licence (PP9886008) according to the UK Animals (Scientific Procedures) Act (1986), The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) Guidance (329) and reported using International Animal Research: Reporting *In Vivo* Experiments (ARRIVE 2.0) Guidelines. (226) C57BL-6 mice were purchased from Charles River

Laboratory, United Kingdom. Mice had undergone no previous procedures or genetic modification prior to starting the study.

The control group contained two subgroups, gel alone and no treatment. This kept the number of mice to a minimum as it was not anticipated that there would be any difference in anastomotic leak between these two groups. (329) Stratification was not required given the C57BL-6 are bred for genetic similarity. A 20% minimum difference in leak rate between groups was considered a meaningful reduction**.** Assuming a 20% minimum difference between treatment groups, 128 mice (64 per group) were required to obtain 80% power at a one-sided significance level of 5%. Adult C57BL-6 mice aged between 10 and 15 weeks were included in the study.

6.3.1 Surgery

6.3.1.1 Isolation and Processing of O-SVCF

All mice in the intervention group received autologous O-SVCF harvested at the start of the laparotomy. Unless specified, all cell harvest and culture products were obtained from Thermofisher Scientific, UK. Mice were anaesthetised using isoflurane (2L/Min, Isoflurane 2%). The abdomen was shaved and hair removed with tape. The skin was prepared using 70% Ethanol / 2% chlorhexidine wipes (GAMA Healthcare, UK) and a 1.5cm incision was made from xiphisternum to mid-abdomen. The omentum was resected (Figure 35) and the caecum delivered. The omentum was washed in 5mls of HBBS 95% and Amphotericin 5% and placed in 5mls of 0.25% EDTA/Trypsin for 20 minutes at 37℃. Trypsinisation of the omental sample was neutralised with 1ml FCS (Thermofisher Scientific, UK). The sample was homogenised and passed through a 100µL cell sieve. The collected O-SVCF filtrate was resuspended in 5mls of complete DMEM (450mls DMEM containing 50mls 1% FCS, 5mls of L-glutamine, and 5mls of

Penicillin / Streptomycin) and centrifuged at 2000rpm at 37℃ for 5 minutes. Media was removed and the cell pellet was re-suspended in 1ml of complete DMEM.

6.3.1.2 Animal Model

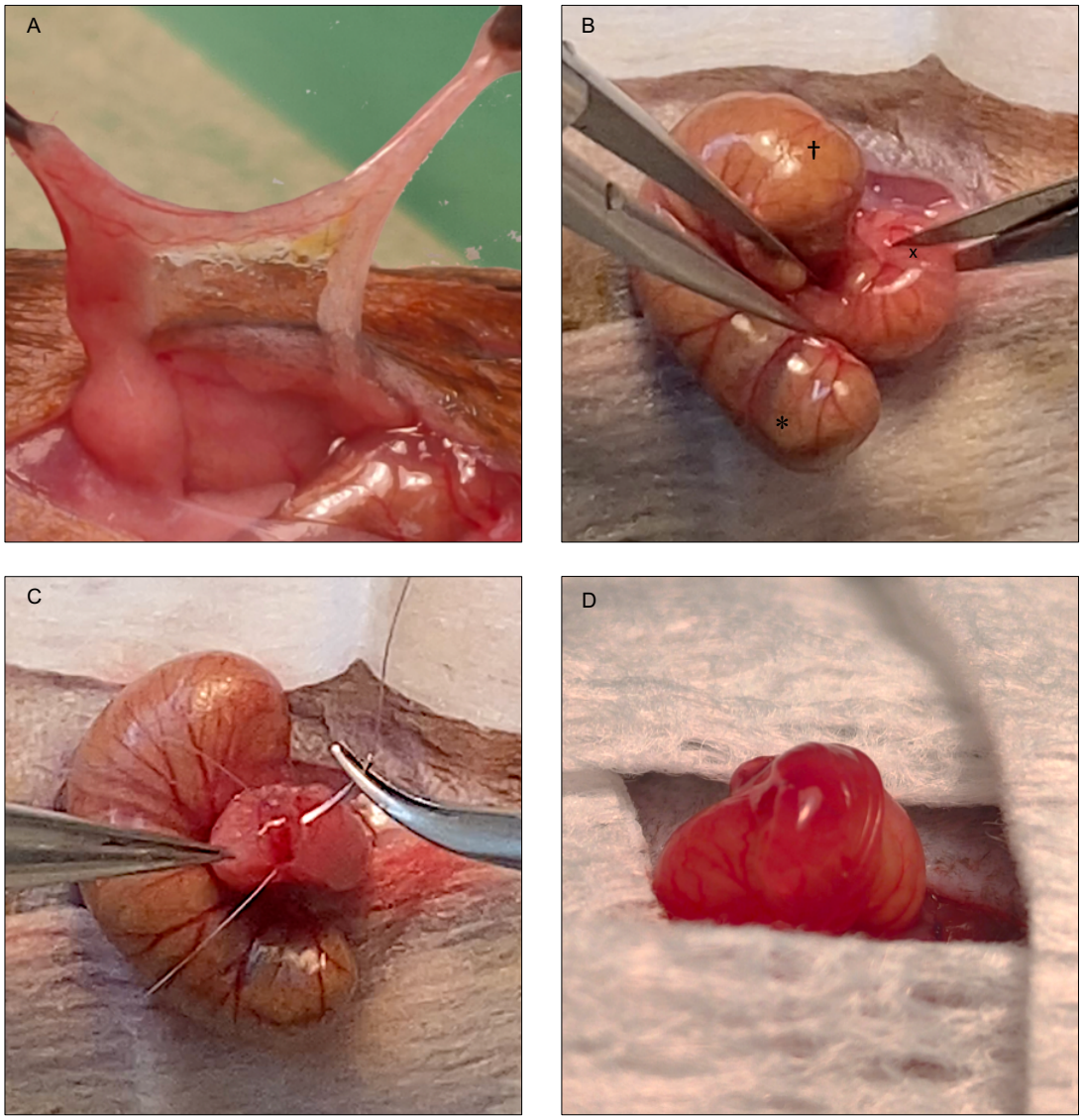
The end-end colonic anastomosis with a 40% leak rate described by Pommergaard *et. al.* (2014) using the C57BL-6 model was chosen. This is in line with current international consensus (229) which describes a mouse model with a known leak rate as acceptable for studying anastomotic leak. (229) Mice were randomised using block randomisation through SealedEnvelope™ (London, UK) at a 1:1 allocation ratio (Intervention and control and at a further 1:1 allocation for the two subgroups (Gel alone and No treatment) within the control arm. They were housed separately 24 hours before operation to allow for acclimatisation and enable monitoring of stool output.

All anastomoses were performed using 3.5x front-mounted Loupes (The Loupes Company, London, UK). The colon was divided using microscopic scissors 1cm distal to the caecum (Figure 35). Where this point fell directly onto mesenteric vessels, the safest point distal to 1cm was chosen as close to the vessel as possible to maintain perfusion. An end-to-end colonic, single layer sub-mucosal anastomosis was fashioned (Figure 35) using four equidistant 8/0 interrupted vicryl sutures (Ethicon, UK).

Following each anastomosis, a leak test was performed where gentle pressure was placed proximal and distal to the anastomosis with four drops of normal saline applied to the anastomotic line. If any air bubbles or faecal content were observed the anastomosis was taken down and refashioned. Normal saline (0.2 ml) was injected subcutaneously to balance fluid losses during surgery. Animals were placed on a warm plate and kept at 37℃ for 30 minutes before being transferred to cages.

Mice were given 83µL of Vetergesic S/C at induction and an oral Vetergesic/Nutella mix for analgesia postoperatively. (289) Cages were cleaned every other day with replacement of soft bedding and a water check. Mice were fed with electrolyte gel (Bio-Serv, London, UK) for the first 24 hours then standard mouse feed thereafter.

Figure 35 | (A) Mouse omentum was harvested taking care not to damage the pancreas medially and the spleen laterally. (B) A primary end-end colonic anastomosis with four 8/0 vicryl suture 1cm distal to the caecal pole. * appendix, † Caecal Pole, X Site of incision. (C) A 4x 8/0 vicryl suture end-end colonic anastomosis was fashioned and (D) the O-SVCF Gel composite applied.



6.3.1.3 Application of O-SVCF Gel

Cell counts were calculated using a haemocytometer following trypan blue application (Sigma-Aldrich, Dorset, UK). O-SVCF cell suspension in 10% FCS complete DMEM (100 µl) was mixed with a 0.15 ml of 3% Complete DMEM / Alginic acid sodium salt from brown algae (Sigma, UK) and placed in one arm of a y-connector with the other arm containing 250 µl of 150mM calcium chloride which was used as a rapid ionic cross-linker. (330) The gel was weighed and applied directly to the anastomosis with forceps (Figure 35). The caecum was then placed back into the abdomen and the abdominal wall closed with a continuous 8/0 vicryl suture and two veterinary clips to the skin.

6.3.1.4 Outcome Measures

Mice underwent regular checks adhering to the humane endpoints defined by a loss of 20% of their starting body weight, a mouse grimace scale of >6 (331), or a score of >3 in any parameter of the Colon Surgery Post-Operative Monitoring Score Anastomotic (Appendix 6). (332) Mice were subject to Schedule 1 killing at day 7 or earlier if they met these criteria. JB and TM performed the surgery and were blinded post-procedure and pre-autopsy during monitoring of the mice and at the point of reaching a humane endpoint prior to day 7. Blinding was achieved through single storage of mice without ear markings and randomisation of cage number hidden from observers. (All mice received a midline laparotomy).

The primary outcome was anastomotic leak at autopsy graded by two blinded independent reviewers (MK and BK) using photographs and processed anastomosis specimens:

International Consensus Anastomotic Complication Score (ACS) for macroscopic outcomes in animal research investigating bowel anastomosis classifies leaks as per Table 15 (210)

Table 15 | Anastomotic complication score and criteria (229)

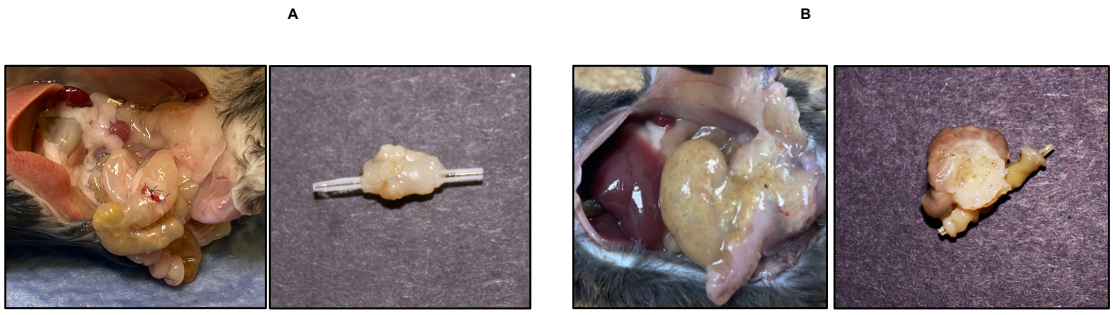
Anastomotic Complication Score (229)	
No adhesion or abnormalities	0
Adhesion to fat pad, clean anastomosis underneath	1
Adhesion to intestinal loop, abdominal wall or another organ	2
Anastomotic defect found underneath adhesion, no other abnormalities	3
Signs of possible contamination (e.g. small abscess)	4
Clear anastomotic complication; spread of pus, obstruction at anastomosis, sign of peritonitis	5
Faecal peritonitis / death due to peritonitis	6

Using this classification 'No Leak' was then defined as an Anastomotic Leak Complication Score (ACS) of 0-3 and 'Leak' as and ACS of 4-6

6.3.1.5 Macroscopic Analysis

Mice were sacrificed by Schedule 1 killing using rising carbon dioxide and cervical dislocation. The abdomen was opened using a 3-sided box incision to avoid damage to any midline adhesions. Anastomoses were photographed in a standardised fashion and adhesions scored. Anastomoses were resected and a 20G Venflon Cannula (BD, New Jersey, USA) was passed through the lumen to maintain the integrity of the tissue during fixation and processing. Photographs and processed samples were then independently reviewed by MK and BK and leak outcomes and percentage coverage of the anastomosis by adhesions documented (Figure 36).

Figure 36 | Standardised autopsy photographs and processed specimens were analysed by two blinded independent reviewers. Example photographs demonstrate an ACS score of 0 - no adhesions (A) and a score of 6, Faecal peritonitis / death due to peritonitis (B). Gel composite was applied circumferentially around the anastomosis



6.3.1.6 Statistical Analysis

All analyses were prespecified and conducted on an intention to treat basis. Observer agreement for both primary and secondary outcomes was calculated using Cohen's Kappa. (333) Statistical analysis was performed using Prism 10 (Graph-Pad software Inc., La Jolla, CA). Data were tested and decisions around which statistical test were used followed the same methodology and statistical principles as described in section 4.3.6.

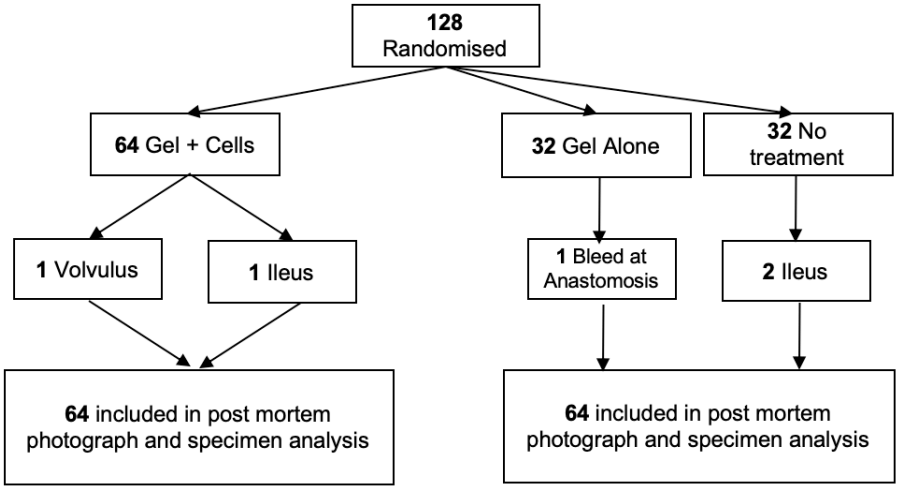
6.4 Results

6.4.1 Randomised Controlled Study

128 mice (male = 64, female = 64), mean age 13.1 weeks (SD,1.2), mean weight 24.1g (SD,4.3) were randomised (Figure 37). The three treatment groups were well balanced in terms of age, weight, sex and gel weight received. The mean anaesthetic time was 19.1 minutes longer in the intervention compared to the control group (mean [SD]

operative time, 39.3 [6.3], vs. 20.3 [9.8] respectively *p=<0.0001*). This was due to the 20-minute enzymatic trypsin degradation of the omentum. Gel production was completed during enzymatic degradation and added minimal additional time. A mean [SD] $3.3x10^3$ cells/mg [$0.3x10^3$] were applied to the anastomoses in the intervention group and mean [SD] 174.7mg [39.4] of gel across both groups.

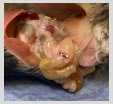
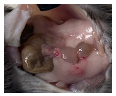
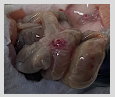
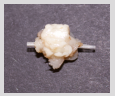
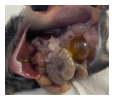
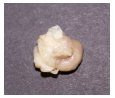
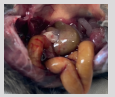
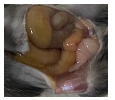
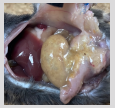
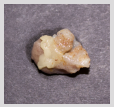
Figure 37 | Diagram of the randomisation of animal subjects



6.4.2 Anastomotic Leak

There was high agreement in inter-rater reliability at 93.8% (124/128) for subjective assessment of anastomotic integrity using the anastomotic complication score, Cohen's Kappa 0.912 (standard error 0.028, 95% confidence interval 0.893-0.986) (Table 16). (333) Four outcome decisions were settled by a 3rd reviewer (JB). None crossed between 3/4 and 5/6 ACS categories meaning inter-rater reliability for subjective assessment of no leak (score of 0-3), abscess (4-5) or faecal peritonitis (6) was 100% (128/128).

Table 16 | Inter-observer outcomes of Anastomotic Complication Score and Leak.

Anastomotic Complication Score (229)		Autopsy	Specimen	Observer 1 (n)	Observer 2 (n)	Observer Agreement (n)	Following 3rd Reviewer Decision	Leak Outcome	Observer 1 (n)	Observer 2 (n)	Observer Agreement (n)
No adhesion or abnormalities	**0**			6	7	6	6	No Leak	65	65	65
Adhesion to fat pad, clean anastomosis underneath	**1**			8	8	8	8				
Adhesion to intestinal loop, abdominal wall or another organ	**2**			48	48	48	48				
Anastomotic defect found underneath adhesion, no other abnormalities	**3**			3	2	2	3				
Signs of possible contamination (e.g. small abscess)	**4**			32	29	29	30	Leak (Abscess)	42	42	42
Clear anastomotic complication; spread of pus, obstruction at anastomosis, sign of peritonitis	**5**			12	13	12	12				
Faecal peritonitis / death due to peritonitis	**6**			21	21	19	21	Leak (Faecal Peritonitis)	21	21	21
Total	**-**	-	-	128	128	126	128	-	128	128	128

All 128 animals were included in the intention to treat analysis. Overall leak rate was 49.2% (63/128) defined by an ACS score of 4-6. There was a numerical but non-significant reduction in leak rates between the intervention group (30/64, 46.9%) and control groups (33/64, 51.5%). Table 17 presents the results from a multilevel logistic regression model and shows no significant effect of sex, age preoperative weight, anaesthetic time, surgeon experience or gel application on odds of anastomotic leak. There were two iatrogenic complications, one volvulus from misplacement of the caecum prior to closure and one bleed from the anastomosis that underwent early Schedule 1 at day 1.

Table 17 | Mouse characteristics, operative details, primary and secondary outcomes and subgroup analysis

VARIABLE	INTERVENTION	CONTROL	*P*	CONTROL (GEL)	CONTROL (NO TREATMENT)	*P*
BASELINE (N)	64	64	-	32	32	-
AGE (W), MEAN (SD)	13.1 (1.2)	13.1 (1.2)	0.88	13.0 (1.3)	13.1 (1.2)	0.75
SEX (N), (%)	64 (50.0)	64 (50.0)	-	32(25.0)	32 (25.0)	-
MOUSE WEIGHT (G), MEAN (SD)	24.1 (4.0)	24.0 (3.8)	0.81	24.4 (3.7)	23.7 (4.0)	0.44
GEL WEIGHT (MG), MEAN (SD)	174.7 (39.4)	-	-	201.0 (25.9)	-	-
ANAESTHETIC TIME (M), (SD)	39.3 (6.3)	20.2 (9.8)	**<0.0001**	19.8 (8.2)	20.5 (11.2)	0.76
MEAN SURVIVAL (D), (SD)	5.3 (2.2)	5.0 (2.3)	0.49	5.4 (2.0)	4.7 (2.6)	0.21
PRIMARY OUTCOME (LEAK)						
NO LEAK (ACS 0-3) (%)	34 (53.1)	31 (48.4)	0.72	15 (46.9)	16 (50.0)	0.99
LEAK (ACS 4-6) (%)	30 (46.9)	33 (51.5)		17 (53.1)	16 (50.0)	
SECONDARY OUTCOME (ANASTOMOTIC COMPLICATION SCORE)						
0 (N), (%)	4 (6.3)	3 (3.7)	0.20	1 (3.1)	2 (6.3)	0.19
1 (N), (%)	5 (7.8)	3 (3.7)		2 (6.3)	1 (3.1)	
2 (N), (%)	23 (35.9)	24 (37.5)		11 (34.4)	13 (40.6)	
3 (N), (%)	2 (3.1)	1 (1.6)		1 (3.1)	0 (0.0)	
4 (N), (%)	17 (26.6)	13 (20.3)		8 (25.0)	5 (15.6)	
5 (N), (%)	8 (12.5)	4 (6.3)		4 (12.5)	0 (0.0)	
6 (N), (%)	5 (7.8)	16 (25.0)		5 (15.6)	11 (34.4)	
SUB-GROUP ANALYSIS (LEAK SEVERITY)						
ACS 4-5 (ABSCESS)	25 (39.1)	17 (26.6)	0.50	12 (37.5)	5 (15.6)	**0.038**
ACS 6 (FAECAL PERITONITIS)	5 (7.8)	16 (25.0)		5 (15.6)	11 (34.4)	

There were 3 mice who were subject to Schedule 1 who were found to have ileus and no leak, one in the intervention group (day 3) and two in the no treatment group (day 3 and day 4). O-SVCF/Gel containing mean $3.3x10^3$cells/mg (SD, $0.3x10^3$) was applied in the intervention group (Table 18).

Table 18 | Rates, Relative Risk and Multi Level Logistic Regression Model of Odds of Anastomotic Leak. [A] Column variables are included in the model as fixed effects. [B] Sex [C] Age [D] Weight [E] Anaesthetic Time [F] Surgeon Experience and [G] Gel Application.

EFFECT[A]	NO. LEAKED / TOTAL NO. (%)	UNADJUSTED RELATIVE RISK (95% CI)	UNADJUSTED ODDS RATIO (95% CI)	*P* VALUE	ADJUSTED ODDS RATIO (95% CI)	*P* VALUE
TREATMENT						
INTERVENTION	30/64 (46.9)	0.91 (0.64-1.69)	0.83 (0.41 - 1.65)	0.72	1 [Reference]	0.99
CONTROL	33/64 (51.6)				1.01 (0.14 - 7.21)	
SEX[B]						
MALE	35/64 (54.7)	1.25 (0.88-1.80)	1.55 (0.78-3.16)	0.29	1 [Reference]	0.78
FEMALE	28/64 (43.8)				0.79 (0.13 - 4.64)	
AGE[C]						
< 12 WEEKS	12/25 (48.0)	0.97 (0.59 - 1.44)	0.94 (0.41 - 2.22)	0.99	1 [Reference]	0.87
≥ 12 WEEKS	51/103 (49.5)				1.09 (0.39 - 3.02)	
PRE-OP WEIGHT[D]						
< 23G	27/62 (43.5)	0.77 (0.54 - 1.09)	0.59 (0.29 - 1.17)	0.16	1 [Reference]	0.79
≥ 23G	36/66 (50.0)				1.25 (0.21 - 7.40)	
ANAESTHETIC[E]						
< 30 MINS	33/65 (50.8)	1.07 (0.75 - 1.53	1.13 (0.57 - 2.27)	0.73	1 [Reference]	0.72
≥ 30 MINS	30/63 (47.6)				0.86 (0.38 - 1.95)	
SURGEON EXPERIENCE[F]						
1ST 64	32/64 (50.0)	1.03 (0.72 - 1.42)	1.07 (0.53 - 2.12)	0.99	1 [Reference]	0.89
2ND 64	34/64 (53.1)				0.95 (0.43 - 2.06)	
GEL[G]						
ANY GEL	47/96 (49.0)	1.10 (0.75 - 1.73)	1.20 (0.56 - 2.60)	0.69	1 [Reference]	0.96
NO GEL	16/32 (50.0)				0.97 (0.38 - 2.49)	

6.4.3 Severity of Anastomotic Leak

Of the mice that leaked (n=63), the number of mice with faecal peritonitis was significantly higher in the control group 16/33 (47.4%) than in the intervention group 5/30 (16.5%) (RR 2.50 [95%CI:1.24-5.78] (*P*=0.009). Within the control group 5/33 (15.2%) that received gel alone and 11/33 (33.3%) that received no treatment developed feculent peritonitis rather than abscess (Relative Risk 0.44 [95%CI: 0.19-0.90]; *P*=0.038).

6.4.4 Adhesion Formation

Gel was visible at day 7 in 100% of cases (n=96) where it was applied. In terms of adhesions, 110/128 (85.9%) mice had two or more organs adherent to the anastomosis site. The most adherent organ to the site of the anastomosis was the small bowel (n=42) (Table 19). Mean subjective anastomosis circumferential coverage with adhesions was 60.7% (SD, 32.0). There was significantly less mean percentage circumference coverage of adhesions in those that leaked versus those that did not (Table 20). There was a significant correlation between mean percentage circumference coverage of adhesions and both mean day survival and ACS score (Table 20).

Table 19 | Frequency of adherent organs to site of anastomosis.

ADHERENT ORGAN	MICE (N)
SMALL BOWEL	42
CAECUM	38
APPENDIX	29
LEFT INGUINAL FAT PAD	26
LIVER	21
SPLENIC ADIPOSE TISSUE	20
ABDOMINAL WALL	18
RIGHT INGUINAL FAT PAD	17
ASCENDING COLON	9
SPLEEN	6
SMALL BOWEL MESENTRY	5
STOMACH	5
BLADDER	4
PANCREAS	3

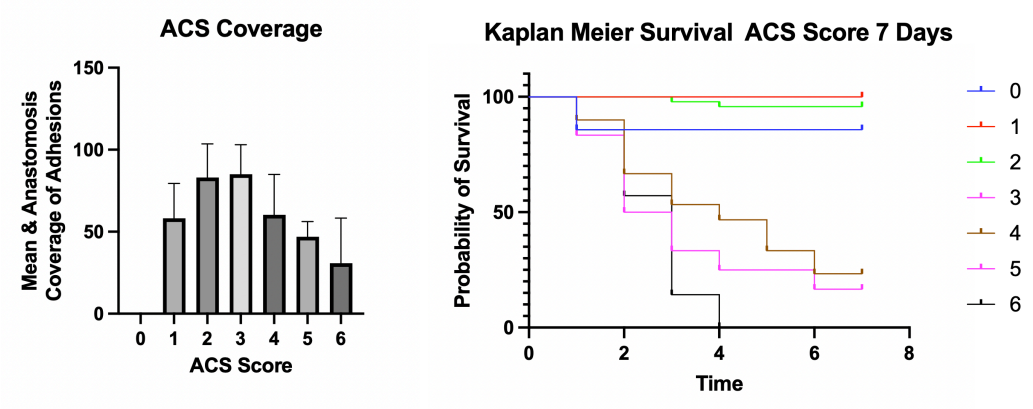
Table 20 | A comparison of mean percentage coverage and mean day survival across primary and secondary outcomes.

Treatment	Mean % Coverage (SD)	Mean Day Survival (SD)
Intervention	61.5 (32.8)	5.2 (2.2)
Control	60.9 (32.2)	5.0 (2.3)
p	0.88	0.46
Control Gel	63.9 (29.1)	5.4 (2.0)
Control No Treatment	57.8 (35.3)	4.7(2.6)
p	0.76	0.25
Primary Outcome		
No Leak (%)	71.1 (47.9)	6.8 (1.0)
Leak (%)	32.4 (26.8)	3.5 (2.0)
p	**<0.0001**	**<0.0001**
Subgroup Analysis		
ACS		
0 (n), (%)	0.0 (0)	6.1 (2.3)
1 (n), (%)	58.1 (21.4)	7.0 (0)
2 (n), (%)	83.0 (20.6)	6.6 (0.7)
3 (n), (%)	85.0 (18.0)	7.0 (0)
4 (n), (%)	60.6 (24.7)	4.2 (2.2)
5 (n), (%)	44.2 (13.2)	3.3 (2.2)
6 (n), (%)	30.8 (27.4)	2.6 (0.9)
p	**<0.0001**	**<0.0001**

6.4.5 Adhesion Coverage Score, % Circumferential Coverage and Survival

ACS score and % circumferential coverage correlated significantly with mean survival. There was significantly less mean percentage circumference coverage of adhesions in those that leaked versus those that did not (Figure 38).

Figure 38 | ACS coverage and Kaplan Meir Survival Curves for ACS Score



Log-Rank Test *P* = < *0.0001*

6.5 Discussion

This is the largest *in vivo* of anastomotic leak in an animal and serves as a standardised model on which to test future interventions. There is insufficient evidence to conclude that O-SVCF Gel composite reduces the risk of anastomotic leak, however the significant findings of leak severity reduction from the application of gel in this model is encouraging. The intervention and control groups were well balanced in terms of characteristics and in subjective outcome measures. There was good inter-observer reliability.

A clear difference in my methodology with that previously reported in the literature is the implantation of autologous SVCF without previous culture. In a recent systematic review investigating application of SVCF to bowel anastomoses, between 1-2x10^6 SVCF cells were cultured to confluence. This is higher than the number applied in this study (5.7x10^5 cells) and may have affected the efficacy of the intervention through production of cytokines and growth factors conducive to wound healing. However, if this were to be translated to humans, the number of cells needed would require culture of SVCF from alternative sources, such as subcutaneous fat or bone marrow, with additional morbidity.

(199) To that end it is assumed that the SVCF cells were present, healthy and survived the process of collaging with alginate and following implantation using the peritoneal fluid as a source of nutrients to proliferate after the cell media constituents had been utilised. The use of cell media within human abdominal cavities is not currently documented within the literature however there are synthetic peritoneal fluids (those used for peritoneal dialysis) on the market which could be a viable alternative. The methodology is also reliant on both the gel and cells surviving the mechanical stress of being implanted within an abdominal cavity (with sheer stress of bowel peristalsis), likely acidic Ph due to even minor faecal leakage from the join and a mobile mouse with effective analgesia day one post operation.

The studies included in the aforementioned systematic review also assume that mesenchymal stem cells become the predominant cell type when in fact this differentiation can be controlled during cell culture. (334) This lack of molecular and phenotypic characterisation is a weakness of the study and all studies within the literature. This is because the cells may undergo differentiation in an unpredictable way within the abdominal cavity and the predominant cell type may not be MSCs. As such another cell may be responsible for any positive (or negative effect). Therefore application of autologous SVCF without culture may be more representative of a translatable solution for anastomotic leak.

Current human research into anastomotic leak is hindered by study heterogeneity and a lack of standardisation of anastomotic technique. This same problem is reflected in the animal literature with many models attempting to mimic theorised causes of leak (e.g. ischaemia at the anastomotic line). The model presented in this study was selected to try and remove these confounders and standardise the anastomosis such that only suture number and tissue apposition affected leak rate. This means that the only Halsted principle being purposefully deficient is tissue opposition. This is a sensible approach as

it is arguably the one which is easiest to standardise and if it goes wrong is probably the most influenced by human performance. This model does not however capture deficiencies in any of the other Halsted principles (e.g. blood supply, tension) and so it could be argued that any conclusions drawn could only be applied to anastomoses where tissue apposition is deficient (as opposed to other factors).

6.6 Summary

The application of gel conferred a reduction in leak rate severity. The severity classification of anastomotic leak has previously been poorly defined however, the grading defined by Rahbari *et. al.* in 2010 is the most widely used but limits grading based on intervention required. (12) The phenotype of leak has seldom been studied in a randomised controlled setting as a primary outcome likely due to a lack of consensus surrounding the possible progression of severity CAL in humans described in the animal anastomotic complication score used in this study (an increasing score is observed from adhesion - either causative or a result of leak - to abscess and finally to faecal peritonitis). (229) Finally, whilst the application of O-VCF did not confer any statistically significant advantage in terms of leak rate the alginate gel provides an exciting medium of which to test future interventions at the anastomotic line.

7. Discussion

7.1 Aims and objectives of this work

The main aim of this PhD was to investigate whether the application of omental derived regenerative cells to a colonic anastomosis improves healing and reduces anastomotic leak in an animal model. Additional specific objectives were:

I. To conduct a systematic review of the literature to identify how omental derived regenerative cells, as a source of mesenchymal stem cells, have been used to augment anastomotic healing (Chapter 3).
II. To investigate the characteristics of omental stromal vascular cell fraction and explore a suitable vehicle for their delivery to an anastomosis (Chapter 4).
III. To develop a reliable and reproducible animal model to study the biological mechanisms of anastomotic healing (Chapter 5).
IV. To evaluate the efficacy of an omental regenerative cell-gel implant to improve anastomotic healing and prevent leak in an animal model (Chapter 6).

The completed body of work provides a robust and comprehensive evaluation of: i) the current literature on the use of omental derived regenerative cells to prevent anastomotic leak, ii) a novel animal model to allow for future investigation of anastomotic healing, iii) characterisation of murine omental regenerative cells, and iv) a randomised controlled study demonstrating a possible benefit to using gel at the anastomosis which might be progressed through human studies.

7.2 Conclusions

7.2.1 Chapter 3: Systematic Review

The systematic review showed that application of MSCs might confer a benefit in anastomotic healing when judged by experimental and histological outcomes. There was consistency in the outcome measures reported but with wide variance in how they were measured. There was generally low compliance to ARRIVE guidelines but good compliance of lower GI AL models to international consensus criteria. If animal models of AL are to serve as valuable tools to understand the pathophysiology of anastomotic leak and to test novel pre-clinical interventions, there is a need to standardise them. In particular the AL leak rate should be known and reproducible. A method for correctly defining mesenchymal stem cell populations across animal species is required. Surrogate outcome measures of wound healing should be reported using validated methods of assessment.

7.2.2 Chapter 4: Characterisation of stromal vascular cell fraction and formulation of delivery vehicle

It has been demonstrated that O-SVCF can be reliably harvested from mouse omentum and incorporated within a rapidly setting alginate gel applied to the murine bowel wall. There were no significant differences between sex or between two different mouse strains.

7.2.3 Chapter 5: Temporal model of anastomotic healing

The qualitative analysis of the mouse samples demonstrated that the broad principles of wound healing observed in human skin are also observed in C57BL/6 mice. Whilst the principles of inflammation and remodelling remain consistent with skin healing in

this qualitative analysis the mechanics of bowel healing may be more complex. This will be investigated further using the rich tissue bank that has been generated,

7.2.4 Chapter 6: The M-omentum Study

O-SVCF gel produced a 9.1% reduction in leak rate, but this did not reach statistical significance. The application of gel to an anastomosis alters the severity of anastomotic leak, favouring abscess formation rather than faecal peritonitis. Application of alginate gel to an anastomosis is a low-cost intervention and an attractive means of modulating bowel healing, providing a vehicle for novel therapeutics.

7.3 Limitations

7.3.1 Chapter 3: Systematic Review

The limitations of this study include the validity of animal model systematic reviews as a predictive modality for human response to prophylactic human interventions. (335) However those who support this modality recommend conclusions be based around methodological quality of experiments, which this review satisfies. This review utilised two simple, novel non-validated scoring systems to quantify compliance to current best practice and use of these scoring systems requires further validation. There was wide study heterogeneity, even amongst the rat colon models, which made up seven of the 12 studies which meant quantitate comparison outcomes measure was not feasible.

7.3.2 Chapter 4: Characterisation of stromal vascular cell fraction and formulation of delivery vehicle

There remains much debate within the literature around the correct markers for murine mesenchymal stem cells. The panel design represents the current literature and understanding of what may define a mesenchymal stem cell population. The implications of this panel not being correct is that some cell types may be miss-classified and the reported cell populations incorrect.

It has been theorised and previously demonstrated in a rat model that omentum becomes “activated” and its cellular composition and morphology changes (expansion of milky spots, increase expression of growth factors and vasculogenesis) in response to an abdominal insult. (336) This study utilised SVCF from ‘non-activated’ omentum i.e. omentum which had not been exposed to a foreign body. The cell profile may be different in the presence of inflammation.

7.3.3 Chapter 5: Temporal model of anastomotic healing

Microscopic histological analysis of bowel healing requires standardised and reproducible anastomoses. Whilst every effort was made to achieve this through simulation prior to conducting the study there was some variance around the amount of tissue incorporated within the anastomosis as observed during qualitative review of the samples. This would represent a real-world scenario of hand sewn anastomoses in humans. Less variance might be seen in stapled anastomoses.

7.3.4 Chapter 6: The M-omentum Study

This study has several limitations. The non-statistically significant reduction in leak rate between the intervention and control groups limits the ability to provide conclusive evidence about the primary research question – whether O-SVCF Gel composite can reduce anastomotic leak rate. But a 9% reduction is leak rate is encouraging and suggests that the study was underpowered. This is possibly due to the overall leak rate being approximately 10% higher than the 40% assumed in the sample size calculation and reported in the original Pommergaard study. (289) The one-sided significance design utilised to keep animal numbers to minimum in line with the 3Rs is also likely to have contributed. The number of anastomoses performed in this study was three times than in the original model. Therefore, the leak rate demonstrated may be closer to the true leak rate of the model. This was mitigated through randomisation and standardisation of anastomotic technique in all groups. Whilst there was no difference observed between anaesthetic times (+/- 30 minutes) those animals undergoing OSVFC application received around 20 minutes longer anaesthetic which could have been controlled for by adding this time to animals not receiving OSVFC. However, this was deemed unethical by the Animals Review Committee and was not included in the study protocol.

The gel remained present at the anastomotic site for up to 7 days. It was visible during specimen review and could have influenced the subjective assessment of leak and Adhesion Complication Score. However, as critically analysed in Chapter 6 the assumption that the SVCF cells were present, healthy and survived the process of collaging with alginate and following implantation and both the gel and cells surviving the mechanical stress of being implanted within an abdominal cavity must be considered here when drawing any conclusions. Previous studies have investigated other outcomes

such as histological analysis and burst pressure. It is the authors opinion that these outcomes have limited translatable significance. Meaningful histological outcomes are unlikely in a model that is designed to leak. Investigating histological outcomes may be more beneficial in a temporal model of anastomotic healing that does not leak. (1)

This study also utilised SVCF from 'non-activated' omentum i.e. omentum which has not been exposed to a foreign body. Whilst 'activating' the omentum may improve its wound healing potential it would add a second procedure and be impractical in the human scenario. There is also the possibility that stromal vascular cell fraction morphology becomes mesenchymal stem cell predominant following implantation. Finally, cell counts observed in this study were a factor of 10 less than applied in current literature as they were applied within the same procedure which did not allow for incubation and culture to confluence.

7.4 Meaning of this work and implications for future research

7.4.1 Adoption of anastomotic leak models

This body of work supports the use of the two murine models for research into bowel healing and anastomotic leak. Histological changes at the anastomotic line are useful in a temporal model of bowel healing, whilst the anastomotic leak model is useful for evaluating possible therapeutic interventions. The dissemination of these works through peer reviewed publications and conference presentations will allow researchers to better investigate the normal healing process in the colon and test pre-clinical interventions.

7.4.2 Characterisation of Stromal Vascular Fraction

Whilst a larger sample size will be required to validate the findings, the characterisation experiments presented suggest that both C57BL/6 and BALB/C mice can be used to harvest stromal vascular cell fraction without the requirement to consider the sex of animals. The flow cytometry panel presented will contribute to the limited literature on markers of mesenchymal stem cells to better inform any future consensus, which is currently lacking.

7.4.3 Translation into Human Trials

The work presented adds to first-in-human clinical trials that are ongoing and investigating alginate gel delivery at the anastomotic line. It will be interesting to see if the results of alginate gel in human anastomoses replicate my findings in a mouse model. Whether the addition of O-SVCF to alginate gel confers an additional benefit in humans needs to be explored.

7.5 Summary

In conclusion, the work in this thesis provides a compelling case for the use of alginate gel to reduce the severity of anastomotic leaks or as a future transport medium for other interventions. It also provides two robust animal models, one that does not leak for the investigation of histological outcomes in bowel healing, and one that has a reliable leak rate of around 50% for investigating therapeutic interventions. This work builds on the evidence base in the literature about the use of mesenchymal stem cells in AL and provides confirmation of a novel source from the greater omentum. This evidence can now be used to inform future studies on local interventions to prevent anastomotic leak.

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9. Appendices

9.1 Appendix 1 | Defining a Mesenchymal Stem Cell Population

The following criteria as defined by Dominici *et. al.* were used to assess correct definition of a human mesenchymal stem cell population (202):

1. Plastic Adherent in Standard Culture Conditions
2. Expression of CD105, CD73 and CD90
3. Lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-D.
4. Differentiate into osteoblasts, adipocytes and chondroblasts.

9.2 Appendix 2 | Raw Scores for compliance to ARRIVE 2.0 Guidelines Checklist (227)

Paper	Authors	Reviewer 1 (/38)	Reviewer 2 (/38)	Median	Median (%)
1	*Xue et. al. (China)*	16	17	16.5	43.4
2	*Komiyama et. al. (Japan)*	19	19	19	50.0
3	*Zhang et. al. (USA)*	14	16	15	39.5
4	*Hara et. al. (Japan)*	18	20	19	50.0
5	*Maruya et. al. (Japan)*	17	18	17.5	46.1
6	*Pascual et. al. (Spain)*	17	17	17	44.7
7	*Adas et. al. (Turkey)*	23	24	23.5	61.8
8	*Yoo et. al. (Korea)*	24	22	23	60.5
9	*Adas et. al. (Turkey)*	24	25	24.5	64.5
10	*Van De Putte et. al. (Belgium)*	23	23	23	60.5
11	*Sukho et. al. (Netherlands)*	26	27	26.5	69.7
12	*Alvarenga et. al. (Brazil)*	24	25	24.5	64.5

9.3 Appendix 3 | Raw Scores for compliance of Lower Gastrointestinal Models to International Consensus (210)

Paper	Authors	Reviewer 1 (/6)	Reviewer 2 (/6)	Median
1	*Xue et. al. (China)*	n/a	n/a	n/a
2	*Komiyama et. al. (Japan)*	n/a	n/a	n/a
3	*Zhang et. al. (USA)*	n/a	n/a	n/a
4	*Hara et. al. (Japan)*	n/a	n/a	n/a
5	*Maruya et. al. (Japan)*	n/a	n/a	n/a
6	*Pascual et. al. (Spain)*	4/6	4/6	4/6
7	*Adas et. al. (Turkey)*	6/6	6/6	6/6
8	*Yoo et. al. (Korea)*	6/6	6/6	6/6
9	*Adas et. al. (Turkey)*	5/6	5/6	5/6
10	*Van De Putte et. al. (Belgium)*	5/6	5/6	5/6
11	*Sukho et. al. (Netherlands)*	5/6	5/6	5/6
12	*Alvarenga et. al. (Brazil)*	5/6	5/6	5/6

9.4 Appendix 4 – Protocol for the Harvest and Isolation of ODRCs from Mouse Omentum

Preparation:

1) 10% Cell Media at room temperature □
    - DMEM (440mls)
    - FCS (50mls)
    - L-glutamine (5mls)
    - Penicillin/Streptomycin (5mls)
2) HBBS with 1% Amphotericin B and Pen Strep 1%. □
3) 50ml Plastic Centrifuge tubes □
4) Sterile dissecting forceps and Scissors (x2) □
5) Animal Shaver / Razors / tape removal □
6) Sterile gloves and drape □
7) 70% Ethanol & Chlorhexidine □
8) Contect Prochlor, stabilised hypochlorous acid (Contect, vannes, France Cat no. FBT102PC) □
9) Cork Board and Needles □
10) Disposable scalpel □
11) Sterile Gauze □
12) Trypsin EDTA 0.25%. 10mls per 50 ml falcon tube / sample, place in incubator □
13) Waste Pot □
14) Sterile 1ml Pipette Tips (Box) + x5 separately sterilised □
15) Turn on Shaking Incubator at 37℃ □
16) Trypan Blue and Haemocytometer / counting slides □
17) 1 extra falcon of FCS to neutralisation □
18) PBS if diluting trypsin □

Omental Harvest

1) Prepare 50ml Plastic Centrifuge conical base sterile pots with 20mls of complete DMEM mesothelial at room temperature in sterile pots. (1 for each sample collected)
2) Take dissecting tools, ethanol spray bottle and falcon tube aliquots of DMEM to Animal House
3) Euthanize mice under schedule 1
4) Shave hair from xiphoid to pubic area using clippers +/- razor
5) Place mouse into Class II hood and wipe hair out with 70% ethanol-soaked gauzes / sticky Tape
6) Clean surgical area with Contec
7) Don sterile gloves
8) Prepare sterile field using drapes
9) Make an incision from Xiphoid process to pre-pubic area using scalpel and 1st set of instruments.
10) Pin back abdominal wall using needle and cork board
11) Change to second set of instruments and change sterile gloves.
12) Dissect mouse omentum and place in warmed media – sterile pots.
13) Transport tissue back to cell culture lab and place falcons into class II hood.
14) Poor the media off with carefully into waste using a sterile pipette tip to secure tissue.
15) Wash with 15ml of warmed 1% Pen/Strep and 5mls of non-diluted Ampho-B HBSS at least two times.

Enzymatic degradation

16) Incubate each omental sample with 10mls Non-diluted 0.25% EDTA Trypsin in the shaking incubator at 37℃, 270rpm for 20min.
17) Take tissue back to the class II cabinet and neutralise each sterile pot with 1ml FCS.
18) Prepare the same number of 50ml falcon tubes with a sterile cell sieve (100µm) placed at top of tube.
19) Whilst using the bottom end of a sterile pipette tip to squeeze the tissue, wash tissue and let cells go down into the falcon tube with 20mls of complete media.
20) Pellet cells at 2000 rpm (440RCF) @ 40 degrees C. Acc 9 Dec 9 for 5min.
21) Remove media re-suspend cells into one 50ml Falcon tube using 1ml of complete DMEM. This will five you cell count / ml when counting.

Cell Count

22) Take 20µL of cell solution and add to 20µL of Trypan blue to a 0.5ml Eppendorf and mix x10.
23) Take up 20µL of this mixture and add 10µL to the haemocytometer / measurement slide.
24) Perform cell count using cell counter

Making Up 6 Well Plates

25) Calculation: (aim for 100,000 cells /cm^2 1xwell in 6 well plate = 9.62cm^2. Therefore aim for 1mls of cell suspension at concentration of $5x10^5$/ml)
26) Photograph Cells, complete a full media change at 72 hours and then every 48 hours until 70% confluent (Day 10-14)

9.5 Appendix 5 – Immunohistochemistry Protocol

1. Dewax sections
2. Rinse in running water for 5 minutes
3. Antigen retrieval with citric acid buffer for 10min in microwave
   [Citric acid=2.1g in 950ml dH_2O, add 13ml 2M NaOH, pH to 6 with NaOH.
   (can pH between 6-6.5, but no lower than 6) – make up to 1lt]
4. Cool for 20 minutes at Room Temp
5. Rinse in running water for 5 minutes
6. Hydrogen peroxide block for 10 mins (200ml Methanol in 2ml H_2O_2)
7. Rinse in running water for 5 minutes
8. Rinse in TBS for 5 minutes
9. Pre-block in antibody diluent (1 min)
10. Add 100µl 1° Antibody for 1 hour (Room Temp)
11. Rinse 2X in 1% TBST for 5 minutes
12. Rinse in TBS for 5 minutes
13. Add 100µl 2° Antibody incubate for 30 mins (Room Temp)
14. Rinse 2X in 1% TBST for 5 minutes
15. Rinse in TBS for 5 minutes
16. Apply DAB substrate to cover sections, incubate for 10 mins
17. Rinse in running water for 5 minutes
18. Counterstain with haematoxylin and mount using DePex

TBS	60ml 2.5M NaCl 20ml 1M Tris HCl pH 7.4 Make up to 1L
TBST	as above Add 1.25ml 10%v/v Tween-20

10mM Citric acid buffer, pH 6.0

Add 2.1g citric acid monohydrate to
800ml of distilled water. Mix well.
Add 13ml of 2M NaOH solution.
pH 6.0-6.5 (not less than pH 6).
Make up to a final 1L volume

OR

10mM Tris-EDTA pH 9.0

Add 1.21g Tris-Base to 800ml of distilled water
Mix well
Add 0.37g EDTA
Adjust pH to 9.0
Make up final volume to 1L

9.6 Appendix 6 – Mouse Outcome Monitoring Charts

Mouse Wellness Score (329)

Date	Weight (g)	Orbital Tightening (0-2)	Nose Bulge (0-2)	Cheek Bulge (0-2)	Ear Position (0-2)	Whisker Change (0-2)	Score (0-10)	Wound
80% weight at Sx:		**Date before Sx:**			**Weight g**		**Cage No.**	
Sx								

Colon Surgery Post-Operative Monitoring Score (332)

Day & Time		Date	Time	Weight	Appearance	Behaviour	Sepsis	Int. Func.
Surgery	**0**							
	2 hrs							
	4 hrs							
	6 hrs							
	8hrs							
	16hrs							
Day 1	AM							
	PM							
	Late							
Day 2	AM							
	PM							
	Late							
Day 3	AM							
	PM							
Day 4								
Day 5								
Day 6								
Day 7								

Colon Surgery Post-Operative Monitoring Score – Key (332)

Score	BODY WEIGHT	
0	Normal	<5%
1	Monitor	5-10%
2	Monitor	10-15%
3	Cull	≥20%

Score	APPEARANCE
0	Glossy coat, bright open eyes
1	Dull coat, squinting or occasionally closed eyes
2	Ungroomed coat, piloerection, intermittent hunched posture, persistently partially closed eyes
3	Soiled coat, piloerection, hunched appearance, continuously closed eyes/discharge, cold at touch, pale

Score	BEHAVIOUR
0	Alert and interested in the environment
1	Alert occasionally interested in the environment
2	Less mobile, little interest in the environment
3	Immobile, unresponsive.

Score	SIGNS OF SEPSIS
0	Normal temperature, normal breathing pattern, normal colour
1	Warm at touch, panting
2	Cold at touch, laboured breathing
3	Cold at touch, abdominal breathing (gasping)

Score	INTESTINAL FUNCTION & FAECAL OUTPUT
0	>3 faecal pellets in 24 hours, no abdominal distention
1	<3 faecal pellets in 12 hours, palpable moderately distended abdomen
2	<2 faecal pellets in 24 hours, palpable moderately distended abdomen
3	0-2 faecal pellets in 48 hours, visibly distended abdomen palpable very distended abdomen

Score	OTHER OBSERVATIONS
4	Diarrhoea >48 hours
4	Bleeding from mouth, nose, or anus
4	Pale mucous membranes
4	Paralysis, ataxia, convulsions
4	Vocalization
4	Large/ ulcerated solid masses, untreatable skin wounds/ infection
4	Signs of pain (MGS score) not alleviated by pain relief for 12-24 hours post-surgery

Score	Action
0	Continue baseline monitoring schedule
1	Increase monitoring (minimum twice a day) provide additional support (mashed food, medication.)
2	Increase monitoring (minimum 4 times per day), provide additional support (mashed food, medication, fluids, additional nesting), cull if no improvement in 24 hours
3	Critical animal, increase monitoring (minimum every 4 hours), provide additional support (mashed food, medication, fluids, additional nesting), cull if no improvement at the end of the day
4	Euthanasia