Early detection of anastomotic leak following elective colectomy

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ABSTRACT

Anastomotic leakage is the most feared complication following colonic surgery. Despite recent improvements in perioperative care and operative technique, anastomotic leakage remains a major clinical complication with a significant mortality rate and significant short and long-term morbidity.

Chapter 1 introduces this topic with a discussion on colonic anastomosis, the pathophysiology of healing, and the role of inflammatory biomarkers in this process. The theory of inflammatory imbalance following colorectal surgery is introduced and outlines the aim of this thesis – to define a biomarker profile for early detection of anastomotic leakage.

A review of the normal inflammatory response in the clinical context *specific* to colonic surgery is explored in Chapter Two. Chapter Three then explores the difficulty surrounding the early diagnosis of anastomotic leakage, followed by a literature review in Chapter Four which discusses the utility of inflammatory biomarkers in the early detection of anastomotic leakage.

Findings from Chapters Two, Three and Four are incorporated in the development of the biomarkers and anastomotic leakage longitudinal (BALL) study. This is a prospective study with the methodology outlined in Chapter Five, and results presented in Chapter Six and Chapter Seven. This prospective study shows that inflammatory biomarkers are useful in the diagnosis of anastomotic leakage. The individual findings from each chapter are then discussed in a unified manner in Chapter 8, followed by a conclusion in Chapter 9.

Dedication

It takes a village to raise a child

I dedicate this thesis to Alavine Su'a, my mother, for her ongoing love and support; to Su'a Tanielu, my father and mentor, whose life story continue to inspire achievements in my own.

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Table of Contents

ABSTRACT II

DEDICATION III

ACKNOWLEDGEMENTS III

LIST OF TABLES XI

LIST OF FIGURES XII

1. INTRODUCTION 15

1.1 BACKGROUND 15

1.1.1 ANASTOMOTIC LEAKAGE 16
1.1.2 DEFINITION OF ANASTOMOTIC LEAKAGE 16
1.1.3 DIAGNOSING ANASTOMOTIC LEAKAGE 16
1.1.4 COMPLICATIONS OF ANASTOMOTIC LEAKAGE 17
1.1.5 ANASTOMOTIC LEAKAGE RATE 17
1.1.6 SURGICAL STRESS 18
1.1.6.1 Reducing surgical stress *18*
1.2 PHYSIOLOGICAL RESPONSE TO HEALING 19

1.2.1 Stages of wound healing *19*
1.2.2 Cutaneous (skin) healing *20*
1.2.3 Gastrointestinal healing *20*
1.3 HEALING AT THE ANASTOMOSIS 21

1.3.1 ANATOMY 21
1.3.1 GASTROINTESTINAL IMMUNOLOGICAL RESPONSE 23
1.3.1.1 M1 and M2 Macrophage subtypes *24*
1.3.1.2 Uncomplicated anastomotic healing *24*
1.4 PATHOPHYSIOLOGY OF ANASTOMOTIC LEAKAGE 26

1.4.1 HISTOLOGY OF ANASTOMOTIC LEAKAGE 27
1.4.2 AETIOLOGY OF ANASTOMOTIC LEAK 27
1.4.2.1 Communication *28*
1.4.2.2 Infection *30*
1.4.2.3 Healing disturbances *32*
1.5 MEASURING THE ALTERED INFLAMMATORY RESPONSE IN ANASTOMOTIC LEAKAGE 34

1.5.1 CYTOKINES AND EFFECTOR PROTEINS 34
1.6 AIM ..... ERROR! BOOKMARK NOT DEFINED.

1.7 SUMMARY 35

1.8 HYPOTHESIS 35

2. SYSTEMATIC REVIEW OF THE PHYSIOLOGICAL RESPONSE AFTER COLORECTAL SURGERY 38

2.1. BACKGROUND 38

2.2 METHODS 38

2.2.1 LITERATURE SEARCH **38**
2.2.2 STUDY SELECTION **39**
2.2.3 DATA EXTRACTION **39**
2.2.4 STATISTICAL ANALYSIS **40**
2.2.5 QUALITY ASSESSMENT **40**
2.3 RESULTS **40**
SCREENING **41**
INCLUDED **41**
ELIGIBILITY **41**
IDENTIFICATION **41**
2.3.1 STUDY CHARACTERISTICS **42**
2.3.2 QUALITY ASSESSMENT **42**
2.3.3 STRESS RESPONSE AFTER SURGERY **42**
2.3.4 HUMORAL RESPONSE **54**
2.3.5 CELL MEDIATED RESPONSE **55**
2.3.6 OXIDATIVE STRESS RESPONSE **56**
2.3.7 ENDOCRINE AND METABOLIC RESPONSE **56**
2.3.8 OTHER INFLAMMATORY BIOMARKERS **57**
2.4 DISCUSSION **57**
3. SYSTEMATIC REVIEW OF CLINICAL PARAMETERS RELATED TO ANASTOMOTIC LEAKAGE AFTER MAJOR COLORECTAL SURGERY **62**
3.1 BACKGROUND **62**
3.2 METHODS **62**
3.2.1 LITERATURE SEARCH **62**
3.2.2 STUDY SELECTION **63**
3.2.3 DATA EXTRACTION **63**
3.2.4 QUALITY ASSESSMENT **64**
3.3 RESULTS **64**
3.3.1 STUDY CHARACTERISTICS **64**
SCREENING **65**
INCLUDED **65**
ELIGIBILITY **65**
IDENTIFICATION **65**
3.3.2 DEFINITION OF ANASTOMOTIC LEAKAGE **68**
3.3.3 CLINICAL PARAMETERS MEASURED **68**
3.3.3.1 Heart rate ***68***
3.3.3.2 Respiratory rate ***68***
3.3.3.3 Fever ***68***
3.3.3.4 Abdominopelvic signs and symptoms ***69***
3.3.3.5 Bowel function ***69***
3.3.3.6 Blood pressure ***70***
3.3.3.7 Urine output ***70***
3.3.3.8 Other signs and symptoms ***70***
3.3.4 ONSET OF CLINICAL PARAMETERS AND THE DIAGNOSIS OF ANASTOMOTIC LEAKAGE **71**

3.4 DISCUSSION 74

4. SYSTEMATIC REVIEW OF BIOMARKERS IN THE DIAGNOSIS OF ANASTOMOTIC LEAKAGE FOLLOWING COLORECTAL SURGERY 77

4.1 BACKGROUND 77

4.2 METHODS 78

4.2.1 LITERATURE SEARCH 78
4.2.2 STUDY SELECTION 79
4.2.3 VALIDITY ASSESSMENT 80
4.2.4 DATA ABSTRACTION 80
4.3 RESULTS 80

SCREENING 81
INCLUDED 81
ELIGIBILITY 81
IDENTIFICATION 81
4.3.1 STUDY CHARACTERISTICS 81
4.3.2 VALIDITY ASSESSMENT 82
4.3.3 DEFINITION OF ANASTOMOTIC LEAKAGE 95
4.3.4 CLASSIFICATION OF BIOMARKERS 97
4.3.5 ISCHAEMIC BIOMARKERS 100
4.3.6 INFLAMMATORY BIOMARKERS 101
4.3.7 MICROBIOLOGICAL BIOMARKERS 103
4.4 DISCUSSION 103

5. MATERIALS AND METHODS: BIOMARKERS AND ANASTOMOTIC LEAKAGE LONGIDUTINAL STUDY 107

5.1 BACKGROUND 107

5.2 HYPOTHESIS AND DESIGN 107

5.3 ETHICS AND SAFETY 108

5.4 FUNDING 108

5.5 PATIENT SELECTION 108

5.6 PATIENT FOLLOW UP 109

5.7 PERIOPERATIVE CARE 109

5.8 PRIMARY OUTCOME MEASURES 109

5.9 MEASUREMENT OF POST-OPERATIVE COMPLICATIONS 109

5.10 DEFINITION OF ANASTOMOTIC LEAKAGE 110

5.11 MEASURE INFLAMMATORY BIOMARKERS 110

5.12 PHLEBOTOMY, INITIAL ANALYSIS AND STORAGE 110

5.13 SAMPLE COLLECTION 110

5.14 LABORATORY PROCESSING 110

5.1.1 CYTOKINE ANALYSIS USING MILLIPLEX ® LUMINEX ® TECHNOLOGY 111

5.1.1.1 Preparation of plasma samples *112*
5.1.1.2 Preparation of reagents for immunoassay *112*
5.1.1.3 Assay characteristics *115*
5.15 SAMPLE SIZE **116**
5.16 STATISTICAL ANALYSIS **116**
5. RESULTS: RECOVERY FOLLOWING COLECTOMY WITHOUT ANASTOMOTIC LEAKGE **119**
6.1 BACKGROUND **119**
6.2 METHODS **119**
6.3 RESULTS **119**
6.3.1 BASELINE CHARACTERISTICS **120**
6.3.2 INFLAMMATORY RESPONSE **124**
6.3.3 BODY MASS INDEX AND INFLAMMATORY RESPONSE **125**
6.3.4 LEFT AND RIGHT COLON SURGERY **126**
6.3.5 PREOPERATIVE STATIN MEDICATION AND NUTRITION **128**
6.3.6 PATIENTS WITHOUT ANY PERIOPERATIVE COMPLICATIONS **128**
6.3.6.1 Inflammatory response *129*
6.3.6.2 BMI *129*
6.4 DISCUSSION **130**
7. RESULTS: RECOVERY FOLLOWING COLECTOMY COMPLICATED WITH ANASTOMOTIC LEAKAGE **134**
7.1 BACKGROUND **134**
7.2 METHODS **134**
7.3 RESULTS **134**
7.3.1 BASELINE CHARACTERISTICS **135**
7.3.2 INFLAMMATORY RESPONSE IN ANASTOMOTIC LEAKAGE **137**
7.3.2.1 C-reactive protein *138*
7.3.2.2 Interleukin-10 ..... *139*
7.3.2.3 Interleukin-6 ..... *140*
7.3.3 EARLY AND LATE ANASTOMOTIC LEAKAGE **140**
7.4 DISCUSSION **141**
8 DISCUSSION **144**
8.1 OVERVIEW OF RESULTS **144**
8.2 APPRAISAL OF LITERATURE FINDINGS **145**
8.3 APPRAISAL OF BALL STUDY FINDINGS **147**
8.4 ALTERED INFLAMMATORY RESPONSE **149**
8.5 LIMITATIONS AND IMPLICATIONS FOR FUTURE RESEARCH **152**
8.6 CLINICAL IMPLICATIONS **154**
9 CONCLUSION **156**

APPENDIX 1: PARTICIPANT INFORMATION SHEET 158
APPENDIX 2 CONSENT FORM 162
REFERENCES.....166

LIST OF TABLES

Table 1: Key differences between cutaneous and colonic wall healing 21
Table 2 Risk factors for anastomotic leakage 27
Table 3 Study characteristics 43
Table 4 Categories of inflammatory biomarkers measured 45
Table 5 Summary of results 47
Table 6 Search terms used 63
Table 7 Study characteristics 66
Table 8 Summary of outcomes 72
Table 9 Onset of clinical symptoms prior to AL 73
Table 10 Search terms and hits per database 79
Table 11 Summary of included studies evaluating intra-peritoneal biomarker(s) 83
Table 12 Summary of included studies evaluating systemic inflammatory biomarker(s) 88
Table 13 QUADAS-2 results 94
Table 14 Study definition of anastomotic leakage 95
Table 15 Group classification of biomarkers 98
Table 16 Laboratory reagent and materials required 111
Table 17 Baseline characteristics 121
Table 18 Summary of measured inflammatory biomarkers 124
Table 19 Perioperative medications 129
Table 20 Baseline demographics 136
Table 21 Summary of measured inflammatory biomarkers 137

LIST OF FIGURES

Figure 1: Stages of wound healing 20
Figure 2: Cross section of colonic wall 22
Figure 3: Macrophage polarisation into M1 and M2 23
Figure 4: Categories for the development of AL 28
Figure 5 Healing disturbances 33
Figure 6 PRISMA flow chart, chapter 2 41
Figure 7 PRISMA flow diagram 65
Figure 8 PRISMA flow chart 81
Figure 9 Preparation of standards 113
Figure 10 Author with MAGPIX readers 115
Figure 11 BALL study patient flow and recruitment 120
Figure 12 CRP trajectory in laparoscopic vs. open approach 125
Figure 13 CRP trajectory in obese and non-obese patients 126
Figure 14 CRP trajectory in left and right colectomy 127
Figure 15 IL-10 levels in left and right colectomy 128
Figure 16 BALL study patient flow and recruitment 135
Figure 17 AUROC CRP on POD 3 139

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Background

The inexorable rise in the incidence of colorectal cancer has led to an increase in major colorectal resections. Following resection of the cancerous segment of bowel, the remaining ends are re-joined to form an anastomosis. To support anastomotic healing, operative techniques have evolved to ensure adequate vascular supply and ensure appropriate tension along the anastomotic edges. Failure of the anastomotic edges to heal results in anastomotic leakage, which usually rapidly deteriorates into a cascade of undesirable clinical consequences. Despite advances in modern perioperative care, anastomotic leakage remains a significant clinical problem.

Biochemical markers are widely utilised in clinical medicine to aid in disease diagnosis and monitoring of progression. Following colorectal resection, a multitude of inflammatory mediators are released as part of the healing process. Many of these can be measured in peripheral blood tests and then can be used as biomarkers. Interest in inflammatory biomarkers in colorectal surgery has grown in the last few years as tools in the monitoring of recovery from resectional surgery. [1, 2] Some, including CRP, IL-6 and procalcitonin have shown promise in the early detection of anastomotic leak but currently lack precision and have proven to be more useful in ruling out anastomotic leak rather than in its early detection. [3-5] This thesis aims to investigate how the use of inflammatory biomarkers can be optimised to detect anastomotic leak early and thus lead to early definitive treatment before the patient deteriorates.

1.1.1 Anastomotic leakage

Surgery is the mainstay treatment for colorectal cancer, recurrent diverticulitis and other bowel conditions.[6, 7] Following resection of the affected bowel segment, the remaining bowel ends are re-joined to form an anastomosis. Dehiscence or breakdown of the anastomosis, anastomotic leakage (AL), results in leakage of bowel contents into the abdominal cavity.

1.1.2 Definition of anastomotic leakage

For the purposes of this thesis, AL is defined as a *defect of the intestinal wall at the anastomotic site leading to a communication between intra- and extraluminal compartments*.[8] It is a devastating complication that causes sepsis and directly impacts overall morbidity and mortality both in the short and long term.[9, 10] Efforts to decrease AL rates have focused primarily on improving operative techniques, perioperative care, and reducing surgical stress.

1.1.3 Diagnosing anastomotic leakage

Although a subset of risk factors has been reported, AL remains difficult to predict and difficult to diagnose early after surgery. In many cases, the course of AL is insidious with ileus, vague abdominal symptoms, and failure to progress, with a mean time to diagnosis of between 6 to 12 days after surgery.[11-13]

Clinically, most surgeons rely on bedside clinical review, haemodynamic parameters, and biochemical markers to develop a risk profile. This is usually followed by cross-sectional imaging or intra-operative findings in high-risk patients. In practice, this often means that AL is detected only after the patient becomes clinically unwell. This

delay almost certainly contributes to poorer patient outcomes.[10, 14, 15] With the advent of Enhanced Recovery after Surgery (ERAS) or optimised perioperative care protocols, patients may be discharged three to four days after surgery and thus patients run the risk of developing AL in the community and readmission with AL or severe sepsis.[16, 17]

1.1.4 Complications of anastomotic leakage

If diagnosed late, AL can progress to overwhelming sepsis, multi-organ dysfunction, and death. Delayed diagnosis and subsequent delay in antibiotic administration from the onset of septic shock has been associated with a decrease in survival of 7.6% per hour.[18] Furthermore, long term consequences of AL include increased colorectal cancer recurrence, reduced quality of life, and decreased long term survival.[19, 20] Thus a timely diagnosis and accurate prediction of AL before clinical symptoms become apparent is of utmost importance.

1.1.5 Anastomotic leakage rate

Since 1960, colorectal AL rates have reduced from 69%, to 6.5% or lower.[9, 21, 22] These improvements have been attributed to advances in surgical techniques and perioperative care. Given that New Zealand has one the highest rates of colorectal cancer among OECD countries, AL will continue to be a significant burden among patients following major colorectal surgery for the foreseeable future.[23]

The following sections present an overview of current knowledge on surgical stress, anatomy and physiology of anastomotic healing, and the potential role of inflammatory biomarkers in the early detection of anastomotic leakage.

1.1.6 Surgical Stress

Major colorectal surgery induces a significant stress response that influences healing and recovery. This response is mediated by a host of immunological, metabolic, and inflammatory mediators locally at the site of injury and systemically.[24] The stress response is characterized by release of stress hormones and activation of the sympathetic nervous system.[25] The overall net effect is to increase systemic catabolism, which is detrimental to recovery in the postoperative period. [26, 27]

In the early postoperative period, this response is initiated by the access incision/s and intraperitoneal injury to peritoneal mesothelial cells and resident mucosal macrophages, and is characterized by pro-inflammatory cytokine release, microcirculatory disturbance, and cell-mediated immune dysfunction. It is then followed by a compensatory anti-inflammatory response that may predispose the patient to opportunistic infection, gut dysmotility and organ dysfunction. [28-30]

1.1.6.1 Reducing surgical stress

Modern optimised, evidence-based, perioperative care, such as ERAS, aims to reduce surgical stress through a multimodal approach.[31] Minimal access operative approaches, such as laparoscopy, have been shown to reduce surgical stress by decreasing the size of the access wound and have been incorporated into ERAS protocols.

At the site of the anastomosis, operative techniques have evolved to improve vascular supply and apposition of colonic wall layers. Full wall thickness colonic anastomosis performed either hand-sewn or stapled, is now considered the gold standard. [32, 33] Despite these improvements, AL remains a dreaded complication following major

surgery. The following sections describes the physiological response to injury, with particular focus on gastrointestinal injury and anastomotic healing.

1.2 Physiological response to healing

Surgery causes significant injury from the initial access wound and from mobilization and resection of appropriate bowel segments from embryological attachments. The traditional stages or phases of wound healing have been extensively studied and are summarized below. [34, 35]

1.2.1 Stages of wound healing

Initially in the haemostasis and inflammation stage, platelets first maintain haemostasis by creating an insoluble fibrin clot. There is a simultaneous brief vasoconstriction period followed by increased vessel permeability that allows an influx of inflammatory mediators such as IL-6 and white blood cells, such as macrophages, into the wound. This is followed by the activation of neutrophils, and later macrophages and lymphocytes. Activated neutrophils remove necrotic tissue and release growth factors that attract and promote proliferation of endothelial cells at the wound site.

The proliferation stage is modulated by fibroblasts and involves the formation of granulation tissue and epithelialisation. Arriving fibroblasts replace the provisional matrix with a collagen-rich granulation tissue, specifically collagen subtypes 1 and 3. Collagen is vital to wound healing as it provides the appropriate scaffold for cells to grow and differentiate within the wound. Epithelial cells migrate to wound edges to form a seal.

Remodeling then occurs to strengthen the collagen and cellular network initially laid out in the proliferation stage. During this phase, thin collagen transforms into thick collagen bundles. Collagen is degraded and re-synthesized simultaneously along tension lines to buffer shear stress, Figure 1.[24]

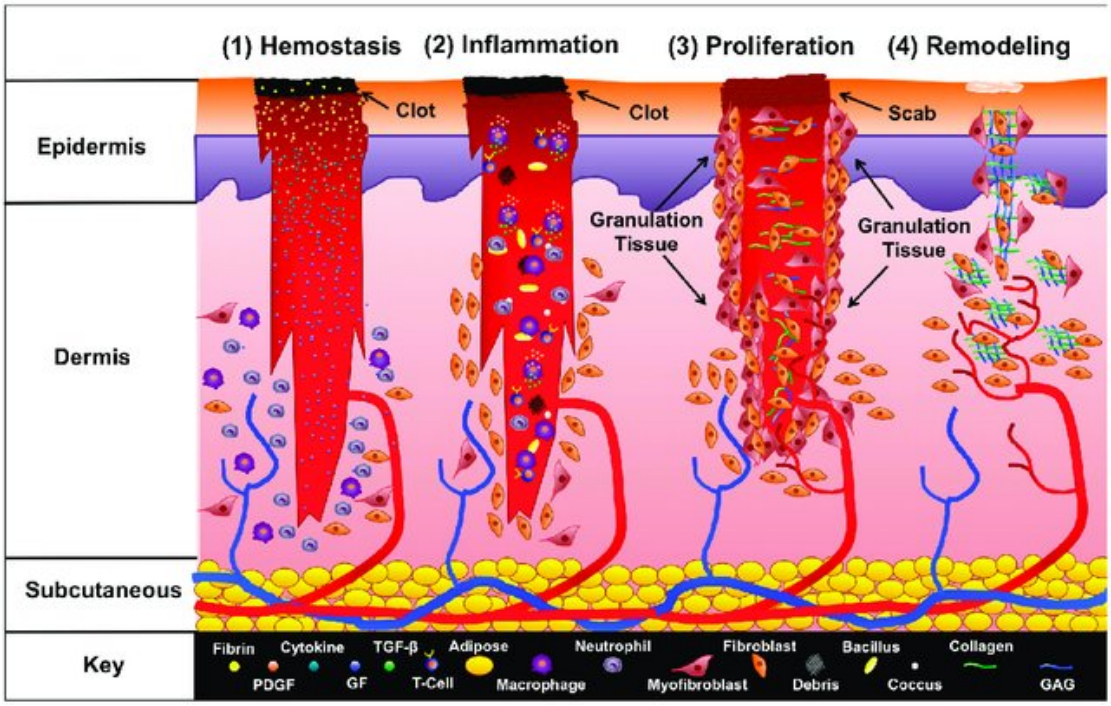

Figure 1: Stages of wound healing

1.2.2 Cutaneous (skin) healing

Intestinal anastomotic healing is often compared with skin healing as it follows the traditional phases of *inflammation, proliferation,* and *remodelling* described above. However, significant differences between the two exist.[25, 26]

1.2.3 Gastrointestinal healing

Observed differences are a result of the different peri-anastomotic and cutaneous wound environments at both the gross and histological levels. Key differences are summarised in Table 1.[27]

Table 1: Key differences between cutaneous and colonic wall healing

Wound environment	Colon	Cutaneous (skin)
Shear stress	Increased, secondary to intraluminal bulk transit and peristalsis	Minimal
Bacteria	Aerobic and anaerobic (may affect anastomotic healing)	Aerobic, unlikely to cause problems
Vascular perfusion	Downregulated in hypovolaemia states	Constant perfusion
Collagenase activity	Increased in first 3 days following surgery (transient decrease in anastomotic strength)	Not significant

1.3 Healing at the anastomosis

As described above, gastrointestinal healing differs from cutaneous healing. In addition to the varied environments, anastomotic healing is further differentiated by varying anatomical, and immunological differences.

1.3.1 Anatomy

The colonic wall consists of four layers: mucosa, submucosa, muscularis propria and serosa, Figure 2. (Uplifted from **https://legacy.cnx.org/content/m45985/1.14/**; Copyright License: **http://creativecommons.org/licenses/by/4.0/**)

The mucosa includes a columnar epithelium, lamina propria, and muscularis mucosa which contains smooth muscle cells. The submucosal layer contains nerves that modulate functions of the colon, and fibroblasts that secrete collagen after surgery to facilitate healing. The submucosa and muscularis propria layers contain the blood vessels and nerve plexi which innervate the mucosal layer to enable peristalsis. The outer serosal layer is a smooth membrane that secretes serous fluid and provides a barrier that separates the abdominal cavity from luminal contents and bacteria.

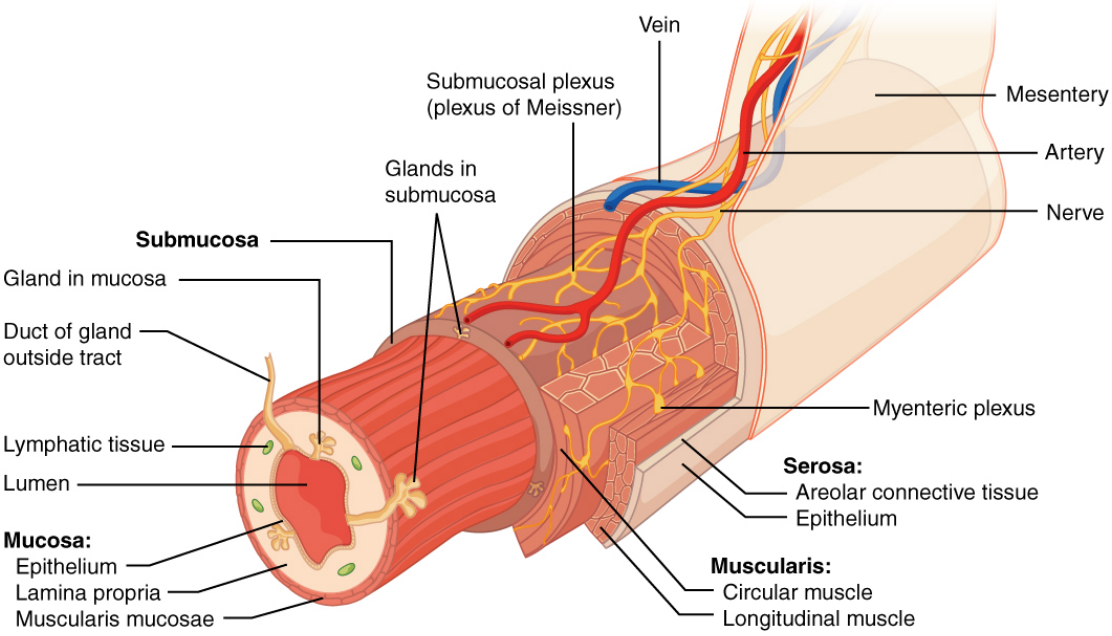

Figure 2: Cross section of colonic wall

Certain colonic layers seem to play a significant role in anastomotic healing. An experimental study by Bosman *et al* [28] in mice with a knocked out mucosal gene showed that these mice are predisposed to development of AL. Furthermore, the high levels of anti-inflammatory leucocytes seen in murine anastomotic mucosa are associated with optimal anastomotic healing.[29] This means that a functional mucosal layer with an appropriate composition of leucocytes is required for optimal anastomotic healing.

In addition to hosting nerve plexi, the submucosa has the highest tensile strength owing to the presence of collagen secreted by fibroblasts. This is thought to provide strength to withstand peristaltic pressures during healing.

1.3.1 Gastrointestinal immunological response

Following surgery, native macrophages found in the mucosal layer, play a significant role in the inflammatory and healing process. Macrophages are myeloid immune cells that ingest and degrade dead cells, debris, and foreign material and facilitate the inflammatory process. [48, 49] In addition to its phagocytic role, macrophages differentiate into two broad classes: M1-type macrophages, and M2-type macrophages, Figure 3.

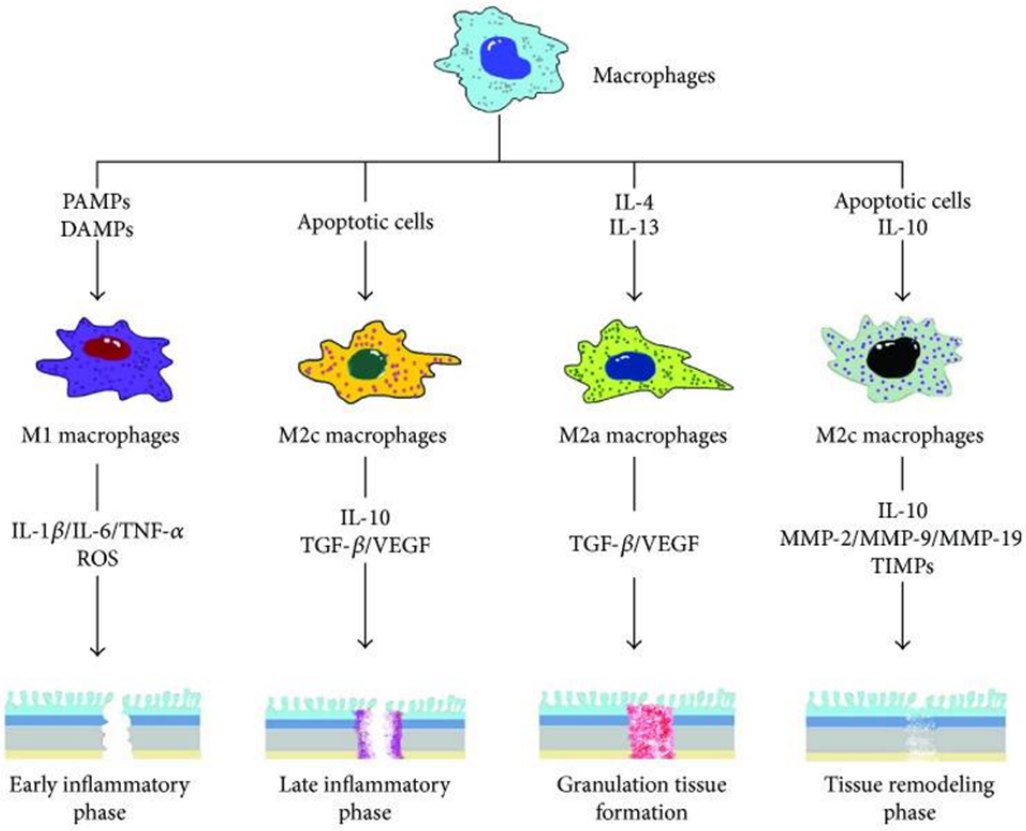

Figure 3: Macrophage polarisation into M1 and M2

1.3.1.1 M1 and M2 Macrophage subtypes

Polarisation or differentiation of macrophages can be divided into two categories: the classically activated which results in M1 type macrophages (M1) and the alternative activation pathway which results in M2 type macrophages.

These two broad macrophage groups perform diverse functional activities in response to microenvironmental changes including cell byproducts, presence of Pathogen associated molecular patterns (PAMP), and Damage associated molecular patterns (DAMP), and damaged cells and cytokines from activated lymphocytes. [50-52]

Some authors have suggested that polarisation of macrophages should be viewed as a continuum spectrum, of which M1 and M2 occupy the opposite ends. Other classifications have also been proposed including a classification based on fundamental functions rather than stimuli. [53] Most M1 macrophages are classically activated and exert host defence and are pro-inflammatory; while M2 macrophages are activated through the alternative pathway and act to preserve tissue homeostasis and resolution of inflammation, and thus are anti-inflammatory. [54, 55]

1.3.1.2 Uncomplicated anastomotic healing

As described earlier, all 4 colonic wall layers need to be apposed and each layer provides different functions to improve healing and act as a barrier between intra-luminal contents and the abdominal cavity. At the mucosal layer, resident macrophage absence or dysfunction impairs anastomotic healing. [56, 57] The submucosa consists of blood vessels and fibroblasts that activate after surgery to facilitate collagen deposition.[58]

Similarly, to skin healing, gastrointestinal anastomotic healing is divided into 3 phases: inflammation, proliferation, and remodelling.

1.4.2.2.1 Inflammation

Inflammation from surgical trauma can be divided into early and late inflammatory responses. In the early phase, polymorphonuclear leukocytes are recruited from circulating blood to local wounded tissue (the anastomotic area) at first. These remove local foreign particles or bacteria and then undergo apoptosis or necrosis. Following this, monocytes are recruited and differentiate into macrophages which are highly phagocytic. They phagocytose impaired neutrophils and other tissue debris to protect from further tissue damage.[59] During this phase, macrophages are classically activated and express the M1 phenotype, designed for microbial killing.[60]

M1 macrophages release high concentrations of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-12 (IL-12); and reactive oxygen species (ROS).[53] Furthermore, M1 macrophages produce collagenase, an enzyme that increase collagen degradation, that results in less anastomotic strength.[61]

In the late inflammatory phase, activated macrophages begin to express anti-inflammatory mediator transforming growth factor (TGF).[62] This change results in the inhibition of proinflammatory cytokines such as TNF-α and IL-1β. [63, 64]. Therefore, the phenotype of macrophages changes from M1 like to M2-like (Figure 3). M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and begin new tissue formation. [65, 66] Furthermore, anti-inflammatory macrophages have been

shown to be entirely IL-10 dependent in sterile environments such as a surgical wound. This results in a feedforward loop by secreting IL-10 to amplify the anti-inflammatory response.[67]

1.4.2.2.2 Proliferation and remodelling

Macrophages produce various growth factors such as Tissue growth factor (TGF). This is a profibrotic cytokine that influences fibroblasts to differentiate into myofibroblasts in wound tissue. They produce extracellular matrix (ECM) components, laying the foundation for collagen and fibronectin to build upon. Monocyte-derived cell populations are key regulators of the fibrotic process. They act as a brake on the processes driving fibrogenesis, and they dismantle and degrade established fibrosis.[68]

Remodelling is a dynamic process of maturation based on the balance of ECM deposition and breakdown.[69] Fibrolytic macrophages also regulate the degradation by synthesizing the tissue inhibitor of metalloproteinases. Moreover, they are responsible for fibroblast apoptosis, and suppression of further inflammation via IL-10 release.[68]

1.4 Pathophysiology of anastomotic leakage

Little is known about the pathophysiology of AL. Several theories have been proposed that incorporate technical aspects, the role of gut bacteria, and inflammatory markers in anastomotic healing. The following sections outline the histology, aetiology, and introduces the theory of altered inflammatory response in the pathophysiology of AL.

1.4.1 Histology of anastomotic leakage

The formation of an anastomosis results in a *prolonged inflammatory reaction* involving the different colonic wall layers. Anastomotic samples biopsied in an uncomplicated recovery years after surgery still show non-specific inflammatory changes.[30] In patients with AL however, histology from beyond 4 days following resection shows features of extensive mucosal necrosis and poor submucosal apposition.[31] This is consistent with the above sections on apposition and mucosal integrity.

1.4.2 Aetiology of anastomotic leak

Though a subset of risk factors for AL, Table 2, have been identified these do not directly cause AL. [11, 32] However, it does seem that they predispose to AL.

Table 2 Risk factors for anastomotic leakage

Risk factors
Preoperative albumin
Operative time
Obesity
Male gender
Ongoing antiocoagulant use
Inflammatory disease

Three broad mechanisms have been proposed to contribute to the development of AL: *communication*, *infection* and *healing* disturbances [33] shown in Figure 4 (Uplifted from Sparreboom *et al* 2016). Preventative strategies for AL have primarily focused on

these aetiological factors. Presumably, the above risk factors work through these mechanisms to predispose to AL.

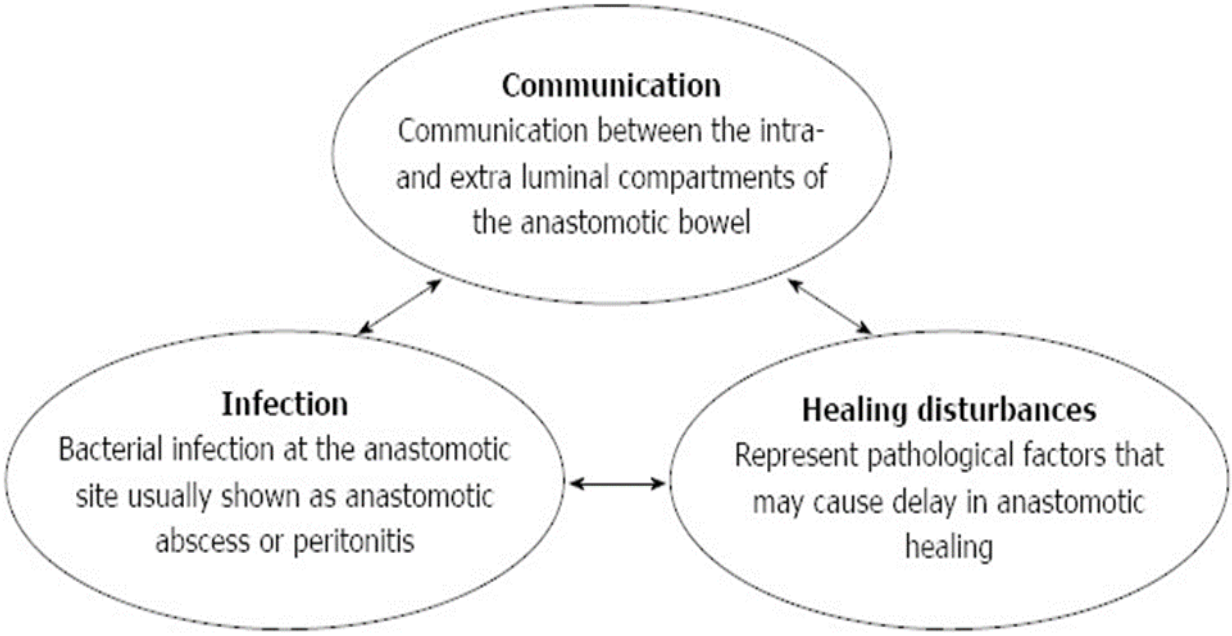

Figure 4: Categories for the development of AL

1.4.2.1 Communication

This represents the definition described above for AL: "*defect of the intestinal wall at the anastomotic site leading to a communication between intra- and extraluminal compartments"*

Early efforts to reduce AL centred around technical aspects to improve healing. Usually, AL that develop within 24-36 hours are a result of technical failure in formation of the anastomosis. Smaller leaks (perhaps best termed micro leaks) are likely to be almost a normal feature of anastomotic healing, as it seems unlikely that anastomoses are completely water/air-tight and are not usually due to stapler or suturing failure. It is only when failure of healing of these microleaks occurs that a clinically important AL arises.

Operative techniques have evolved to ensure vascular supply and appropriate apposition of colonic layers. Apposition of different layers of the colon were investigated in the early 19th century to optimise healing. Apposing the serosa only (Lembert 1826), serosa and mucosa only (Czerny 1881) or submucosa only (Halstead 1887), produced varying success. [34]Full thickness colonic anastomosis involving all 4 layers performed either hand-sewn or stapled, is now considered the gold standard approach.[35, 36]

Different methods have been employed by surgeons to check for *communication*. One example is the intraoperative air leak test. [37] The air leak test (ALT) is most frequently used intraoperatively to identify a technically failed anastomosis especially following rectal excision with anastomosis. The rate of this intraoperative test varies greatly in studies evaluating ALT. [38] Even with this employed, a recent meta-analysis did not show any significant reduction in AL with the use of intraoperative leak testing. [39]

Similar to ALT, intraoperative endoscopy (IOE) is another intraoperative test which, may allow both immediate diagnostic and therapeutic interventions. However, studies on this topic are limited due to small numbers,[40] or its use is restricted to oesophageal or left sided colonic anastomoses.[41] Further, this method seems inadequate to predict late anastomotic complications.[42] Routine use of IOE does not reduce AL rates compared with selective use.[42]

Surgeons may also prevent *communication* by reinforcing the anastomosis. One strategy is to perform a second layer of sutures over the anastomosis. However, multiple studies have found that a single layer continuous anastomosis has a similar rate of complications compared with a two-layered technique.[43, 44]

More recently, tissue adhesives such as fibrin tissue glue have been used for anastomotic reinforcement and sealing [45-47]. Experimental data showed initial promise, however a recent review has shown no difference in AL rates.[48]

The use of a temporary diverting stoma seems to be the only definitive method to reduce AL, particularly in left sided anastomoses. [49, 50] However it is probable that the rate of AL is not decreased greatly and the more important role is to decrease the consequence of an AL. However, stomas are associated with significant morbidity rates and their reversal requires a further anastomosis, and thus their use is limited to a select group of patients and situations at high risk for AL. [51, 52]

Despite these improvements in operative technique in reducing communication between intra- and extra-luminal compartments, AL rates have remained unchanged in recent years. This suggests a multifactorial aetiology and requires further research and work in improvement in early detection and diagnosis.

1.4.2.2 Infection

Another preventative method is reducing *infection* at the site of anastomosis. Historically peritoneal drains were placed near anastomoses in order to eliminate infected fluid left behind, and prevent accumulation of fluid that may develop into an intra-abdominal collection.[53, 54] Drain placement is seldom utilized currently as no clear advantage has been demonstrated in their use for colectomy.[55-57]

1.4.2.2.1 Local intra-operative contamination and infection

As mentioned above there is likely to be a degree of leakage for every anastomosis formed whether handsewn or stapled. This inevitably results in some contamination with *E coli* or *E faecalis*. If the response to this, perhaps due to the predisposing factors mentioned above, is altered then instead of controlling the acute phase of infection, a prolonged M1 response (pro-inflammatory) may be detrimental.[58] In other experimental studies, M1 type macrophages upregulate the expression of nitric oxide (NO), which in addition to its defence role, plays a significant role in collagen deposition.[59, 60] Excessive NO results in impaired wound healing.[61]

1.5.2.2.1.2 Preoperative antibiotics and mechanical bowel preparation

Multiple murine studies have suggested a positive effect of antibiotics on anastomotic healing.[62-64] A study by Alverdy *et al*, found virulent bacteria such as *E faecalis,* with high collagenase activity, led to AL.[65]

Preoperative selective decontamination of the digestive tract (SDD) aims to eradicate pathogenic enteric bacteria with oral antibiotics before resection. It is a method utilized since 1983, [66] and has been utilized in reducing infectious complications in intensive care patients.[67] A recent trial in 2019 showed that SDD reduced post-operative infectious complications but did not significantly reduce AL rates.[68]

Other strategies such as mechanical bowel preparation with or without antibiotics (MBP) may help to reduce infection by removing the bacterial load within the colon prior to resection.[69]

Recent use however has reduced due to conflicting evidence with reducing infectious complications and increasing evidence of MBP side effects such as dehydration and

electrolyte imbalance.[70] When combined with oral antibiotics, three large retrospective studies and a non-randomised prospective study have shown a reduction in infectious complications including AL.[69-73] However significant limitations and heterogeneity including the type of MBP and antibiotics used, type of surgery, and definitions used raise doubts as to the utility of this approach.[74]

1.4.2.3 Healing disturbances

Common *healing disturbances* risk factors have been identified in recent reviews. These include smoking, diabetes, poor nutrition, and perioperative medications that influence immunity.[32, 75-77] In most cases, these are managed with lifestyle changes, or medical optimisation prior to surgery.

Under physiological conditions, anastomotic healing begins with the *inflammation* phase with clearance of debris and phagocytosis by M1 macrophages and PMN leucocytes. In high risk patients, such as diabetics, chemotherapy, perioperative steroid and other immunosuppressants, phagocytotic ability may be reduced, resulting in an accumulation of necrotic and apoptotic cells at the anastomotic site, s in Figure 5.[78, 79] This can prolong the inflammatory phase, and delay the anti-inflammatory release from M2 macrophages. [80]

Other healing disturbances such as hypoxia from poor anastomotic vascularity, result in increased secretion of proinflammatory cytokines, and further differentiation to the M1 phenotype.[81, 82]

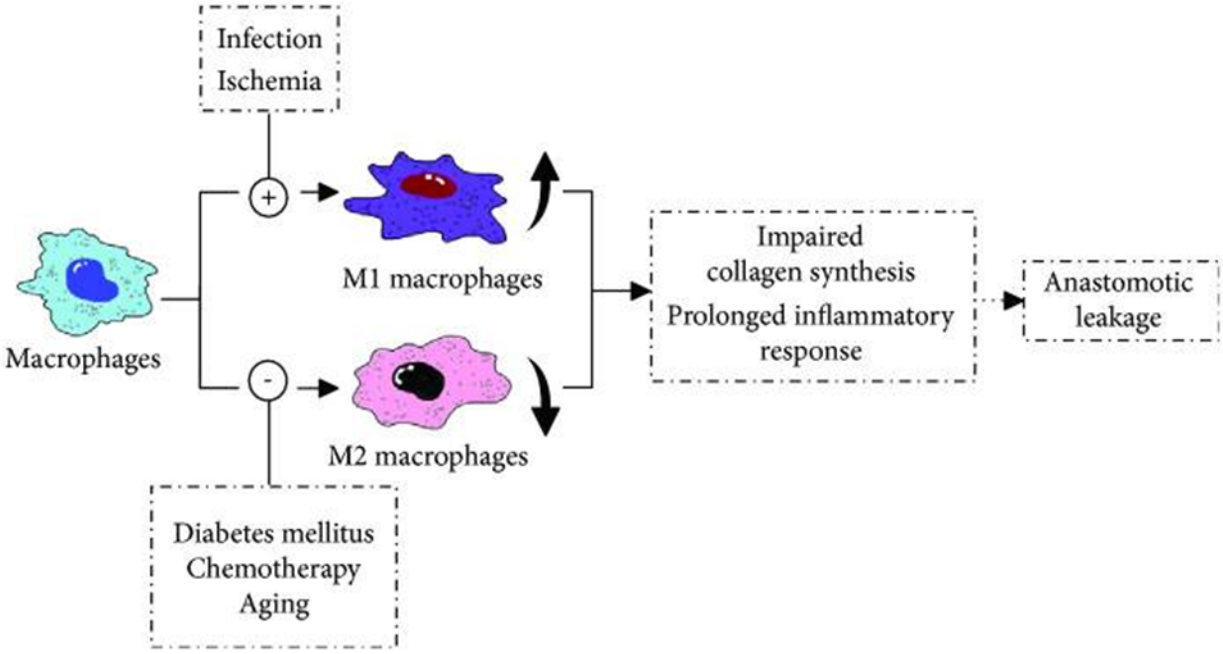

Figure 5 Healing disturbances

1.5.2.3.1 Altered inflammatory response and healing

The theory of an altered inflammatory response contributing to the development of AL has garnered interest recently. This is based on an apparent deviation from normal physiological healing, which results in poor healing at the site of anastomosis.

As discussed above, AL develops when there is a direct *communication*, *infection*, or *healing disturbance*. [33]. In the development of a clinically significant AL, all three factors interact with each other and are altered or exacerbated by predisposing factors noted above. For instance, a microleak will lead to a local infection, with or without collagenase producing bacteria, at the site of the anastomosis. This then stimulates an inflammatory response, which interrupts collagen deposition as part of the remodelling phase.[59] This then leads to enlargement of the previously small and clinically insignificant leak. Bowel contents are then released into the abdominal cavity

with resulting sepsis that, left untreated, leads to death. If any of these factors are disordered in any way, then a clinically relevant AL is more likely.

Thus, a poorly constructed devascularised anastomosis will leak more, an altered inflammatory response due to predisposing conditions will make the leak worse, and aggressive bacteria will slow healing. Any one of these may predominate or all may interact together.

1.5 Measuring the altered inflammatory response in anastomotic leakage

1.5.1 Cytokines and effector proteins

Inflammatory cytokines are heterogenous polypeptides that include chemokines, interferons, tumour necrosis factor and lymphokines.[83] They are synthesized by activated macrophages and endothelial cells at the site of surgery during the *inflammation* phase of healing. Cytokines modulate the inflammatory response through various messengers and inhibitors on surface receptors of target cells.[84] Even without the presence of trauma, cytokines modulate key intestinal functions including intestinal secretions.[85, 86]

In an uncomplicated recovery, cytokines, and their effector proteins, seem to follow a predictable trajectory in the local peri-anastomotic environment.[87] Pro-inflammatory biomarkers such as tumour necrosis factor (TNFα), Interleukin (IL)-1β, and IL-6 play a critical role in promoting immunomodulatory effects of receptor cells (including leucocytes) and downstream inflammatory proteins such as C-reactive protein (CRP). [88] Anti-inflammatory cytokines, such as IL-10, play a significant role in buffering this pro-inflammatory effect to prevent unintentional injury as a result of the inflammatory response, and help facilitate *remodelling* phase.[88]

These inflammatory mediators escape from the peritoneal cavity into the bloodstream where they can be measured as biomarkers. A number of these have been shown to potentially be of some value in early detection of AL.

IL-6 has garnered significant interest in recent studies. It peaks systemically at 24-48 hours after surgery, and its levels may correlate with the degree of the stress response.[89] IL-6 modulates other proteins downstream such as C-reactive protein (CRP) which is widely used in modern clinical medicine.[90] Accumulating evidence suggests measuring intra-peritoneal cytokine levels may aid in diagnosis of AL in the postoperative period.[91] A balance of this inflammatory response may be necessary for optimal anastomotic healing.[92, 93]

1.6 Summary

An appreciation of the composition of the peri-anastomotic environment is crucial for understanding the pathophysiology of AL. As described above, the local perianastomotic environment is awash with inflammatory mediators in a complex inflammatory milieu as part of this healing process. M1 and M2 macrophages and subsequent pro-inflammatory and anti-inflammatory cytokines work in tandem to facilitate this process. In the normal course of healing after surgery, this balance seems to follow a predictable course. In the presence of AL however, this inflammatory balance is altered, and it is unsure why or when this occurs.

Detection of these changes in inflammatory response in the systemic circulation have potential to detect AL early, prior to clinical symptoms and sepsis arise.

1.7 Hypothesis and Aim of the Thesis

It is hypothesized that the early detection of inflammatory biomarkers, that represent the local peri-anastomotic environment, in the systemic circulation can aid in the early detection of clinically significant AL after colorectal surgery.

This thesis aims to test this hypothesis by defining a systemic inflammatory profile of anastomotic leak following elective colectomy.

CHAPTER 2: SYSTEMATIC REVIEW OF THE PHYSIOLOGICAL INFLAMMATORY RESPONSE IN COLORECTAL SURGERY

2. SYSTEMATIC REVIEW OF THE PHYSIOLOGICAL RESPONSE AFTER COLORECTAL SURGERY

2.1. Background

A recent systematic review evaluated the humoral response following laparoscopic versus open colorectal surgery, [94] providing a selective window into the postoperative inflammatory response. Recent developments in understanding of potential stress mediators involved with the cell mediated, oxidative, metabolic, and endocrine stress responses have not yet been systematically evaluated.

Furthermore, the influence of laparoscopy on these inflammatory markers needs to be understood as currently the literature in this area is controversial. Additionally, the difference in inflammatory response between colectomy and rectal resection may also be significantly different. If so, this will guide the selection of patients for the study described in chapter 6.

This systematic literature review therefore aims to analyse results from studies comparing inflammatory responses in patients undergoing laparoscopic versus open colorectal resection and where possible, comparing colonic and rectal surgery. Results will be used to guide the inclusion of relevant inflammatory markers in the prospective study outlined in chapter 5 and chapter 6.

2.2 Methods

2.2.1 Literature search

A comprehensive review of the literature was performed according to the guidelines in the Preferred Items for Systematic review and Meta-analysis (PRISMA)

statement.[95] The following databases were searched from 1997 to January 1st 2018: Medline, Embase and PubMed. The following keywords were used: (Colorectal OR colon OR rectal) AND (Anastomosis OR resection$) AND (Inflam$ response OR physiological response OR systemic response OR local response OR immunological response OR haematological response OR surgical stress). Reference lists of all relevant papers including other literature reviews were searched manually to identify further relevant studies. Studies were restricted to humans and the English language.

2.2.2 Study selection

The primary outcome of interest was the measured levels of stress biomarkers following colorectal surgery. All randomized controlled trials (RCTs) evaluating the stress response through an objective measurement of biomarkers following colorectal surgery that involved a resection were included. Studies were assessed independently by two researchers (Bruce Su'a, Wiremu MacFater) and any disagreements were resolved by consensus with candidate's supervisor, Prof. Andrew G Hill.

2.2.3 Data extraction

Data extraction was carried out by the candidate and entered into predefined tables. Study design, randomization method, disease process and inflammatory biomarker values following surgery were recorded. For the meta-analysis, standard deviations of the outcome data were used if provided by the authors. Missing data were derived from p-values, confidence intervals, data ranges, or from an online image plot digitizer, if they were not published or provided by the corresponding author.[96, 97]

The corresponding author for each study was contacted if further information was required.

2.2.4 Statistical analysis

For included randomised controlled trials, a meta-analysis was completed using the Review Manager version 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) using a random effects model. Statistical results were assessed by graphical presentations of standardized mean differences. Weighted mean differences with 95% confidence intervals (CI) were calculated to assess the size of effect for each approach. $P<0.05$ was considered statistically significant. Statistical heterogeneity was evaluated using I^2 statistics. Funnel plots were used to screen for publication bias.

2.2.5 Quality assessment

Quality assessment was applied independently by three researchers (Bruce Su'a and Wiremu MacFater) with one researcher (Wiremu MacFater) blinded to study title, journal, and authors of the publication.

Assessment was performed using the JADAD score, which focuses on the randomization process, the appropriateness of blinding used, and whether all included patients were accounted for.[98] Any disagreements were resolved by consensus among the researchers.

2.3 Results

A total of 22 studies were included in the final analysis. A PRISMA flow chart showing the selection of articles for this systematic review is presented below, Figure 6.

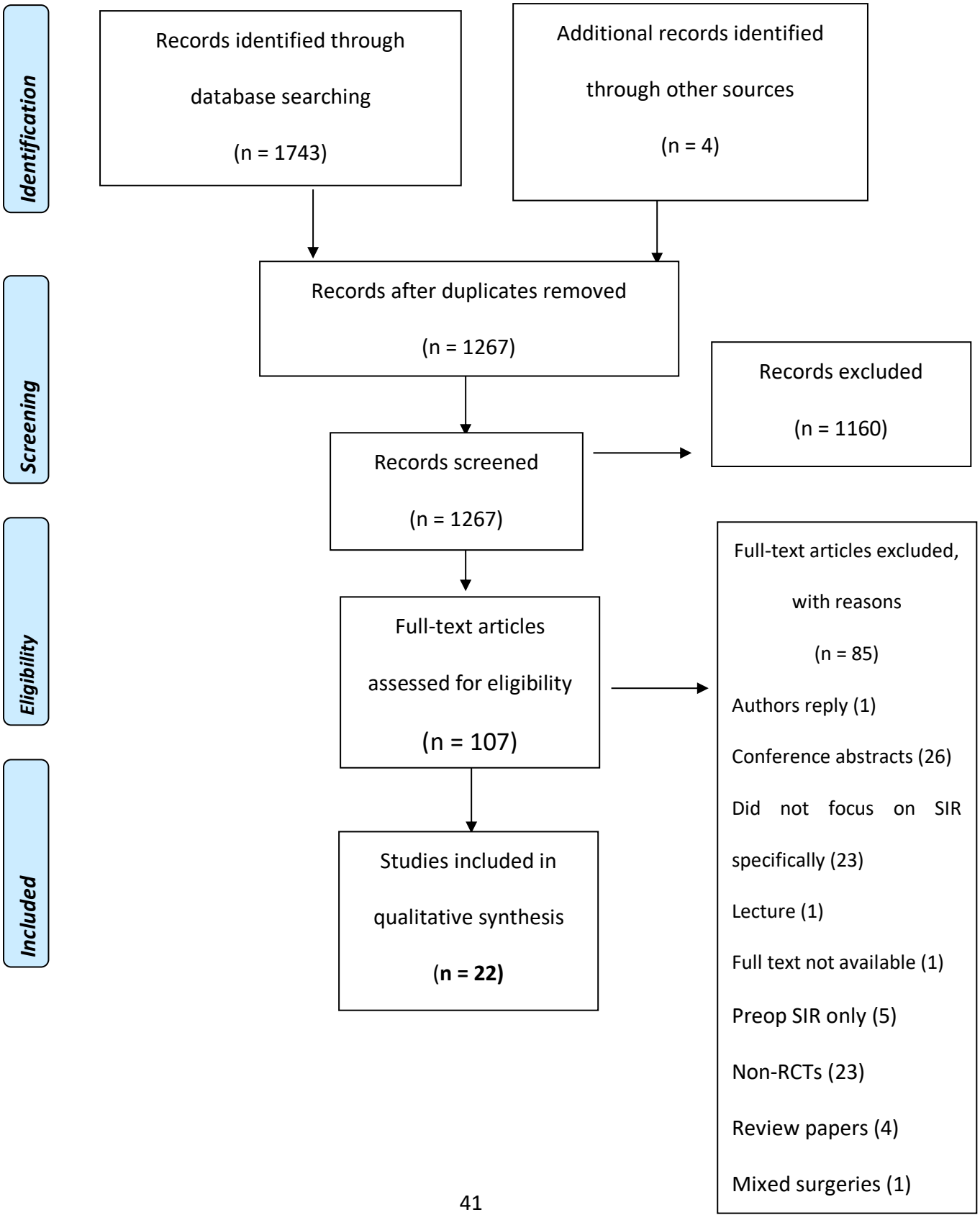

Figure 6 PRISMA flow chart, chapter 2

2.3.1 Study characteristics

Study characteristics are summarized in Table 3. The majority of RCTs combined both colon and rectal surgical procedures. Two RCTs included rectal surgery only. Four studies evaluated recto-sigmoid procedures only, and 8 studies evaluated only colectomies.

2.3.2 Quality assessment

The results from the JADAD tool are shown in Table 3. Only four studies reported blinding. Overall, the quality of included studies ranged from poor to good, with the highest score being 3. Only ten studies reported power calculations.

2.3.3 Stress response after surgery

Twenty-two studies measured 43 systemic inflammatory biomarkers listed in Table 4. Systemic mediators were classified based on their function or role in the stress response after surgery. A summary of the results of each study is presented in Table 5. Meta-analysis is presented where reported data were sufficient and appropriate.

Table 3 Study characteristics

Authors	Randomisation method	JADAD score	Blinding	Colon or rectum	Disease process	Lap (n)	Open (n)	Inflam mediators measured
Braga [99]	Computer generated block randomisation	2	no	Rectum	Benign	40	39	Lymphocyte count, CD4, CD8 subset, cortisol, lactate, and CRP
Delgado[100]	Computer generated	2	no	Colon	Cancer	39	58	Cortisol, Prolactin, CRP, and IL-6
Hasegawa[101]	No details	1	no	Colon	Cancer	29 (5)	30	NK cells, CRP, leucocytes, IL-6
Hewitt[102]	No details	1	no	Both	Cancer	8	8	IL-6, lymphocyte, HLA-DR
Kvarnstrom[103]	No details	1	no	Rectum	Cancer	12	12	IL-1α, IL-6, IL-8, IL-10, TNF-α, CRP, ICAM-1, VCAM-1
Pappas-Gogos[104]	No details	2	Double†	Both	Cancer	30	30	8-isoprostanes, protein carbonyls, 8-hydroguanosine, 3-nitrotyrosine
Liang[105]	Block randomisation	1	no	Sigmoid	Complex polyps	18	26	CRP, ESR, lymphocyte count, CD4/CD8
Ordemann[106]	No details	1	no	Both	Cancer	20	20	WBC, CD4+, CD8+, TNF-α, IL-6

Schwenk[107]	No details	1	no	Both	Cancer	30	30*	IL-1, IL-6, CRP, IL-10
Svendsen[108]	No details	1	no	Colon	Cancer	23 (4)	30	VEGF, VEGFR1
Tang[109]	Computer generated	2	no	Colon	Cancer	118 (15)	118	T cell, B cell, CD4, CD8, NK cells, IgG, IgM, IgA, C3, C4
Tsimogiannis [110]	No details	2	Single	Both	Cancer	20	20	IL-6, hs-CRP, alpha-defensins
Tsimogiannis [111]	Surgeon decision on allocation	2	Single	Both	Cancer	20	20	hs-CRP, IL-6, TLR-2, TLR-4, TNF-alpha
Wang[112]	Random selection of envelope by patient	1	no	Colon	Cancer	40	41	CRP, CD3+ lymphocytes, CD4+, CD8+
Pascual[113]	Computer generated	2	no	Colon	Cancer	60 (7)	60	IL-6, VEGF
Leung [114]	Computer generated	2	no	Rectosigmoid	Cancer	17 (1)	17	IL-1b, TNF-α, IL-6, CRP
Veenhof [115]	Block randomisation	1	no	Colon	Cancer	19	17	IL-6, Prolactin, Cortisol, GH, HLA-DR, CRP
Veenhof [116]	Computer generated	1	no	Rectum	Cancer	22 (2)	18	IL-6, CRP, IL-8, WCC + HLA-DR, WCC, Cortisol, Prolactin, GH
Wu [117]	Computer generated	3	no	Both	Cancer	12	14	VEGF, endostatin

Wu [118]	Computer generated	3	no	Both	Cancer	12	14	IL-6, IL-8, TNF-alpha, CRP
Madsen[119]	Computer generated	3	Double*	Sigmoid	Cancer	11	8	Malondialdehyde, ascorbic acid, dehydroascorbic acid
Stage[120]	No details	3	no	Colon	Cancer	15 (3)	14	IL-6, CRP

Table 4 Categories of inflammatory biomarkers measured

Table 4 Categories of inflammatory biomarkers measured		
Oxidative Stress	Malondialdehyde Ascorbic acid Dehydroascorbic acid 8-isoprostanes	Protein carbonyls 8-hydroguanosine 3-nitrotyrosine
Endocrine / Hormone	Cortisol Prolactin	Growth hormone
Complement Pathway	C3 C4	

Humoral Response	IL-1 IL-6 IL-8 IL-10 TNF-a VEGF VEGFR1 Endostatin α-defensins	TLR-2 TLR-4 B cells IgG IgM IgA Th1 Th2 NK cells
Cellular Response	CD3+ CD4+ CD8+ T cells CD4 CD8	HLA-DR Lymphocytes WBC
Other	lactate CRP ESR	

C3: Complement; Ig immunoglobulins; Th: T-helper cells; VEGFR: vascular endothelial growth factor receptor; CD: cluster of differentiation; ESR: erythrocyte sedimentation rate; TLR: toll like receptor

Table 5 Summary of results

Author	**Time measured**	**Stress mediator**	**Significant results**	**Significant Difference**
Braga [99]	D1, D3, D7	Total lymphocyte		No diff
		CD4		No diff
		CD8		No diff
		CD4/CD8	D1 **Lower** in open	
		Cortisol		No diff
		Lactate		No diff
		CRP	D7 earlier return to baseline in lap.	
Delgado[100]	4h,12h, D1, D3	Cortisol		No diff
		Prolactin		No diff
		CRP	D3 **Lower** in lap.	
		IL-6	4h,12h, and 24hrs **Lower** in lap.	

		HLA-DR		No diff
Hasegawa[101]	D1, D4	IL-6		No diff
		NK cells		No diff
		CRP	D1 and D4 **Lower** in lap.	
		Leukocyte		No diff
Hewitt[102]	4h, 8h, 20, D2	IL-6		No diff
		Lymphocyte sub.		No diff.
		HLA-DR		No diff
Pappas-gogos [104]	6h, D1	8-isoprostanes	6h and D1 **Lower** in lap.	
		Protein carbonyls		No diff
		8-hydroxyguanosine	D1 **Lower** in lap.	
		3-nitrotyrosine	6h and D1 **Lower** in lap.	
Ordemann[106]	1h, 4h, D1, D2, D4, D7	WBC	D1-D4 **Higher** in open	
		CD4		No diff
		CD8		No diff
		CD4/CD8		No diff
		TNF-α	1h-D2 **Higher** in open	
		IL-6	4h **Higher** in open.	

		HLA-DR	D4 **Lower** in open	
Schwenk[107]	0hr, 1h, 4h, D1, D2, D4, D7	IL-1		No diff
		IL-6	Overall **higher** in open	
		IL-10		No diff
		CRP	Overall **higher** in open	
Svendsen[108]	1,2,6h, D1, D2, D8, D30	IL-10		No diff
		VEGF		No diff
		VEGFR1		No diff
Tang[109]	D3	T cell		No diff
		B cell		No diff
		CD4/CD8		No diff
		NK cells		No diff
		IgG		No diff
		IgM		No diff
		IgA		No diff
		C3		No diff
		C4		No diff
	6h, D1	IL-6	6h and D1 **Lower** in lap.	

Tsimogiannis [110]		hs-CRP	D1 **Lower** in lap.	
		α-defensins	D1 **Lower** in lap.	
Tsimogiannis [111]	6h, D1	hs- CRP	D1 **Lower** in lap.	
		IL-6	D1 **Lower** in lap.	
		TNF-α		No diff
		TLR-2	D1 **Lower** in lap	
		TLR-4		No diff
Wang[112]	D1, D3, D5	IL-6	D1 and D3 **Lower** in lap.	
		CRP	D1,3,5 **Lower** in lap.	
		CD3	D1,3,5 **Lower** in lap.	
		CD4/CD8	D1,3,5 **Higher** in lap.	
Pascual[113]	4h, 12h, D1, D2, D4	IL-6	4h **Lower** in lap.	
		VEGF	D2 and D4 **Lower** in lap.	
Leung [114]	2h, 8h, D1, D2, D3, D7, D28	IL-1β	2h **Lower** in lap.	
		TNF-α		No diff
		IL-6	2h **Lower** in lap.	
		CRP	D2 **Lower** in lap.	
	2h, D1, D3	IL-6	6h **Lower** in lap.	

Veenhof [115]		IL-8		No diff
		CRP		No diff
		Prolactin		No diff
		Cortisol		No diff
		Growth hormone		No diff
		HLA-DR	2h **Better** preserved in lap.	
		WBC		No diff
Wu [117]	2h, D1, D4	VEGF		No diff
		Endostatin		No diff
Wu [118]	D1, D4	IL-6		No diff
		IL-8		No diff
		TNF-α		No diff
		CRP	D1 and D4 **Higher** in open	
		HLA-DR		No diff
		WBC		No diff
		Lymphocyte subpop.		No diff
		NK cells		No diff

		CD4		No diff
		CD8		No diff
		CD4/CD8		No diff
		CD14		No diff
Madsen[119]	1h,6h, D1, D2, D3	Malondialdehyde		No diff
		Ascorbic acid		No diff
		Dehydroascorbic acid		No diff
Stage [120]	D1, D3, D10	IL-6	Overall **Higher** in lap.	
		CRP	Overall **Higher** in lap.	
Veenhof [116]	2h, D1, D3	IL-6	2h **Lower** in lap.	
		IL-8		No diff
		CRP		No diff
		WCC + HLA-DR	2h **better** preserved in lap.	
		WCC/monocyte		No diff
		Cortisol		No diff
		Prolactin		No diff

		Growth hormone		No diff
Liang[105]	D1	CRP	**Lower** in lap.	
		ESR	**Lower** in lap.	
		Lymphocyte count	**Higher** in lap.	
		CD4+/CD8+	**Higher ratio** in lap.	
Kvarnstrom [103]	0h, 2h, 4h, D1. D3-5	IL-1α		No diff
		IL-6	2h and 4h **Lower** in Lap.	
		IL-8		No diff
		IL-10	2h **Lower** in Lap.	
		TNF-α		No diff
		CRP	D1 **Lower** in Lap.	
		ICAM-1		No diff
		VCAM-1		No diff

D: Post-operative day; No diff: No significant difference; VCAM: vascular cell adhesion molecules; Lap: laparoscopy

2.3.4 Humoral response

Cytokines, Immunoglobulins (Ig), Toll-like receptor (TLR)-2, TLR-4, α-defensin, complement, and growth factors

Interleukin (IL)-6 was evaluated in 14 studies. Nine studies reported significantly reduced levels of IL-6 in laparoscopic or laparoscopic colorectal surgery compared to open surgery from as early as 2 hours through to postoperative day (POD) 4 after surgery.[100, 106, 107, 110, 112-115] Three studies however found no difference in IL-6 levels between surgical approaches.[101, 102, 118] Of note, Stage et al reported significantly elevated levels of IL-6 in the laparoscopic group compared to open.[120] Although heterogeneous in reported results and at various time points, 9 studies provided sufficient data to be included in the meta-analysis for POD 1 (Figure 2), and 4 studies for POD 3 (Figure 3). On POD 1, patients in the laparoscopic group (n = 343) had significantly lower levels of IL-6 (-0.58 [-0.90, -0.25] P <0.05, I^2 = 75%). Similarly, on Day 3, patients within the laparoscopic group (n = 93) had lower IL-6 levels compared to equivalent open resectional surgery (-0.82 [-1.62, -0.04] p <0.05; I^2 = 83%). The Funnel plot for publication bias is shown in Figure 4.

In contrast, TNF-α levels were not significantly elevated in three of four studies.[110, 114, 118] Ordemann et al reported significantly lower levels of TNF-α in the laparoscopic group from 1 hour after surgery.[106] Other cytokines such IL-1,[107] IL-8,[115, 118, 121] and IL-10[107, 108] were not statistically different between operative approaches.

Similarly, immunoglobulin levels (IgA, IgG and IgM) and complement components (C3 and C4) were not statistically significant between open and laparoscopic

colectomy.[109] Other proteins such as α-defensin, were significantly lower in the laparoscopic group compared to the open group at POD1.[110]

The growth factor VEGF was significantly lower in the laparoscopy group on POD 2 and POD 4 in a study comparing laparoscopic to open colon surgery.[113] No differences however, were reported in a study comparing open and laparoscopic surgery,[108] and another study comparing laparoscopic and open colorectal surgery.[117]

VEGF receptor-1 (VEGFR1) expression was no different between open and laparoscopic colectomy.[108] The receptor TLR-2 expression was down-regulated significantly in the laparoscopic group compared to the open group at 24 hours after surgery. TLR-4 showed a similar down-regulated expression, however this was not significant compared to the open group.[110] Meta-analysis was not possible for other markers of the humoral response.

2.3.5 Cell mediated response

Activated total leucocyte count, lymphocyte, T cell, activated T cells and Human Leukocyte Antigen-antigen D related (HLA-DR), Natural killer (NK) cells, leukocyte count, Cluster of differentiation (CD) immunophenotyping

Total leukocyte count was evaluated in five studies.[101, 106, 115, 118, 121] Only one study, however, showed an elevated leucocyte count in the open approach group.[106] Lymphocyte, lymphocyte subtypes, sub-fractions, associated surface glycoproteins, and other receptor proteins, were evaluated in 8 studies.[99, 101, 102, 105, 106, 109, 112, 118] Only two studies found a significant difference between surgical approaches.[105, 112] Liang showed that the total lymphocyte count was significantly higher in the laparoscopic group at POD 1; and Wang in 2003 showed a

significant reduction of CD3 in the laparoscopic group at POD1, 3 and 5. When comparing CD4/CD8 ratios, three studies showed a significantly lower ratio in the open approach group from POD1.[99, 105, 112] Wu *et al* however found no difference in the CD4/CD8 ratio.[118]

HLA-DR, a protein receptor involved in the cell mediated response, was evaluated in 6 studies.[100, 102, 106, 115, 118, 121] Three studies showed an increased expression of HLA-DR in the laparoscopic compared to the open group.[106, 115, 121]

2.3.6 Oxidative stress response

8-isoprostanes (8-epiPFG$_{2\alpha}$), protein carbonyls (PC), 8-hydroguanosine (8-OHG), 3-nitrotyrosine (3-NT), malondialdehyde (MDA), ascorbic acid (AA), and dehydroascorbic acid (DHA)

Two studies evaluated species of oxidative stress between laparoscopic and open surgery.[104, 119] 8-epiPFG$_{2\alpha}$, PC, 3-NT, and 8-OHG were measured following colorectal surgery, whilst MDA, AA and DHA were measured after sigmoid surgery only. Laparoscopic surgery showed statistically significantly lower levels of *8-epiPFG$_{2\alpha}$*, and 3-NT at 6 hours and 24 hours after surgery; and statistically significantly lower levels of 8-OHG at 24 hours after surgery. There were no reported differences for PC, MDA, AA and DHA.

2.3.7 Endocrine and metabolic response

Cortisol, prolactin, and growth hormone

Two studies evaluated the metabolic response following colon surgery,[100, 115] and one study following rectal surgery.[99] The hormones were measured from 2 hours to

7 days after surgery. There was, however, no reported difference between laparoscopic and open surgery for both colonic and rectal surgery.

2.3.8 Other inflammatory biomarkers

C-reactive protein (CRP) and erythrocyte sedimentation ratio (ESR)

CRP, a protein modulated initially by the release of IL-6 and produced by the liver, was measured in 13 studies.[99-101, 105, 107, 110, 112, 114, 115, 118, 120, 121] All but four studies showed a reduction of CRP with the laparoscopic approach. Three studies showed no difference,[115, 120, 121] whilst Stage et al, reported a significant increase of CRP in the laparoscopic group compared to open.[120] Ten studies provided sufficient data for meta-analysis (Figure 5). Patients in the laparoscopic resectional group (n = 252), had a statistically lower level of CRP on Day 1 compared to equivalent open resectional surgery (-1.06 [-1.62, -0.50] p <0.05; I^2 = 88%).

In a single study, ESR, a non-specific measure of inflammation based on the rate of red blood cells sediment, was also significantly lower in the laparoscopic group at POD 1.[105]

2.4 Discussion

This systematic review critically appraised 22 randomised controlled trials (RCTs) evaluating the inflammatory stress response according to approach after colon and rectal surgery. Included RCTs were heterogeneous in the type of biomarker measured, time point of marker measurement, surgical indication, and surgical approach and follow up. The most evaluated inflammatory biomarkers were IL-6 and CRP. Meta-analysis and qualitative analysis showed that the laparoscopic approach is associated

with a reduced humoral and inflammatory response compared with equivalent open surgery.

Recent studies have also shown improved clinical outcomes with improved outcomes with the laparoscopic approach attributed to the reduced inflammatory response. The smaller access incision seems to be the primary producer of inflammatory biomarkers, thus dwarfing the intra-abdominal response resulting in a reduced overall response. [112, 118, 122] This theory explains, albeit simply, the apparent clinical benefits. Dunker *et al* however, compared groups based on incision size (<8cm and >8cm) and found no demonstrable difference between them. [122] Similarly, Stage *et al* found significantly elevated CRP and IL-6 in the laparoscopic group. To explain this phenomenon, the study authors reported that cyclo-oxygenase inhibitors had been administered perioperatively, which have been shown to reduce the immunomodulatory effects of prostaglandin, resulting in compensatory IL-6 and CRP synthesis.[123]

The clinical benefits of a smaller access incision with AL, however, remains inconclusive. As discussed in Chapter 1: Introduction, most large prospective studies did not show improvement in AL rates with laparoscopic and open approach surgery. [99, 105]. Robotic-assisted surgery also showed comparable anastomotic leak rates with laparoscopic surgery. [124]

Recent studies have been conducted in an enhanced recovery (ERAS) perioperative setting. ERAS protocols are designed to attenuate the inflammatory response following surgery. These protocols have been shown to improve perioperative outcomes. In this literature review, four studies compared laparoscopic with open surgery in an ERAS setting. Anastomotic leakage rates were similar in these studies.

Two studies went further comparing ERAS to conventional perioperative care. [112, 121] Both studies found that the ERAS groups had a reduced systemic inflammatory response compared to non-ERAS groups, however this did not translate to a reduced anastomotic leakage rate. Another recent study by Teeuwen *et al*, directly compared ERAS and conventional postoperative care in colorectal surgery patients. They too found similar anastomotic leak rates despite improved overall morbidity and a reduced inflammatory response. [125]

CRP and IL-6 were the two most evaluated inflammatory biomarkers in this literature review. CRP especially has been increasingly used as an adjunct to aide in the early diagnosis of AL following surgery. A recent meta-analysis indicates that a low serum CRP on post-operative days 3 to 5 is a useful negative predictive test for AL.[3] The studies included in that meta-analysis however, included predominantly open approaches, and results therefore may not apply specifically to laparoscopic surgery as the post-operative inflammatory response may be altered.

There are several limitations to this literature review. There was significant heterogeneity with the included studies. This is particularly evident when attempting to summarize individual studies reporting variable outcomes in conjunction with the measured variable. The timing of blood tests and methods of analysis varied considerably between studies. The quality of studies ranged from poor to good with only three studies reported blinding. Furthermore, the definitions of colon and rectal surgery varied among included studies.

A recent study showed a reduced cytokine response in colon resection compared with rectal surgery [87]. Andethersson *et al,* directly compared the inflammatory response between colonic and rectal resections. [126] They showed that Interleukin-10 was

significantly increased in the rectal group on day 0 and day 3. Other significant differences were detected for albumin, prealbumin, and total iron-binding capacity (TIBC) in the early postoperative period. It is unclear what the clinical significance of this is for the development or early detection of AL. Interestingly CRP, IL-6, IL-8, glucose, and cortisol did not show any differences between rectal and colon groups. Future studies should consider differentiating these two groups and studying them separately given the apparent inflammatory response differences observed.

Laparoscopic colorectal surgery is associated with reduced IL-6 and CRP on POD 1 compared with equivalent open colorectal surgery. The response between colon and rectal surgery appears to be different but more work is required in the future to clarify this.

Chapter 3

SYSTEMATIC REVIEW OF CLINICAL PARAMETERS RELATED TO ANASTOMOTIC LEAKAGE AFTER MAJOR COLORECTAL SURGERY

3. SYSTEMATIC REVIEW OF CLINICAL PARAMETERS RELATED TO ANASTOMOTIC LEAKAGE AFTER MAJOR COLORECTAL SURGERY

3.1 Background

Even with the advent of ERAS protocols, the introduction of AL calculators, and the utility of biomarkers, the early diagnosis of AL remains a significant diagnostic challenge.[89, 127-130] As discussed in the introduction this is often due to its non-specific clinical presentation and confusion with other common postoperative complications.

Traditionally, the diagnosis of AL begins by the piecing together of clinical signs and symptoms, the interpretation of a variety of blood tests, and a high index of clinical suspicion supplemented by radiological investigations. Compared with the utility of inflammatory biomarkers, the diagnostic value of clinical features has infrequently been investigated following colorectal surgery. Clinical findings when correlated with measured inflammatory markers in the post-operative period may improve the detection of AL. This literature review aims to collate clinical symptoms and signs in the context of AL following major colorectal surgery. Results from this literature review will help to identify relevant clinical parameters to be prospectively collected in the BALL study described later in the thesis.

3.2 Methods

3.2.1 Literature search

A comprehensive review of the literature was conducted according to the guidelines in the Preferred Reporting Items for Systematic review and Meta-Analysis (PRISMA)

statement. [95] The following databases were search from 1947 to April 2018: MEDLINE, Embase and PubMed. The search terms used are shown in Table 6. Reference list of all relevant papers were search manually to identify further studies. Studies restricted were restricted to Humans.

Table 6 Search terms used

Vital sign$ OR clinical sign$ OR blood pressure OR heart rate OR temperature OR resp$ rate OR clinical measure$ OR pain OR abdom$ discomfort OR abdom$ pain OR peritonitis AND Anastomotic leak$ OR anastomotic dehiscence

3.2.2 Study selection

All published studies measuring patient clinical parameters such as vital signs including abdominal pain, in the context of AL after colorectal surgery, were included in this review. Studies were limited to colorectal surgery leaks only. Studies were assessed independently by the PhD candidate, and another researcher, Weis Xia. Any disagreements over inclusion and exclusion were resolved by discussion with the candidate's primary supervisor, Prof Andrew G Hill.

3.2.3 Data extraction

Data extraction was carried out by the candidate with another researcher, Weis Xia, and entered into predefined tables. The outcome of interest was AL as defined as

reported in the included studies. Study design, clinical parameter measurements, and behaviour in the context of AL, were recorded.

3.2.4 Quality assessment

Quality assessment of the studies were applied independently by the candidate and fellow researcher, Weis Xia using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS)-2 tool. [131] This assesses risk of bias and concerns about applicability by evaluating four key domains: patient selection, index test, reference standard, flow of patients through the study, and timing of tests.

3.3 Results

A PRSIMA flow chart showing the selection of articles for this systematic review is presented in Figure 7. In total, thirteen studies met the inclusion criteria.

3.3.1 Study characteristics

The characteristics of the included studies are shown in Table 7 below. Most included studies were prospective observational studies. The majority of included patients underwent elective colorectal surgery for colorectal cancer through an open surgical approach. Using the QUADAS-2 tool, the applicability of the included studies was average to good.

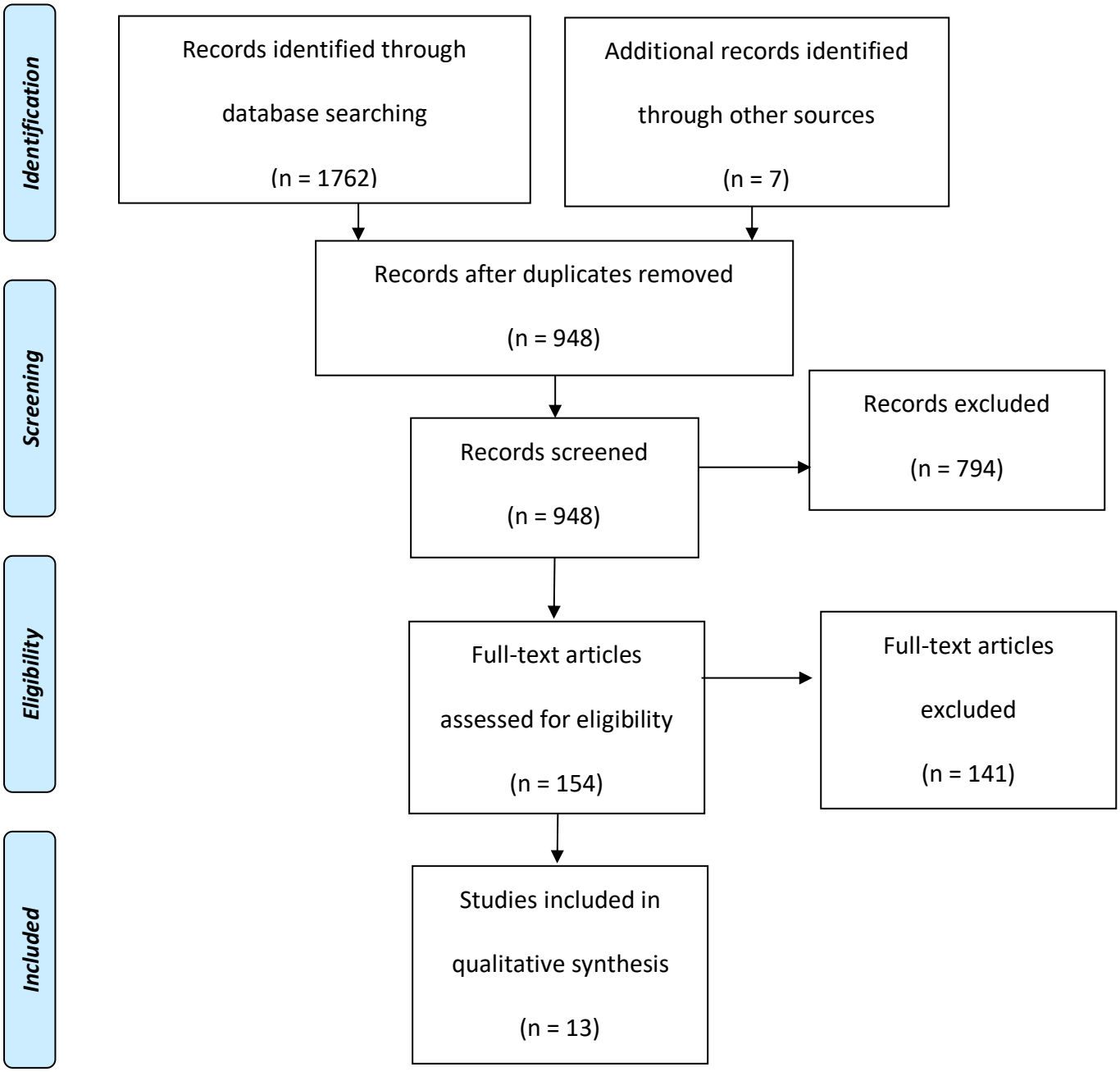

Figure 7 PRISMA flow diagram

Table 7 Study characteristics

Author, year	Study design	Study interval	Acuity	Disease process	Colorectal	Approach	n	AL rate
Alves, 1999 [132]	Retrospective	1990-1997	Elective (100%)	Cancer (39.5%)	Colorectal	-	655	6%
Aziz, 2016 [133]	Case controlled	2006-2013	Elective (100%)	FAP	Colorectal	Laparoscopic (100%)	95	10.5%
Bellows, 2009 [134]	Retrospective	2000-2005	Elective (95%)	Cancer 71%	Colorectal	-	311	8%
Doeksen, 2007 [135]	Retrospective	2000-2003	-	-	Colorectal	-	289	12.45%
Eckmann, 2004 [136]	Prospective	1992-2000	Elective (100%)	Cancer (100%)	Rectal	Open (100%)	306	9.8%
Facy, 2015 [137]	Prospective	2011-2014	Elective (100%)	Cancer (69.5%)	Colorectal	Open (63.5%)	501	11.8%
Floodeen, 2012 [138]	Case matched	1999-2005	-	Cancer (100%)	Rectal	-	234	19.23%

Garcia-Granero, 2010 [139]	Prospective	2008-2010	Elective (100%)	Cancer (73%)	Colorectal	Open (79%)	205	8.29%
Guo, 2015 [140]	RCT	2013	-	Cancer (100%)	Colorectal	Laparoscopy (100%)	57	7%
Mileski, 1988 [127]	Retrospective	1978-1986	Elective (100%)	Cancer (53.1%)	Colorectal	Open (100%)	405	4%
Nesbakken, 2005 [141]	Prospective	-	-	Cancer (100%)	Rectal	-	115	9%
Ortega-Deballon, 2010 [142]	Prospective	2007-2008	Elective (100%)	Cancer (61.6%)	Colorectal	Open (88%)	133	15.5%
Post, 2012 [143]	Prospective	(24-month period)	Elective (92%)	Cancer (61%)	Colorectal	Open (63.9%)	285	5.26%
Sutton, 2003 [144]	Prospective	1994-1998	-	Cancer	Colorectal	-	379	6%

3.3.2 Definition of anastomotic leakage

Included studies had varying definitions of AL ranging from clinical signs of peritonitis, free faeces, or pus in the drain fluid, to extravasation of contrast on computer tomography scan, endoscopy, or on re-laparotomy. Reported AL rates ranged from 4%[127] to 15.5%.[142]

3.3.3 Clinical parameters measured

A summary of the measured clinical parameters, and when they were first detected leading up to the diagnosis of AL is shown in Table 8, and Table 9, respectively. Key observations are outlined below.

3.3.3.1 Heart rate

Three studies evaluated the presence of tachycardia (described as HR > 90 beats per minute) in the presence of AL. [133, 135, 139] All three studies found tachycardia to be a feature of AL. [133, 139]

3.3.3.2 Respiratory rate

Three studies evaluated RR or the presence of tachypnoea (defined as RR>20 breaths per minute). [133, 134, 139] Although median RR was significantly higher in AL compared to non-AL patients [133, 139], Bellows found that tachypnoea was present in only 9% of patients with AL. [134] Oxygen saturation was not significant between the two groups. [133]

3.3.3.3 Fever

Ten studies measured patient temperature or recorded the presence of fever (defined as temperature >38°C) in the context of AL. [127, 132, 134-136, 138, 139, 141, 142, 145]. The proportion of AL patients with fever ranged from 19% [132] to 94%. [127] One study reported a significantly higher proportion of AL patients with fever, [142] whilst another study reported a markedly higher median temperature (37.4°C vs. 36.8°C). [139]. Comparing between minor and major AL, Paliogiannis *et al* reported a higher proportion of major AL with fever (100% major AL vs. 55.6% minor AL). [145]

3.3.3.4 Abdominopelvic signs and symptoms

Seven studies reported the presence of abdominopelvic pain and/or peritonitis among AL patients.[127, 134-136, 141, 144, 145] The presence of increased abdominopelvic pain varied from 20% [141] to 100% of AL patients.[127] Bellows et al,[134] found a significantly higher proportion of AL patients complaining from increased abdominal pain compared to the non-AL group (52% vs. 17.5%). On a time analysis, Ortega-Deballon *et al* showed that the presence of increased abdominopelvic pain was statistically significant but only from Day 6 after surgery.[142] In one study, signs of diffuse peritonitis were only present in 55.6% of major AL, whilst completely absent in minor AL.[145]

3.3.3.5 Bowel function

When evaluating time to first bowel motion after surgery, both Ortega-Deballon *et al* and Bellows *et al* showed no differences between the two groups.[134, 142] In one study, the absence of bowel activity was significantly higher in AL patients by (Postoperative day) POD 6 compared with non-AL group (48% vs. 5.6%).[134] Alves *et*

al showed that AL patients had significant transit disturbances such as absence of bowel movement on POD4 (49% vs. 18% in non-AL), diarrhea before POD 7 (36% vs. 13% in non-AL). Furthermore, a significantly higher proportion of AL patients exceeded 400mls peritoneal drain fluid output compared to non-AL patients (47% vs. 20%). Nesbakken *et al* reported none of the AL group exhibited bowel paresis, whilst it occurred in 12.2% of non-AL patients.

3.3.3.6 Blood pressure

Two studies evaluated preoperative, intraoperative and postoperative blood pressures.[133, 146] Preoperatively, Post *et al* found that a diastolic blood pressure greater than 90mmHg is significantly associated with the development of AL. Similarly, an intraoperative reduction of diastolic BP greater than 40% is significantly associated with AL.[146] Aziz *et al* showed a higher postoperative diastolic BP, and a higher mean arterial pressure (MAP) is associated with AL.[133]

3.3.3.7 Urine output

Mean urine output in one study was shown to be significantly lower in AL patients.[133] Alves *et al* similarly showed a higher proportion of AL patients had a UO of < 1L/24 hours (37.5% vs. 18.8% in non-AL).

3.3.3.8 Other signs and symptoms

Neurological symptoms such as an altered mental state, was significantly more common in AL patients compared to non-AL (24% vs. 3%).[134] In a series of 379

patients, 30 patients suffered cardiac type symptoms only that were investigated, of which 13 (40%) later developed an AL.[144]

3.3.4 Onset of clinical parameters and the diagnosis of anastomotic leakage

Seven studies directly compared onset of clinical symptoms and signs with the diagnosis of AL.[132-135, 137, 138, 142] Median AL diagnosis ranged from POD 6 to POD 10. A variety of symptoms and signs were analyzed. None were consistently present within the first 6 days after surgery.

Table 8 Summary of outcomes

Clinical parameters	**Study**	**Reported outcome**
Tachycardia* or reported median heart rate	Aziz	Median HR higher in AL group (sig.)
	Doeksen	61% of AL
	Garcia-Granero	Median HR higher in AL (sig.)
Tachypnoea** or RR	Aziz	Higher median RR in AL (sig.)
	Bellows	Tachypnoea present in 9% of AL
	Garcia-Granero	Higher median in AL (sig.)
Fever (>38°C)	Alves	19% in AL vs 4.9% in non-AL
	Bellows	32% in AL, median 37.8 in AL
	Doeksen	66.7% of AL
	Eckmann	93% of AL
	Floodeen	Median 37.4°C $
	Garcia-Granero	Median 37.4°C in AL vs 36.8°C non-AL (sig.)
	Mileski	94% of AL
	Nesbakken	50% of AL vs. 22% in non-AL
	Ortega-Deballon	22.2% of AL vs. 5% in non-AL (sig.)
Abdomino-pelvic pain and/or presence of peritonitis	Bellows	52% in AL vs. 17.5% in non-AL (sig.)
	Doeksen	28% in AL
	Eckmann	93% in AL
	Mileski	100% in AL
	Nesbakken	20% in AL vs. 0% in non-AL
	Sutton	32% in AL
	Ortega-Deballon	Presence is sig. on POD6
Blood pressures	Aziz	Higher DBP in AL (sig.)
		Higher MAP in AL (sig.)
	Post	Higher preoperative DBP in AL (sig.)

		>40% diastolic relative hypotension in AL (sig.)

*defined as >90 beats per minute

**defined as >20 breaths per minute

RR: respiratory rate

Sig.: statistically significant

$: early leaks

DBP: Diastolic blood pressure; MAP: mean arterial pressure

POD: postoperative day

Table 9 Onset of clinical symptoms prior to AL

Study	**AL diagnosed**	**Onset of symptoms**
Alves [132]	8.1 +/- 4 days (4-25 days)	Fever D2 (sig.)
		Absence of bowel movement D4 (sig.)
		Diarrhoea before D7 (sig.)
		Drain fluid >400mls D0-D3 (sig.)
		Oliguria D4 (sig.)
Aziz [133]	>4.5 D	HR higher D4 (sig.)
		Oral intake D1 higher in AL (sig.)
		Stool volume lower in AL (sig.)
		MAP, DBP, RR, UO
Bellows [134]	10.2 +/-1.1 Day (4-28 days)	Time to flatus D4
		Time to BM D5
		Fever in 32% AL D4
		Temp 37.8 (mean) before Dx

Doeksen [147]	D7 +/- 4.1 (3-24 days) median	Tachycardia D4
		Fever D5
		Peritoneal reaction D6
Facy [137]	D7 (5-12days interquartile) (median)	Fever 16.9% AL D4
Floodeen [138]	D8 (3-18) (median) early AL	37.4 median (36.4-38.5) in AL
Ortega-Deballon [142]	D6 median (4-12 days)	Fever higher proportion AL D6 (sig.)
		Time to BM D4
		Abdominal tenderness D6 (sig.)

3.4 Discussion

This literature review has identified several clinical signs and symptoms leading up to the diagnosis of anastomotic leakage. These include tachycardia, diastolic hypertension, fever, increased abdominopelvic pain, increased pelvic drain output, and reduced urine output. However, none was consistently abnormal leading up to the diagnosis of AL.

The non-specific nature of clinical features of AL is a direct result of the systemic inflammatory response. The type of leak, whether contained or free, is likely to dictate the clinical features seen.[148] Patients with a contained AL in rectal anastomoses can have ambiguous symptoms such as perianal pain and scrotal swelling, compared with free AL, where peritonitis is a common feature. As a result, contained leaks are often detected later in the postoperative period.

Most of the signs or symptoms showed heterogenous results between studies. This may be explained by the subjective interpretation of bedside clinical assessment,

particularly abdominal pain and tenderness.[149] Another reason is the heterogeneity in study populations. The included studies included both colonic and rectal resections and did not differentiate between the two. As discussed in Chapter 2, these two groups of patients appear to have different inflammatory responses, and this may be reflected in clinical features. Furthermore, there was significant heterogeneity in defining different signs and symptoms. Fever, tachycardia, or abdominal pain definitions varied between study populations.

This literature review has identified several non-specific clinical signs and symptoms associated with the diagnosis of AL after colorectal surgery. These include tachycardia, diastolic hypertension, fever, increased abdominopelvic pain, increased pelvic drain output, and reduced urine output. However, none were consistently abnormal leading up to the diagnosis of AL.

Chapter 4

SYSTEMATIC REVIEW OF BIOMARKERS IN THE DIAGNOSIS OF ANASTOMOTIC LEAKAGE FOLLOWING COLORECTAL SURGERY

4. SYSTEMATIC REVIEW OF BIOMARKERS IN THE DIAGNOSIS OF ANASTOMOTIC LEAKAGE FOLLOWING COLORECTAL SURGERY

4.1 Background

Recovery after surgery for patients with colorectal disease has improved markedly with the advent of minimal access surgery and enhanced recovery after surgery protocols (ERAS). [16] Despite these advances, AL remains as one of the most dreaded complications following colorectal surgery, with a reported AL rate of 3% to 27%.[150, 151]

As noted above although a subset of risk factors has been reported, AL remains difficult to predict and diagnose in the early phase after surgery.[152-154] In many cases, the course of AL is insidious with ileus, vague abdominal symptoms, and failure to progress, with a mean time to clinical diagnosis of 6 to 12 days after surgery.[12, 13] Moreover, in the early postoperative period, intra-abdominal sepsis can be difficult to distinguish from the physiological systemic inflammatory response to surgery.[155, 156]

With the advent of ERAS, patients may be discharged three to four days after surgery and thus patients run the risk of readmission with AL or severe sepsis.[16, 17] If diagnosed late, AL can progress to overwhelming sepsis, multi-organ dysfunction, and death. Delayed diagnosis and subsequent delay in antibiotic administration from the onset of septic shock has been associated with a decrease in survival of 7.6% per hour.[18] Furthermore, long term consequences of AL include increased colorectal cancer recurrence, reduced quality of life, and decreased long term survival.[10, 19,

20] Thus a timely diagnosis of AL before clinical symptoms become apparent is of utmost importance.

A biomarker is defined as a characteristic that is objectively measured as an indicator of a pathogenic process. Following colorectal surgery, biomarkers involved in the healing process have been identified and have shown promise in detecting various stages of early ischaemia, inflammation, and necrosis. [157, 158] Previously, studies evaluating colorectal infective complications have focused primarily on non-specific systemic markers of inflammation such as CRP [159, 160] or analysing peritoneal drain fluid.[161] Larger studies in an ERAS environment have since been conducted to evaluate a broader range of biomarkers involved in the different stages of healing following major colorectal surgery. This systematic literature review therefore aims to assess the potential of biomarkers for pre-clinical detection of AL following colorectal surgery.

4.2 Methods

4.2.1 Literature search

A comprehensive review of the literature was conducted according to the guidelines in the Preferred Reporting Items for Systematic review and Meta-analysis (PRISMA) statement.[162] The following databases were searched from 1990 to June 2016: MEDLINE, Embase and PubMed. The search terms used are shown in Table 10. Reference lists of all relevant papers were searched manually to identify further relevant studies. Studies were restricted to Humans and English language with reviews excluded.

Table 10 Search terms and hits per database

Database **(number of results)**	**Search terms**
Medline (77 results)	biomark$ OR marker$ OR inflam$ biomark$ AND colorect$ OR rectal OR gastrointestinal OR bowel AND anastomo$ leak$ OR anastomo$ dehisce$
PubMed (83 results)	
EMBASE (102 results)	

4.2.2 Study selection

Search results were entered into a unified database on the EndNote Reference software. Title and abstracts were screened. All published studies evaluating biomarkers, both systemic and peritoneal drain, in the context of AL following colorectal surgery were included in this review. Exclusion criteria were animal studies, studies without an English translation, inclusion of gastro-intestinal anastomosis other than colorectal, and studies that did not measure plasma, serum, or peritoneal levels of biomarkers.

Clinical studies were assessed independently by three authors (Bruce Su'a, Hinetamatea Mikaere, Jamie-Lee Rahiri) and any disagreements over inclusion and exclusion were resolved by consensus. Consultation with doctoral supervisor, Prof. Andrew G. Hill if consensus was not reached.

4.2.3 Validity assessment

In included clinical studies and where appropriate quality assessment was applied independently by BS, SM and JLR using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS)-2 tool [131]. This assessed risk of bias and concerns about applicability by evaluating four key domains: patient selection, index test, reference standard, flow of patients through the study, and timing of tests.

4.2.4 Data abstraction

All included studies' characteristics, and data on evaluated biomarkers were collated and summarised in predefined tables. The outcome of interest was AL defined as reported in the included studies. Study design, objective methods for analysing biomarker(s), biomarker behaviour in the context of AL, diagnostic utility and if reported, predictive indices for AL were recorded.

4.3 Results

A total of 262 studies were included in the unified database. A PRISMA flow chart showing the selection of articles for this systematic review is presented in Figure 8 below. In total, thirty-six studies met the inclusion criteria.

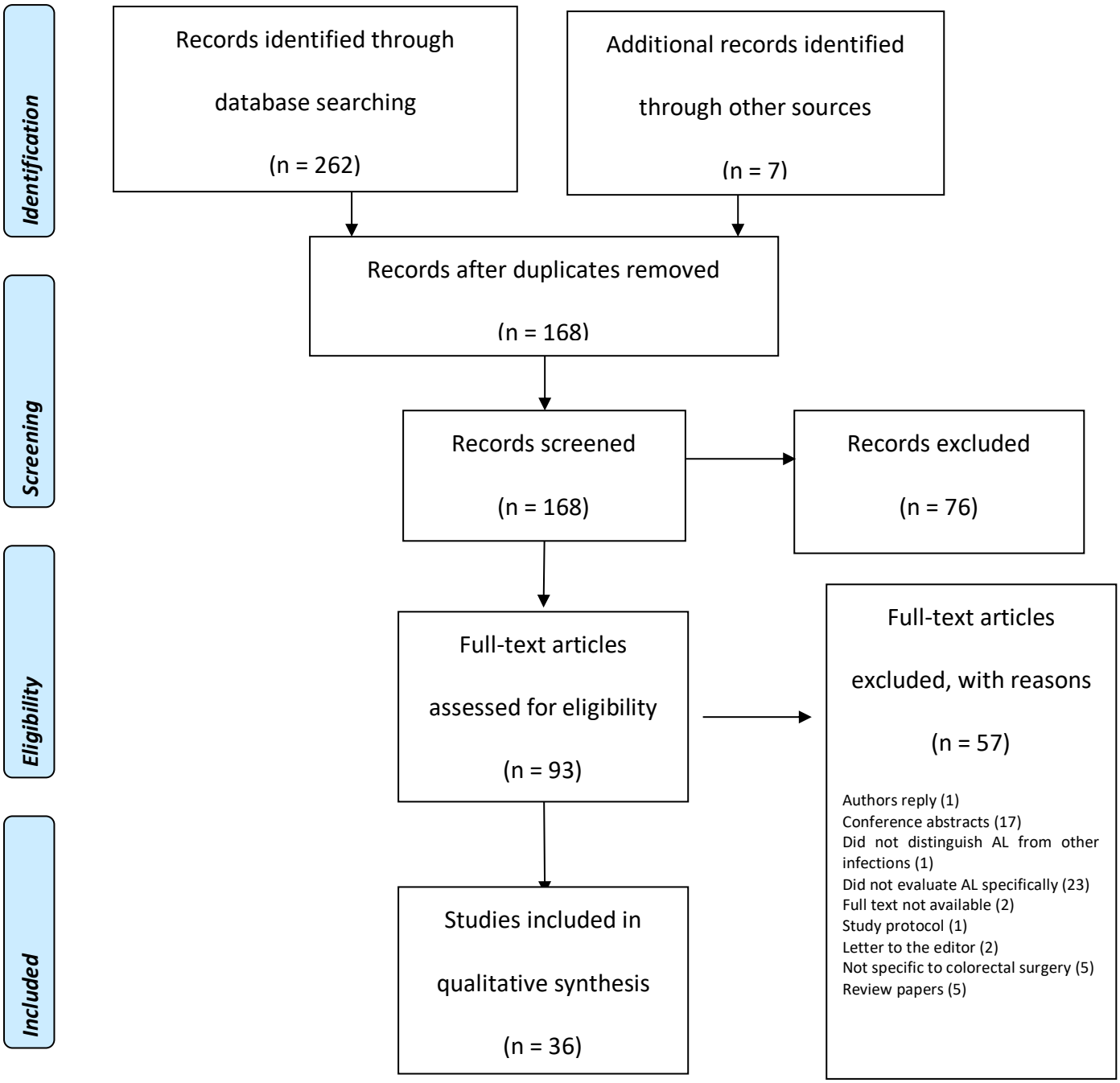

Figure 8 PRISMA flow chart

4.3.1 Study characteristics

The included studies were divided into peritoneal drain biomarker and systemic biomarker studies. Study characteristics peritoneal and systemic biomarkers are summarized in Table 11 and Table 12 respectively below.

Fifteen studies evaluated peritoneal drain biomarkers [18, 89, 101, 138, 158, 163-171], and 24 studies analysed systemic biomarkers.[13, 137, 139, 142, 146, 150, 151, 163, 169, 172-185] Three studies evaluated both peritoneal and systemic biomarkers.[89, 163, 169] Eleven of the 14 studies that evaluated peritoneal drain biomarkers were prospective studies. Most patients included in studies that evaluated peritoneal drain biomarkers underwent elective surgery for colorectal cancer through an open approach. Enzyme-linked immunosorbent assay (ELISA) was the most commonly used method to measure peritoneal drain biomarkers.

In studies evaluating systemic biomarkers, 12 were prospective studies (52%). Four studies evaluated rectal surgical patients only. Similarly, most included patients in studies that evaluated systemic biomarkers underwent elective colorectal surgery for colorectal cancer via an open approach.

4.3.2 Validity assessment

The results from the QUADAS-2 tool are shown in Table 13. Overall, the applicability of included studies was average to good. Only two studies reported blinding.[142, 186]

Table 11 Summary of included studies evaluating intra-peritoneal biomarker(s)

AUTHOR, YEAR	STUDY DESIGN	STUDY PERIOD	ELECTIVE	OPEN	OPERATION FOR CANCER	N	AL RATE	BIOMARKER(S)	BIOMARKER STUDY METHOD
ALONSO, **2015 [163]**	Prospective matched	3 years	100%	87%	100%	280	10.7%	Interleukin (IL)-6 Vascular endothelial growth factor (VEGF)	Enzyme-linked immunosorbent assay (ELISA)
BERTRAM, **2002 [164]**	Prospective	6 months	100%	100%	100%	25	12 %	IL-6 Tumour necrosis factor (TNF)-α	Chemiluminescence immunometric assay
ELLEBAEK, **2009 [165]**	Prospective	-	-	100%	100%	45	9%	Lactate, pyruvate, glucose, and glycerol	Peritoneal micro dialysis
FOUDA **2011 [187]**	Prospective	33 months	100%	-	100%	56	14.3%	IL-6, IL-10 TNF-α	ELISA for cytokines

								Klebsiella, E. coli, and *Pseudomonas* spp	Culture for microbiology
HERWIG, **2002 [188]**	Prospective matched	13 months	100%	-	38.4%	24	50% (matched)	IL-1β, IL-6 and TNF-α	ELISA
JUNGER, **1996 [167]**	Prospective randomised study	-	-	-	100%	43	18.6%	Lysozyme	Testomar® lysozyme kit
KOMEN, **2014 [168]**	Prospective	35 months	100%	62%	84%	243	7.8%	C-reactive protein (CRP) Lipopolysaccharide binding proteins (LBP) Procalcitonin (PCT)	LBP using the IMMULITE 1000. CRP (Tina-quant assay on the Hitachi 912. PCT (LUMI-test PCT).

KOMEN, **2014 [189]**	Prospective	2 years	100%	62%	84%	243	7.8%	*E. coli* *E. faecalis*	Polymerase chain reaction (PCR)
KOSTIC, **2015 [169]**	Prospective	19 months	100%	-	100%	150	10%	Matrix metalloproteinase (MMP)-9	ELISA
MATTHIESSEN **2007 [190]**	Prospective	23 months	100%	-	100%	23	30%	Glucose, Pyruvate, Lactate, IL-6, IL-10 and TNF-α	Enzyme-labelled chemiluminescent sequential immunometric assay
PASTERNAK, **2010 [158]**	Prospective	31 months	-	10%	100%	29	34%	Matrix metalloproteinase (MMP)-1, MMP-8 MMP-13, MMP-2	Particle-based flow-cytometry sing Flourkine Multi Analyte Profiling (F-MAP) kits.

								MMP-9, MMP-3, MMP-7. Tissue inhibitor metalloproteinase (TIMP)-1 and TIMP-2	TIMP-1 and TIMP-2 were analysed using ELISA
SAMMOUR, **2016 [191]**	Prospective	7 years	100%	-	65%	206	8.3%	IL-6, IL-10 TNF-α	ELISA
UGRAS **2007 [170]**	Prospective	12 months	100%	-	100%	34	11.7%	IL-6, IL-10 TNF-α	ELISA
YAMAMOTO, **2011 [192]**	Prospective	-	100%	100%	100%	100	7%	IL-1β, IL-6 and TNFα	ELISA
YANG **2013 [171]**	Retrospective	7 years	-	88%	100%	753	7.6%	pH levels	pH meter pp-15

Table 12 Summary of included studies evaluating systemic inflammatory biomarker(s)

AUTHOR, YEAR	Study Design	Study duration	Elective	Open approach	Colon operation	Operation for cancer	n	AL rate	Biomarker(s)
ALMEIDA, 2012 [146]	Retrospective	22 months	95%	82%	80%	75%	173	13.9%	C-reactive protein (CRP)
ALONSO, 2015 [163]	Prospective matched	3 years	100%	87%	80%	100%	280	10.7%	Interleukin (IL)- 6, vascular endothelial growth factor (VEGF)
ELLEBAEK, 2014 [172]	Prospective matched	-	100%	100%	0%	100%	50	8%	Granulocyte macrophage colony-stimulating factor, interferon-γ, IL-1,1β,2,5,6,8,10, TNF-α, mannin binding lectin and Membrane attack complex

FACY, 2015 [137]	Prospective	29 months	100%	70.3%	73.6%	69.5%	510	11.5%	CRP, Procalcitonin (PCT)
GARCIA-GRANERO, 2013 [139]	Prospective	17 months	100%	79%	58.5%	73.2%	205	5.4%	CRP, PCT
GIACCAGLIA, 2015 [173]	Prospective	21 months	100%	25%	65%	100%	504	5.6%	CRP, PCT
GIACCAGLIA, 2014 [174]	Prospective	12 months	100%	88%	76%	94%	101	8.9%	CRP, PCT, Leukocytes (WCC)
IANCU, 2008 [151]	Retrospective	4 years	77%	-	100%	100%	993	3.22%	Protein, haemoglobin

IVERSEN, 1999 [175]	Prospective matched	21 months	100%	100%	0%	-	161	10.6%	Prothrombin fragment 1 + 2, Thrombin anti-thrombin complex, Soluble fibrin, tPA, PAI-1
KASER, 2014 [176]	Retrospective	60 months	-	-	-	43%	1106	7.3%	Na^+ WCC
KORNER 2009 [177]	Retrospective	12 months	77%	76%	-	63%	201	8.9%	CRP WCC
KOSTIC, 2015 [169]	Prospective	20 months	100%	-	56.7%	100%	150	10%	CRP
LAGOUTTE, 2012 [13]	Prospective	13 months	100%	65%	68%	55%	100	13%	CRP, PCT
MATTHIESSEN, 2008 [150]	Prospective	19 months	100%	-	0%	32/33	33	27.2%	CRP, WCC

ORTEGA-DEBALLON, 2010 [142]	Prospective	11 months	100%	88%	42%	61.7%	133	15.5%	CRP, WCC
PEDERSEN, 2012 [178]	Retrospective	12 months	-	0%	78%	-	129	18%	CRP
PLATT, 2012 [179]	Retrospective	10 years	87%	100%	66.1%	100%	454	5.7%	CRP Albumin WCC
RAMANATHAN, 2013 [193]	Prospective	7 years	100%	92%	63%	100%	357	3.9%	CRP

REISINGER, 2014 [186]	Prospective	26 months	100%	57.1%	77.3%	100%	84	10%	CRP, Calprotectin, IL-6, fatty acid binding proteins
SLOTWINSKI, 2002 [181]	Prospective matched	-	100%	-	32%	100%	22	9%	IL-1b,1α,1ra,6,8,10. TNF-α, soluble TNF receptor 1
SAMMOUR, 2016 [89]	Prospective	7 years	100%	-	-	65%	206	8.3%	IL-6, IL-10, TNF-α
WARSCHKOW, 2011 [182]	Retrospective	12 years	91.8%	100%	33.4%	100%	1115	8%	CRP WCC
WELSCH, 2007 [183]	Retrospective	4 years	-	100%	0%	100%	96*	5.7%	CRP WCC Platelets

WOESTE, 2010 [184]	Retrospective	3 years	88%	72.8%	83.6%	48%	342	7.6%	CRP WBC

Table 13 QUADAS-2 results

Study	Risk of Bias				Applicability		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Almeida, 2012							
Facy, 2016							
Garcia-Granero, 2013							
Giaccaglia, 2014							
Giaccaglia, 2015							
Iancu, 2008							
Kaser, 2014							
Korner, 2009							
Kostic, 2015							
Lagouette, 2012							
Ortega-Deballon, 2010							
Platt, 2012							
Warschkow, 2011							
Welsch, 2007							

Reisinger, 2014							
Sammour, 2016							
 Low risk  unclear  risk High risk							

4.3.3 Definition of anastomotic leakage

Included studies had varying definitions of AL, Table 14 below. Reported median times of AL diagnosis ranged from POD 6 to 12. AL rate in the included studies ranged from 3% [151] to 27%.[150]

Table 14 Study definition of anastomotic leakage

Study	**Definition**
Almeida [146]	Clinical signs of peritonitis and/or clinical evidence of free faecal fluid within the abdomen or emerging from the drain site. Diagnosis confirmed by abdominal and pelvic CT using IV and anorectal contrast.
Garcia-Granero [194]	Anastomotic leak were classified as major - need for reoperation or percutaneous radiological drainage, Clavien-Dindo grades III-V. Minor - conservative medical treatment, Clavien-Dindo grades I-II

	Confirmed by X-ray enema with hydro-soluble contrast performed or with CT, by endoscopy, or intraoperatively.
Giaccaglia [173]	Postoperative peritonitis found at reoperation, faeces in drain, faecal material from wound, extravasation of contrast on enema or presence of air or fluid in the anastomotic region visualised by CT. Classified as major (need for reoperation or percutaneous drainage) or minor (medical management)
Kaser [176]	Confirmed by CT with water soluble contrast enema, conventional contrast enema, proctoscopy, and/or surgical reintervention. Abscess in proximity of the anastomosis without extravasation of water-soluble contrast were accounted as leakage.
Korner [177]	AL confirmed by radiology (contrast enhanced multi-detector CT scan or conventional radiology with water soluble contrast enema), endoscopy or during surgical exploration. Intra-abdominal infection defined as infection, either diffuse or abscess, within the abdominal cavity or the presence of an anastomotic leak.
Lagoutte [13]	The presence of one of the following criteria: postoperative peritonitis found at re-operation, purulent or faecaloid wound drainage, the presence of air or fluid collection in the anastomotic region on CT scan

Ortega-Deballon [142]	One of the following criteria: Pus or enteric contents within the drains, presence of abdominal or pelvic collection in the area of the anastomosis on post-op CT scan, leakage of contrast through anastomosis during enema, or evident anastomotic dehiscence at reoperation for post-op peritonitis.
Platt [179]	Radiologically verified fistula to bowel anastomosis or diagnosed by relaparotomy
Warschkow [182]	Presence of an intra-abdominal abscess with confirmation by rectal exam, sigmoidoscopy, and extravasation of endoluminal administered water-soluble contrast on radiography or CT or confirmation upon return to the operating room.
Welsch [183]	Verified either by radiographic enema performed with CT scan, X-Ray, or by endoscopy.
Reisinger [186]	Extraluminal presence of contrast fluid on contrast-enhanced CT scans and/or leakage when relaparotomy was performed, requiring reintervention.

4.3.4 Classification of biomarkers

A list of analysed systemic and peritoneal drain biomarkers is shown in Table 15. Biomarkers were divided into three main categories: ischaemic, inflammatory, and microbiological biomarkers. Only three studies evaluated a preoperative biomarker

for AL after colorectal surgery.[151, 172, 186] Postoperative biomarker levels were elevated, although at different time points and with significant variance.

Table 15 Group classification of biomarkers

Ischaemic biomarkers	Matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13 Lactate Pyruvate Glycerol Fatty acid binding proteins pH levels
Inflammatory biomarkers	Interleukin (IL)-1,1α,1ra,2,4,5,6,8,10, Tumour necrosis factor (TNF)-α and sTNF-ra Granulocyte macrophage colony-stimulating factor Interferon-γ Membrane attack complex Mannin binding lectin Vascular endothelial growth factor Calprotectin Leukocytes Neutrophils

	C-reactive protein Procalcitonin Na+ Prothrombin fragment 1 and 2 Thrombin anti-thrombin complex Soluble fibrin Tissue plasminogen activator Plasminogen activator inhibitor 1 Platelets Albumin Haemoglobin Serum total protein
Microbiological biomarkers	*E. coli* *E. faecalis* *Pseudomonas spp* *Klebsiella spp*

4.3.5 Ischaemic biomarkers

Fatty acid binding proteins, pyruvate, glycerol, lactate, matrix metalloproteinase, and pH levels

Six studies evaluated ischaemic peritoneal and systemic biomarkers in the context of AL following colorectal surgery.[138, 158, 165, 186] Matthiessen [138] reported a statistically significant increase in the lactate/pyruvate (L/P) ratio on POD 5 and POD 6 in 4 patients with AL diagnosed up to POD 14 (median day 6), and another 3 AL diagnosed after discharge at a median POD 20 (range 18-22). Similarly, Pedersen in a study with 4 AL patients, the L/P ratio increased on POD 1-5 before signs of AL were evident in patients diagnosed >10 days with AL after surgery. Glucose tended to decrease in the early postoperative period before AL when compared to non-AL patients.

Reisinger [186] evaluated liver-, intestinal, and ileal-fatty acid binding proteins, and provided predictive analyses. A pre-operative intestinal-FABP (I-FABP) level greater 882pg/mL had a sensitivity of 50%, and specificity of 100% for predicting AL. Derived NPV and PPV from reported data, were 95% and 100% respectively. Other FABPs were not useful.

Pasternak [158] showed that matrix metalloproteinase (MMP)-8 and MMP-9 measured four hours after surgery showed statistically significant increased levels in patients who later developed AL. Diagnosis of AL was made between POD 2 and POD 13 (median POD 5). Although this showed promise, in a subsequent study Kostic did not find any statistically significant changes in MMP-9 levels in patients with AL at POD 1,3,5 and 7.[169]

Yang [171] measured consecutive peritoneal drain fluid pH levels in patients with AL. They found that mean pH values were significantly lower from POD 3 in patients with AL. A cut-off pH value of 6.98 on POD 3 yielded a sensitivity 98.7% and specificity of 94.7% for AL.

4.3.6 Inflammatory biomarkers

Cytokines, receptors, coagulation markers, fibrin fragments, and electrolytes

Thirty-two studies evaluated thirty different inflammatory biomarkers including cytokines, cytokine receptors, fibrinolytic and coagulation biomarkers, and complement. Ten studies evaluated peritoneal inflammatory biomarkers only. The 6 most commonly evaluated inflammatory biomarkers were CRP, procalcitonin, leucocytes, interleukin-6, interleukin-10, and tumour necrosis factor- alpha (TNF-α).

CRP was evaluated in 18 studies, however only 12 studies provided predictive data specific to AL after colorectal surgery. In general CRP was significantly elevated days before the clinical diagnosis of AL. The reported cut-off values for CRP ranged from 94mg/L to 190mg/L for POD 3 and POD 4. Negative predictive values (NPV) ranged from 95.8% to 98%, positive predictive values (PPV) were up to 22%, and area under receiver operating characteristic (AUROC) values ranged from 0.716 to 0.88 for AL.

Procalcitonin was evaluated in six studies, with five studies providing predictive analysis specific to AL.[13, 137, 139, 173, 174] The optimal cut-off values for PCT ranged from 0.25ng/mL up to 680ng/mL from POD 3 to 5, giving a NPV from 96.7% to 100% for POD 3 to 5, and a PPV of up to 34%. AUROC ranged from 0.68 to 0.88. PCT levels tended to normalize on POD 4 and 5 in uncomplicated cases.

Leucocytes and neutrophils were evaluated in 8 studies, with 4 studies reporting diagnostic indices for AL. [139, 176, 177, 182] Reported sensitivities for WCC ranged from 58% to 74%, with AUROC values ranging from 0.63 to 0.77 for POD 5 to 7. In general, WCC peaked at the time of

AL diagnosis. In one study[139], a neutrophil cut-off value of 5910x10^9/L, gave a sensitivity of 91%, specificity 77%, NPV 99%, PPV 19%, and AUROC 0.817.

Interleukin (IL)-6, -10 and TNF-α were evaluated in twelve studies. Only one study reported predictive indices specific to AL.[89] Seven studies focused primarily on peritoneal drain cytokine levels. All but two studies reported that IL-6, IL-8 and TNF-α levels in patients with AL were statistically significantly elevated in the early postoperative period (POD 1-4) in patients diagnosed with AL at a median time of POD 6.[164, 186]

Calprotectin had a cut-off value of 541ng/mL on POD 4 with 100% sensitivity, 91% specificity, a positive likelihood ratio (LR+) of 11, a negative likelihood ratio (LR-) of 0, and an AUROC value of 0.96.[186] NPV and PPV derived from reported data were 100%, and 55% respectively. Hyponatraemia, as a marker of inflammation, was defined as <136mmol/L by Kaser et al [176]. They reported that hyponatremia on POD 4 had a sensitivity of 10%, specificity 94%, NPV 96%, PPV 7%, and AUROC 0.508 for AL.

Three studies combined 2 or more inflammatory biomarkers. Two studies [176, 186] showed that a combination of CRP and serum calprotectin on POD 3 elicited a sensitivity of 100%, a specificity of 89%, LR+ of 9.09, LR- of 0, and an AUROC of 0.93 based on a linear function: (Calprotectin (ng/mL)] + 0.83 x [CRP (mg/L)] = 707). A positive result was defined when the linear function produced a numerical value greater than 707 on POD 3. NPV and PPV derived from reported data were 100%, and 50% respectively. The combination of hyponatremia (<136mmol/L) and leucocytosis (>10x10^9/L) on POD 5, resulted in a sensitivity of 68%, a specificity of 75%, PPV 18%, and NPV 97%. Giaccaglia et al, [173] reported an AUROC of 0.901 when PCT was added to CRP for POD 5.

One study [175] found levels of PT fragments 1 + 2, thrombin antithrombin complexes, SF and PAI-1 were significantly higher in patients with AL on POD 1 and 2. Other protein biomarkers such as platelets, albumin, and serum protein levels were reported as not being of clinical utility.

4.3.7 Microbiological biomarkers

Escherichia coli, Escherichia faecalis, Pseudomonas species and Klebsiella species

Only two studies evaluated microbiological biomarkers, both of which were from peritoneal fluid. Fouda [166] reported that, in patients with AL diagnosed between POD 2 and POD 8, there were significant levels of *E coli, Klebsiella* and *Pseudomonas spp* on POD 1, 3 and 5. [166] Similarly, Komen [168] isolated statistically significant levels of *E faecalis* and *E coli* from POD 1 to 3 in patients with AL. Komen showed that increased *E faecalis* levels from POD 1 to POD 3 h a sensitivity and specificity of 92.9%, and 70.9% respectively, and a positive predictive value of 30.2% and a negative predictive value of 98.7%.[168]

4.4 Discussion

This systematic review has identified 49 biomarkers used to detect AL following colorectal surgery. The most analysed biomarkers were non-specific inflammatory biomarkers. The measured biomarkers had good negative predictive values for AL but were poor positive predictors of AL following colorectal surgery. This systematic review has identified a variety of intra-peritoneal and systemic biomarkers that are significantly elevated in the presence of AL, though at different time points (POD 1-5) and with significant variance. Those that were elevated prior to clinical evidence of AL included cytokines (IL-1β, IL6, IL-10, TNFα), CRP, procalcitonin, colonic bacterial species, fibrin degradation proteins, complement and matrix metalloproteinase.

Following abdominal surgery, the abdominopelvic cavity is awash with intra-peritoneal biomarkers, both pro-inflammatory and anti-inflammatory, involved in the healing process. In the normal course of healing, the composition of the intra-abdominal milieu follows a predictable course over a period of several weeks.[87, 156, 195, 196] In the context of AL however, this inflammatory milieu is altered, and this is thought to occur prior to the clinician being aware. Thus, a systemic inflammatory biomarker or a combination of biomarkers that reflects the peri-anastomotic intra-peritoneal environment may be a useful, minimally invasive objective predictive tool for AL.

As a result of the non-specific nature of evaluated biomarkers, their clinical utility currently appears to be localised to facilitating early discharge after surgery. Three recent studies, however, have identified a renewed potential of this approach by combining two non-specific biomarkers. Reisinger et al [186] described a combination of serum calprotectin and CRP on POD 3 that resulted in a sensitivity of 100%, specificity of 89%, and AUROC of 0.93 based on a linear function. Comparable results were also reported for POD 4. By adding PCT to CRP on POD 5, Giaccaglia [173] similarly report a markedly improved AUROC of 0.901. Of note however, predictive indices did not improve when combining hyponatremia and leucocytosis.[176]

There are a number of limitations to this review. Studies used a variety of definitions for AL and this has been previously noted by other authors.[159, 197] Definitions varied from clinical symptoms to contrast extravasation on CT scan. In analysing peritoneal fluid, studies used a variety of methods and the location and type of drain used was often not described. Drain location can influence the composition of drainage fluid.[198] Most patients included in the review underwent open surgery. The results therefore, may not apply to laparoscopic patients,

which have been shown above to produce a reduced inflammatory response after surgery.[199, 200] Additionally, the timing of blood tests varied between studies that analysed systemic biomarkers, from daily to selective days. Furthermore, none of the included studies distinguished biomarker levels between colonic and rectal resections, or disease process, nor considered medications that may alter the inflammatory response such as statins and steroids. [19, 201] With the advent of ERAS protocols the extended use of peritoneal drain remains a contentious issue, with recent studies showing no benefits of peritoneal drains. [57, 202]

This systematic review has identified a variety of peritoneal drain fluid and systemic biomarkers that are altered in the presence of AL, though at different time points (POD 1-5) and with significant negative/positive predictive values. The consistent use of peritoneal drains following elective colectomy is controversial and thus systematic biomarkers are of more practical use in early detection of anastomotic leak.

Chapter 5

MATERIALS AND METHODS: BIOMARKERS AND ANASTOMOTIC LEAKAGE LONGITUDINAL STUDY

5. MATERIALS AND METHODS: BIOMARKERS AND ANASTOMOTIC LEAKAGE LONGIDUTINAL STUDY

5.1 Background

The BALL (Biomarker and Anastomotic Leakage Longitudinal) study is a prospective observational study that measured inflammatory biomarkers following elective colonic surgery. In this chapter, the methods that were employed during the implementation of the BALL study are outlined. The BALL study was developed and implemented based on the STROBE (Strengthening the Reporting of Observational studies in Epidemiology) guidelines, version 4. [203] Common laboratory and clinical methods are described below. A comprehensive list of materials and reagents used is presented at the end of this chapter.

The inflammatory biomarkers selected were based on findings from the preceding chapters. Chapter 4 identified a variety of intra-peritoneal and systemic biomarkers that are significantly elevated in the presence of AL. For practical reasons, described in chapter 4 systemic biomarkers were measured only. In addition to routine biomarkers including CRP, leucocytes and neutrophil count, four cytokines were selected based on their pro-inflammatory or anti-inflammatory properties – IL-6, a well-known potent pro-inflammatory cytokine that directly controls the release of CRP into the systemic circulation; IL-10 an anti-inflammatory cytokine released in response either to an exaggerated inflammatory response or presence of a pathogen; IL-1β, a potent pro-inflammatory cytokine that is produced by endothelial cells in response to endotoxins and micro-organisms; and TNFα, a cytokine that enhances inflammatory cell recruitment to the site of injury.

5.2 Hypothesis and design

It was hypothesized that a biomarker profile specific to anastomotic leakage following colon surgery can be determined. This was tested in a prospective observational cohort study across public and private hospitals in Auckland and Christchurch, New Zealand.

5.3 Ethics and Safety

Ethics approval was obtained from the Health and Disability Ethics Committee (HDEC), reference number *14NTB173*. Locality approval was initiated by submitting the HDEC approval letter together with the study protocol to each hospital site in Auckland and Christchurch. Approval from the local Maori Research committee was acquired as per locality guidelines. All approved documents were then submitted to the locality site research office. Approval from Head of Department, Service manager and colorectal surgeons were obtained prior to recruitment commencement.

5.4 Funding

The following sources were used to fund the BALL study:

1. Health Research Council Pacific PhD scholarship Project grant ($10,000)
2. Health Research Council Pacific Clinical Research training fellowship ($167,000)
3. Maurice and Phyllis Paykel Trust grant ($10,000)
4. Colorectal Surgical Society of Australia and New Zealand (Australian $30,000)
5. Royal Australasian College of Surgeons, Foundation, Project grant ($10,000)

5.5 Patient selection

Patients undergoing elective colonic resection with anastomosis were eligible. Exclusion criteria for the study were as follows:

1. Patients undergoing surgery with no bowel-to-bowel anastomosis
2. Age less than 16 years
3. Patient refusal.
4. Rectal only surgery (lesion less than 15cm from anal verge)

Patients were recruited either from surgical pre-admission clinics or the preoperative surgical unit. Informed consent was gained, and consenting patients were added to a prospective study database.

A pilot study of 50 patients at Middlemore Hospital and Manukau Surgery Centre had a 98% recruitment rate for patients undergoing colonic surgery. As this was an

observational study with minimal active participant involvement, the recruitment rate was expected to be similarly high in other centres. To assist recruitment from other sites, a research nurse at Christchurch Public Hospital, and a research fellow based at Auckland Hospital were enlisted. Patient recruitment at Middlemore Hospital and Mercy Ascot Hospital was carried out by the PhD candidate.

5.6 Patient follow up

Patients were followed up for 30 days after surgery, with initial daily clinical review by the PhD candidate or research nurse. The occurrence of AL and other post-operative complications were prospectively recorded from re-admission data or GP letters/referrals.

5.7 Perioperative care

Perioperative care was not standardized across the included hospitals. ERAS protocols, unless otherwise stated by the attending surgeon, were carried out as per hospital protocol.

5.8 Primary outcome measures

The primary endpoint for this study was the difference in the levels of plasma biomarkers in patients who developed anastomotic leakage compared to patients who did *not* develop anastomotic leakage.

Secondary endpoints included biomarker differences among patients undergoing open versus laparoscopic surgery, differences between left and right colon surgery.

5.9 Measurement of post-operative complications

Complication data were pre-defined and graded prospectively on the index admission and any re-admissions. Complications were measured up to POD 30, with day 0 being defined as the date of the operation.

5.10 Definition of anastomotic leakage

Anastomotic leakage was defined as a defect of the intestinal wall at the anastomotic site leading to a communication between the intra- and extraluminal compartments. This was confirmed either on cross-sectional imaging or intraoperatively.

5.11 Measure inflammatory biomarkers

All included patients had pre-operative blood tests to measure the following inflammatory biomarkers – interleukin (IL)-1β, IL-6, IL-10, tumour necrosis factor alpha (TNFα), full blood count (FBC), C-reactive protein (CRP) and albumin levels.
After surgery, IL-1β, IL-6, IL-10, TNFα, FBC, and CRP were measured through daily blood tests for the first five days after surgery, or until discharge.

5.12 Phlebotomy, initial analysis, and storage

Blood tests were timed to coincide with the patient's routine pre- and post-operative blood tests done as part of standard clinical monitoring. All collected samples were sent to the hospital laboratory, where they were centrifuged at x1000g, supernatant removed, aliquoted and stored at or less than -20-degree Celsius. Samples were then analyzed for biomarker levels in duplicate using a commercially available immunoassay kit. Respective hospital laboratories measured non-specific inflammatory biomarkers such as CRP, FBC and albumin levels.

5.13 Sample collection

Samples were collected at 6 timepoints: Preoperative, and POD 1 to 5, or until discharge. Two purple top EDTA (Ethylenediaminetetraacetic acid) tubes and a green top (heparin) tube were used for collection. The first EDTA tube used for FBC levels, later analyzed according to hospital assays; and the second EDTA collected for the plasma supernatant required for cytokine analysis. The green top Heparin tube was used to collect samples for CRP and albumin levels assays.

5.14 Laboratory processing

Upon reception of collected blood samples, one EDTA tube and the heparin tube were analyzed according to each hospital's assay for FBC, CRP and albumin levels.
The second EDTA tube was processed according to Millipore multiplex instruction for ELISA (enzyme-linked immunosorbent assay) measurement (Appendix 4).
All laboratory compliances including in-house UOA laboratory safety training and containment course completed prior to cytokine analysis commencement.

5.1.1 Cytokine analysis using MILLIPLEX ® Luminex ® technology

Cytokine analysis was performed using the Milliplex ® magnetic bead panel, 4-plex. (EMD Millipore 2013 © Millipore Corporation, Billerica, MA 01821 USA). The contents of each MILLIPLEX ® kit are shown in Table 16. Additional reagents and materials required but not provided by Millipore ® are also shown in Table 16 below.

Table 16 Laboratory reagent and materials required

Table 16 Reagents and materials required for cytokine analysis

Reagents supplied in kits	**Additional reagents and materials required**
Cytokine standard	Luminex ® Sheath fluid (Provided by Millipore for running of this study)
Cytokine quality controls	Multi-channel pipettes
Serum matrix	Reagent reservoirs
96 well plate with 2 sealers	Polypropylene microfuge tubes
Assay buffer	Rubber bands
10X wash buffer	Aluminium foil
Detection antibodies	Absorbent pads
Streptavidin-Phycoerythin	Laboratory vortex mixer
Bead diluent	Sonicator
Mixing bottle	

Pre-mixed beads	Titer plate shaker Luminex ® MAGPIX ® with xPONENT ® software 5.1 (provided by Millipore team) Automatic plate washer or handheld magnetic separation block.

Plasma samples from the second EDTA tubes were centrifuged at x1000g within 30 minutes of blood collection and stored at < 20 degrees Celsius at each hospital freezer. Samples were thawed completely, mixed well by vortex, and centrifuged prior to use in the assay to remove particulates. Other than the initial sample collection and storage, cytokine analysis was carried out on the 5th Floor Pharmacy Lab, Building 505, School of Pharmacy, Grafton Campus, The University of Auckland. Full details on equipment and software settings are shown in the Appendix. An outline of the steps taken are shown below.

5.1.1.1 Preparation of plasma samples

1. Thaw samples completely, mix well by vortexing and centrifuge prior to use to remove particulates.
2. Centrifuge for 10 minutes at 100xg within 30 minutes of thaw. Remove plasma and assay immediately or aliquot and store additional samples at <20 deg Celsius.

5.1.1.2 Preparation of reagents for immunoassay

1. Plate reagents were prepared

a. Included antibody-immobilized beads were sonicated for 30 seconds. Bead diluent was then added, and vortexed again for 1 minute.
b. Quality controls were reconstituted and labelled appropriately in polypropylene microfuge tubes. Unused portions were stored at <20 deg Celsius for up to one month.
c. Wash buffer was prepared by diluting the provided 10X wash buffer with 540mL deionized water.
d. Serum matrix was prepared by adding 1mL of deionized water to the lyophilized Serum Matrix reagent provided. This was left to sit for at least 10 minutes to allow complete reconstitution. Left over serum matrix stored in <20 deg Celsius for up to 1 month.
e. Human cytokine standard was reconstituted with 250µL deionized water for the initial 10,000pg/mL concentration. It was inverted several times and vortexed for up to 10 seconds and transferred to appropriately labelled polypropylene microfuge tubes. Working standards were then prepared by adding 200µL of Assay buffer to 5 polypropylene microfuge tubes labelled 2000, 400, 80, 16 and 3.2pg/mL. 50µL of the 10,000pg/mL was transferred to the 2000pg/mL labelled tube and inverted several times. This process was repeated down the 3.2pg/mL concentration, Figure 5.1.

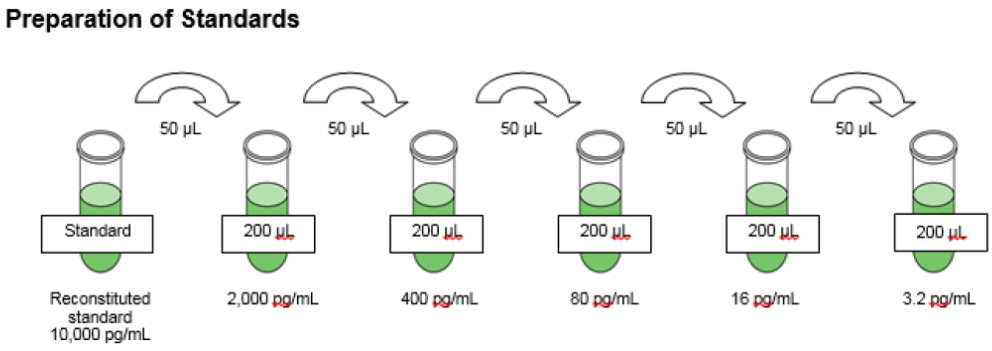

Figure 9 Preparation of standards

2. Immunoassay procedure
    a. Wash buffer was used to clean the plate prior to use. This involved a repeated cycle of washing and decanting.

b. Standard and Controls were transferred to the appropriate wells on the plate.
c. Assay was then transferred to the background and samples wells.
d. Matrix solution was then added to the background, standards, and control wells.
e. Samples were then added to the sample wells, followed by the Beads to all wells.
f. Plates were then incubated overnight at 4 deg Celsius with shaking on a plate shaker.
g. Plate contents were then removed and washed while using a magnetic plate.
h. Detection antibodies were transferred to each well, followed by an incubation period of 1 hour.
i. Streptavidin-Phycoerythrin was then transferred to each well and incubated for a further 30 minutes.
j. Plate contents were again washed 3 times while using the magnetic plate.
k. Sheath fluid or drive fluid was then applied to each well and ready to be read.

3. Plate reading
    a. Each plate was read on the Magpix ® reader provided and serviced by MILLIPORE ® company. (Figure 10)

Figure 10 Author with MAGPIX readers

5.1.1.3 Assay characteristics

There was negligible cross-reactivity between antibodies and other analytes in the provided 4-plex panel. Assay sensitivities were based on minimum detectable concentration using MILLIPLEX ® analyst software 5.1, by calculating empirical minimal detection concentrations. Sensitivity and precision data for these assays are included in the Appendix

5.1.1.4 MAGPIX ® settings

Specifications such as gate settings for both MAGPIX ® and xPONENT software were followed according to type of analyte/cytokine analysed. Settings were finalised and

2 trial run throughs with help from Millipore ® team were completed prior to commencement with study samples.

Bead calibration kits and performance verifications kits were used weekly to ensure both the plate reader and analyst software remains trouble-free.

5.15 Sample size

As this was an observational study, a formal power calculation was not possible as no intervention effect was being evaluated. Based on calculations however, with biostatistician input, the area under the curve (AUC) for two commonly evaluated biomarkers (C-reactive protein and Procalcitonin) was evaluated for an appropriate power analysis. This showed that at least 24 patients with AL would be required. This number of patients would enable appropriate sub-group analysis. With a reported AL rate of approximately 8.3%, [89] the BALL study aimed to recruit 300 patients over 3 years. To counterbalance patients who did not have a planned anastomosis, addition of a stoma, and those with at least 2 missing laboratory values, estimated at around 20%; an additional 60 patients were added. Therefore, the total number of patients targeted was estimated at 360 patients. The study was designed to have 80% power with an alpha of 0.05.

5.16 Statistical analysis

Statistical analysis was performed using the Statistical package for the Social Sciences (SPSS ver. 25 Inc, Chicago, IL) software. Measured means, percentages and standard deviations were calculated and are shown where appropriate. Comparison of categorical variables were performed using Chi-square and Fisher's exact test, with

strength of association using Phi and Cramer V test. The student t test and Mann-Whitney U were used for non-parametric variables.

Univariate analysis was performed between the measured inflammatory biomarkers and the occurrence of AL. Logistic multivariate analysis was performed to correct possible confounders where univariate analysis was significant. Significance of differences were tested by analysis of variances (one-way ANOVA, general linear model); multiple regression analysis (linear model) for significance of independent variables on primary outcome; and receiver operating characteristic area under the curve (ROCAUC) analysis for the diagnostic utility and optimal cut-off values of above measured inflammatory biomarkers. Statistically significant differences were defined as $p<0.05$.

Chapter 6

RESULTS: RECOVERY FOLLOWING COLECTOMY WITHOUT ANASTOMOSTIC LEAKAGE

5. RESULTS: RECOVERY FOLLOWING COLECTOMY WITHOUT ANASTOMOTIC LEAKAGE

6.1 Background

Several published study limitations were identified in the preceding chapters. Previous studies included both colon and rectal surgery, despite studies having shown important differences in inflammatory responses and clinical issues, between the two. One study which aimed to address these issues lacked numbers, while another lacked consistent measurement of biomarkers in the postoperative period. Another key issue with the current literature was the varying degree of timing of systemic blood test measurements.

Therefore, the BALL study, a prospective study, was conducted to evaluate the diagnostic utility of inflammatory biomarkers in diagnosing and predicting anastomotic leak in the early postoperative period following elective colectomy.

However, before inflammatory biomarkers are evaluated in the context of AL, they were assessed in a recovery from colectomy without the complication of AL. The uncomplicated results are outlined in this chapter.

6.2 Methods

Patient selection, recruitment and follow up is discussed in Chapter 5. Laboratory processes and materials used are also outlined in Chapter 5.

6.3 Results

A total of 405 patients undergoing colectomy were identified over 3 years across four public hospitals in Auckland and Christchurch, New Zealand. Patient flow including exclusions are shown in Figure 11. Overall, 251 patients without AL with sufficient data were included in the final analysis.

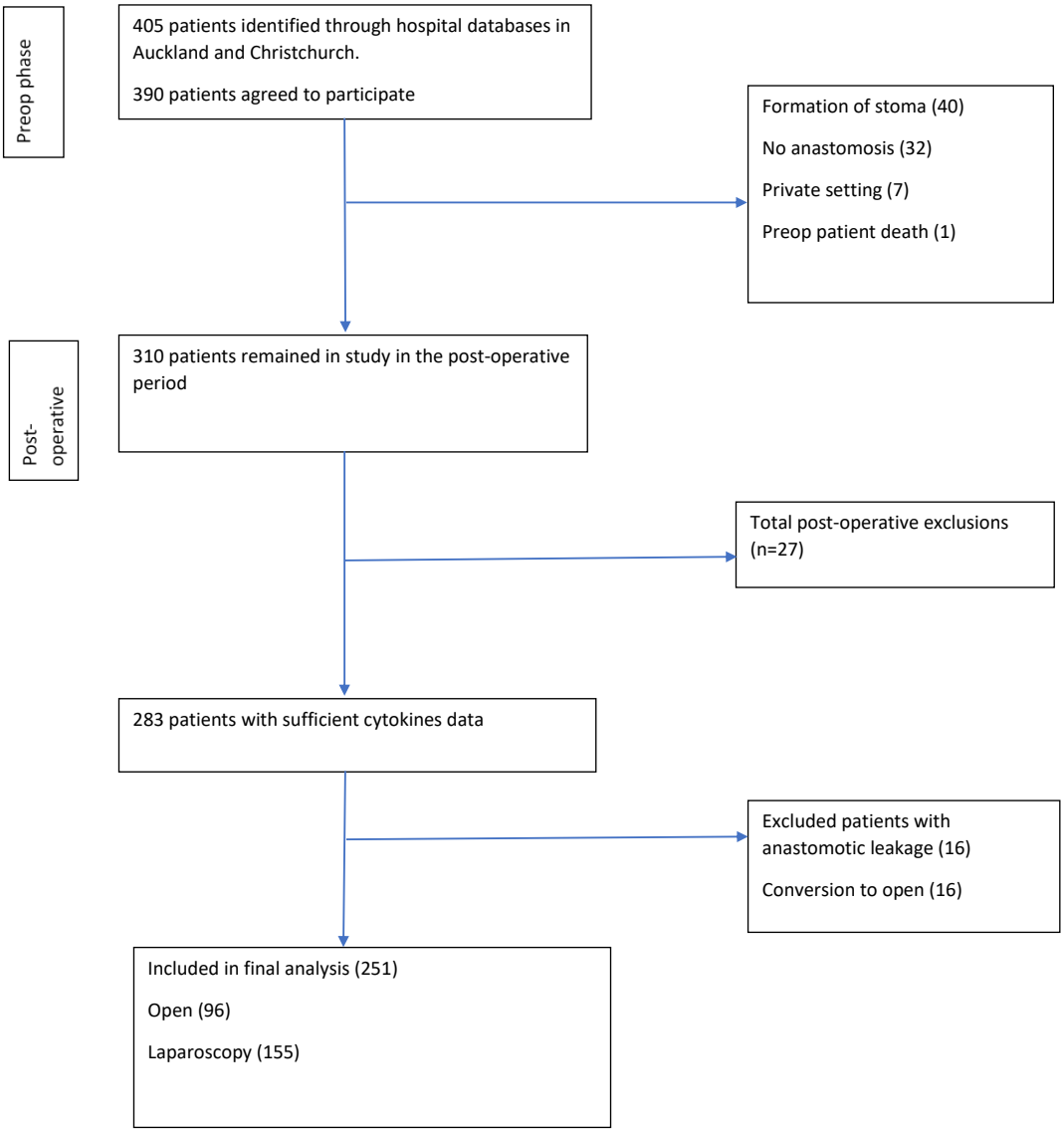

Figure 11 BALL study patient flow and recruitment

6.3.1 Baseline characteristics

The open approach group had a significantly higher rate of Reversal of Hartmann procedures, type 2 diabetes mellitus (DM), longer operative times, and longer hospital

day stay, compared with the laparoscopic group. The open approach group also had significantly lower pre-operative albumin and total protein levels. Further details are shown in Table 17.

Table 17 Baseline characteristics

Baseline characteristics			
	Open (n=96)	Laparoscopy (n=155)	p value
Demographics			
Age (mean years, SD)	68.6 (13)	66.1 (15)	n/s
BMI (mean, SD)	33.7 (34)	27.3 (4)	n/s
Non-obese (% approach)	60	40	n/s
Obese (% approach)	73	23	n/s
Gender (male % approach)	44	71	n/s
Previous abdominal surgery (% approach)	49*	26*	0.001
Comorbidity			
Current smoker (% approach)	9	5	n/s
Hypertension (% approach)	58	53	n/s
Respiratory disease	20	18	n/s

(% approach)			
Dyslipidaemia (% approach)	20	23	n/s
Liver disease (% approach)	1.1	0.7	n/s
Type 2 Diabetes mellitus (% approach)	22*	10*	0.014
Cardiovascular disease (% approach)	37	30	n/s
Renal disease (% approach)	13	7	n/s
Preoperative cancer diagnosis (% approach)	88*	96*	0.045
Medications			
Statins (n, % approach)	43	33	n/s
Anticoagulation (n, % approach)	13	17	n/s
Operative details	Open	Laparoscopic	
ASA			n/s
1	4	13	

2	53	88	
3	37	52	
4	1	2	
Time in OT (mean in minutes, SD)	162 (74)	175 (63)	n/s
Type of surgery (%)	Open	Laparoscopic	
High anterior resection	21*	64*	0.001
Left hemicolectomy	5	4	n/s
Right hemicolectomy	52	82	n/s
Reversal of Hartmann	14*	2*	0.001
Sub/total colectomy	4	3	n/s
Length of stay Median (range)	8 (43) *	6 (16) *	<0.001
Preoperative			
Pre-operative albumin (g/L)	34.7*	37.1*	<0.001
Total protein levels (g/L)	69.2*	72.3*	0.01

(*) Denotes direct comparison between open and laparoscopic group showing a statistically significant result ($p<0.05$)

6.3.2 Inflammatory response

Only CRP was statistically significantly elevated before surgery in the open group, (F (1,99) = 13.273, $p < 0.0005$). On POD 1, IL-6 (F (1,132) = 5.017, $p = 0.017$), neutrophil count (F (1,197) = 5.24, $p = 0.023$), and CRP (F (1,163) = 16.504, $p < 0.0005$) were significantly elevated in the open group. On POD 2, all inflammatory markers including CRP (F (1,73) = 27.28, $p < 0.0005$), except IL-1β were significantly elevated in the open approach group. Other significant results are highlighted in Table 18. Figure 12 highlights the perioperative CRP trends in the open and laparoscopic approach groups.

Table 18 Summary of measured inflammatory biomarkers

Summary of measured inflammatory biomarkers						
	Preop	POD 1	POD 2	POD 3	POD 4	POD 5
CRP (mg/L)						
Open	16.8*	87.1*	144.4*	150.2*	118.9*	106.7
Lap	6.2	63.3	92.6	93.9	91.2	91.1
Leucocyte ($x10^9$ /L)						
Open	7.9	11.7	10.6*	9.1	7.9	7.6
Lap	7.4	10.9	9.7*	8.5	7.8	7.8
Neutrophils ($x10^9$ /L)						
Open	5.2	9.4*	8.4*	7*	5.9	5.4
Lap	4.9	8.5	7.2	6.2	5.5	5.5
IL-10 (pg/mL)						
Open	23.9	31.3	25.5*	27	30.8	24.9
Lap	22.3	23.5	19.4	23	33.8	35.4

IL-6 (pg/mL)						
Open	10.1	54.4*	41*	36.9*	32.4*	38.3
Lap	10.6	33.8	22.9	18.1	18.1	22
IL-1β (pg/mL)						
Open	6.8	6.1	6.1	6	7.2	6.3
Lap	6.6	4.8	4.5	4.5	8.4	7
TNFα (pg/mL)						
Open	29	29.9	32.4*	35.1	35.2	33.6
Lap	28.8	26.5	25.5	28.5	36.1	34.6

(*) denotes a statistically significant result. Lap: laparoscopic group; pg: picogram; POD: post-operative day.

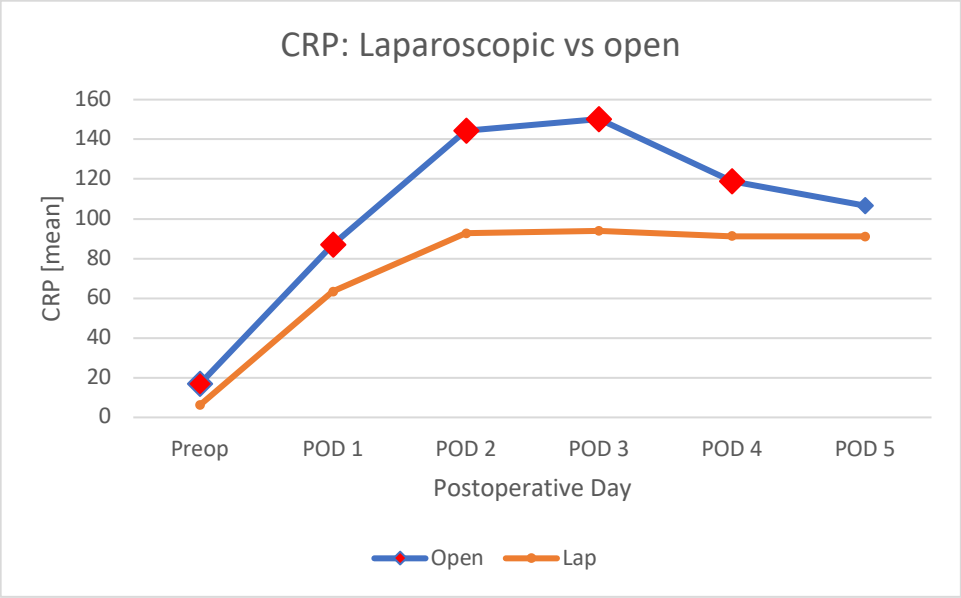

Figure 12 CRP trajectory in laparoscopic vs. open approach

(♦) indicate a statistically significant result

6.3.3 Body Mass Index and inflammatory response

Patients were separated into two BMI groups: obese (>30kg/m^2) and non-obese (<30kg/m^2), with inflammatory biomarkers compared between the two groups.

C-reactive protein levels were significantly higher in obese patients compared to non-obese on POD 4 (p<0.05). No differences were found for leucocytes, neutrophils, IL-10, IL-1β, and TNFα. Figure 13 illustrates CRP levels among different BMI groups in the perioperative period.

A multiple regression analysis was performed to predict POD 4 CRP from BMI and surgical approach. These variables statistically significantly predicted POD 4 CRP F (2, 101) = 3.66, p = 0.029, R^2 = .068, albeit with an average model fit. Only the BMI, added statistically significantly to the prediction, p < .05.

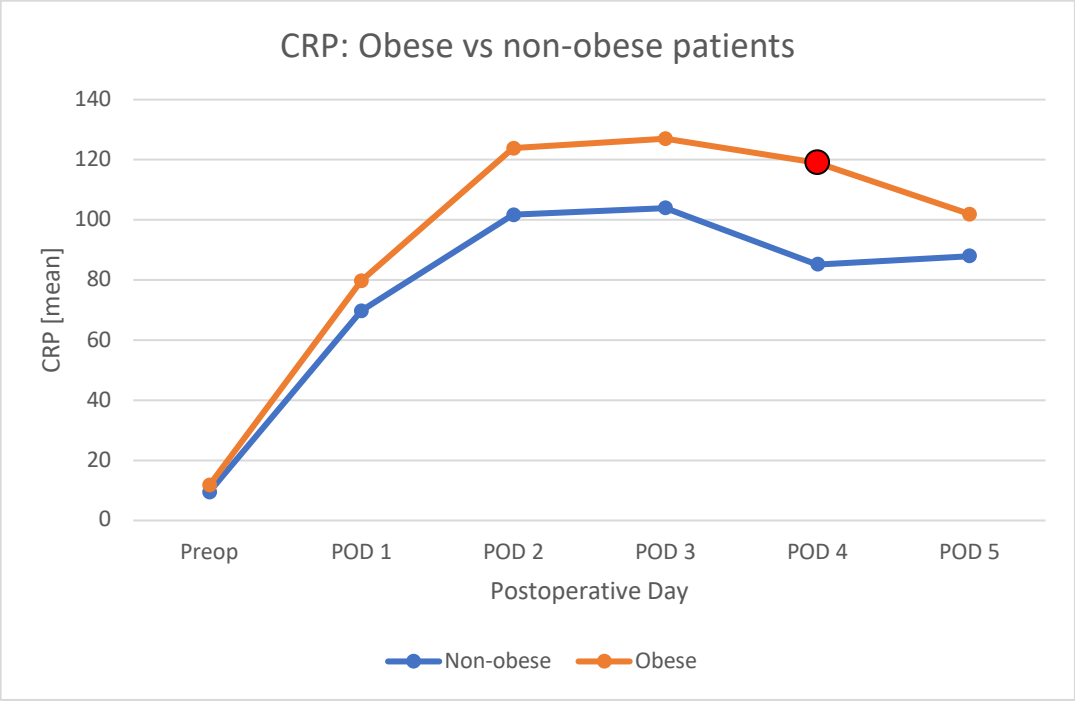

Figure 13 CRP trajectory in obese and non-obese patients

(shows a significant result)

6.3.4 Left and right colon surgery

Left and right colon groups were compared to identify differences in inflammatory responses between the two colonic operations. Right hemicolectomy and extended right hemicolectomy were grouped together as “right colon”. The “Left colon” group comprised left hemicolectomy, high anterior resection, and reversal of Hartmann’s procedure. Total and subtotal colectomy patients (n=7) were excluded from this analysis.

In the preoperative period, CRP was significantly higher in patients undergoing right colectomy, while IL-10 was higher in the left colon group. Only IL-10 was significantly higher on POD 1 in the left colon group. On POD 2, both leucocytes and neutrophil counts were significantly higher in the right colon group. CRP was the only significantly elevated biomarker on POD 4. No significant levels on POD 5. Figures 14 and 15 shows CRP and IL-10 trends for both left and right colon groups.

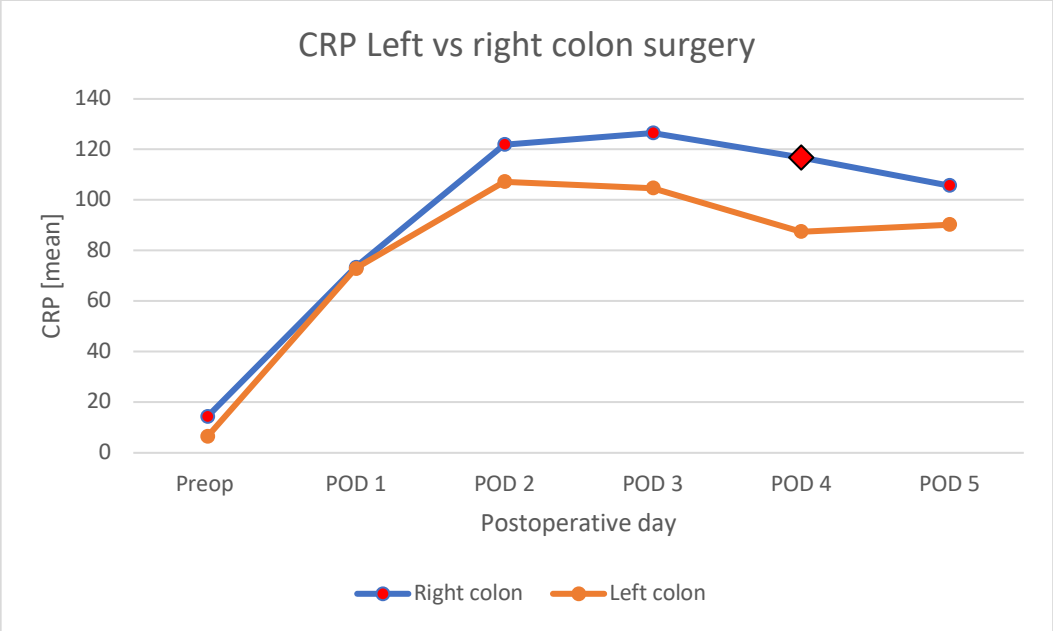

Figure 14 CRP trajectory in left and right colectomy

(◆) denotes a significant result

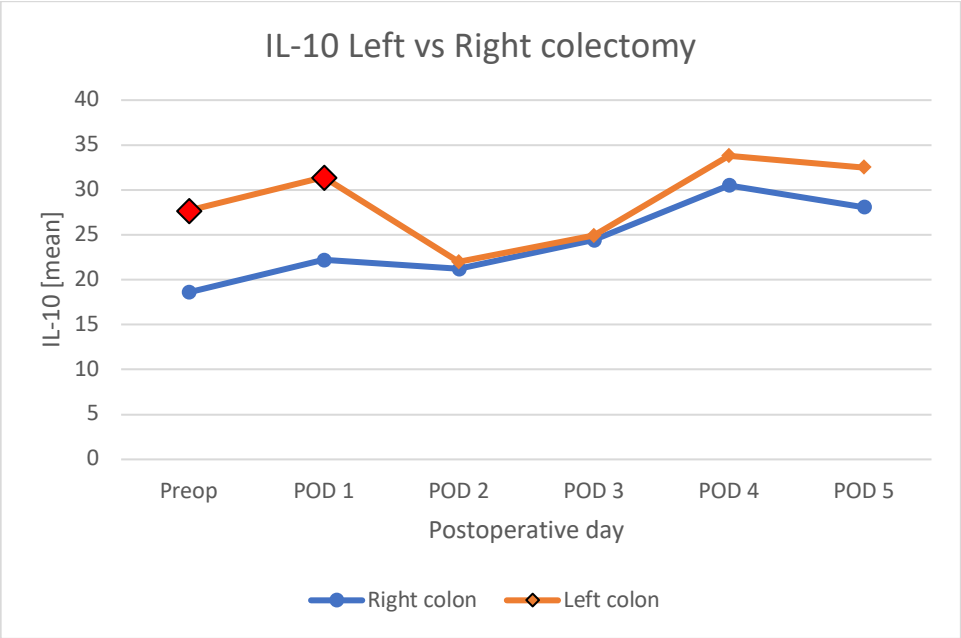

Figure 15 IL-10 levels in left and right colectomy

( ) denotes a significant result

6.3.5 Preoperative statin medication and nutrition

Forty-three percent of patients in the open group were taking preoperative statins compared with 33% in the laparoscopic group (p = 0.16). Preoperative levels of IL-1β, IL-6 and IL-10 were significantly lower in patients taking statins (p<0.05). CRP levels however were significantly higher on POD 1 and POD 2 in the statin group.

When evaluating nutrition levels, patients who underwent open colectomy had significantly lower pre-operative albumin and total protein levels compared with laparoscopic group (p <0.01).

6.3.6 Patients without any perioperative complications

A total of 167 patients (59.4%) did not have any reported perioperative complications following their elective operation. Complications are listed in Table 19.

Table 19 Perioperative complications

Perioperative complications	
Other complication	**n (%)**
SSI	23 (9)
Death	4 (2)
Cardiovascular (MI, CHF, CVA)	7 (2.8)
Intra-abdominal collection	4 (2)
Renal (UTI, AKI, UR)	20 (8)
Respiratory (LRTI, PE)	14 (6)
Ileus	51 (20)

SSI: Surgical site infection; MI: myocardial infarction; CCF: congestive heart failure; CVA: cerebral vascular accident; UTI: urinary tract infection, AKI: acute kidney injury, UR: urinary retention; LRTI: lower respiratory tract infection; PE: pulmonary embolism

6.3.6.1 Inflammatory response

Preoperatively, albumin, CRP, and total protein levels were also statistically significantly reduced in the open approach group ($p < 0.05$). Following surgery, patients with an open approach showed statistically significantly elevated CRP on POD 1 to 4 (p <0.05); leucocytes on POD 2 (p <0.05); neutrophil count on POD 1 to 3 (p <0.05); and IL-6 on POD 1 to 3 (p <0.05).

IL-10 and TNFα which were both raised on POD 2.

6.3.6.2 BMI

CRP levels were significantly elevated in obese patients on POD 4. A multiple regression analysis was performed from BMI and surgical approach. These variables statistically significantly predicted POD 4 CRP (F (3, 85) = 3.4, p = 0.029, R^2 = .07), albeit with an average model fit. Only BMI added statistically significantly to the prediction, p = .038.

6.4 Discussion

In recovery from colectomy uncomplicated by anastomotic leak, this prospective study has shown that patients undergoing laparoscopic colectomy have a reduced inflammatory response compared with equivalent open colectomy. CRP, neutrophil count, and IL-6 showed significant differences from POD 1 to POD 3. Preoperative statin use, and BMI also impacted the inflammatory response following major elective colon surgery regardless of operative approach.

The open approach group had higher rates of previous abdominal surgery including a higher rate of reversal of Hartmann's procedures. Re-operation is a known risk factor for longer operative times, increased risk of bowel injury during adhesiolysis, and a longer period of convalescence.[204, 205]

Results of this prospective study are consistent with smaller studies performed in an ERAS environment. Doubt however remains on whether the postoperative care provided by ERAS is the key contributor to this measured benefit seen in laparoscopy patients. A large RCT, the LAFA-study, suggested that the accelerated perioperative recovery was related more to the surgical approach rather than postoperative care.[206] Interestingly, Stage et al, [120] found significantly elevated CRP and IL-6 levels in their laparoscopy group. This result was attributed to perioperative utilization of NSAIDs (non-steroidal ant-inflammatory drugs). Other perioperative medications have been shown to attenuate the inflammatory response following surgery. A recent RCT by Singh et al, showed a reduced postoperative inflammatory response with perioperative statin use.[207] Similarly, in the current study, statin use was associated with a reduced preoperative inflammatory level.

Other patient factors such as BMI and region of gut resection may affect inflammatory response as shown in the results in this study. Human adipose tissue secretes pro-inflammatory cytokines such as IL-6, likely inducing a low pro-inflammatory state in the obese patient. This is evident in a recent study that showed an increase in systemic CRP levels in patients with an elevated BMI. [208] In the current study, CRP was significantly elevated in obese patients compared with non-obese patients. Multiple regression analysis further outlines the higher-than-normal inflammatory response after surgery in obese patients regardless of operative approach.

ERAS and laparoscopy seem to attenuate the inflammatory response. However, this does not necessarily translate to improvement in the post-operative AL rate. In a study by Teeuwen *et al*, [125] AL rates were comparable between ERAS and non-ERAS postoperative care patients, despite a reduced inflammatory response. This suggests that the inflammatory response to surgery itself is not the only major contributor to AL development. A comparison of inflammatory biomarkers of patients with AL in both laparoscopy and open colectomy may shed further light on the behaviour of inflammatory biomarkers.

Though the exclusion of AL helped lessen study heterogeneity, perioperative medications including dexamethasone on induction, utilization of NSAIDs and other opioids use may have affected the observed results.

In conclusion, this prospective observational study has shown that the inflammatory response following elective colectomy is affected by surgical approach, BMI, region of surgical resection, and preoperative medications, in an ERAS environment. This has implications for the detection of AL and other surgical complications following elective

colectomy. A normal inflammatory result for an obese patient undergoing an open procedure may well represent a significant complication in a non-obese patient who has had a laparoscopic procedure. Evaluating these baseline differences in the presence of AL and other complications may further highlight these differences.

Chapter 7

RESULTS: RECOVERY FOLLOWING COLECTOMY WITH ANASTOMOTIC LEAKAGE

7. RESULTS: RECOVERY FOLLOWING COLECTOMY COMPLICATED WITH ANASTOMOTIC LEAKAGE

7.1 Background

Chapter 6 outlined the inflammatory response in an uncomplicated recovery and identified important factors to be considered in the interpretation of results. In this chapter, the inflammatory response in the context of AL is explored in detail. As noted in earlier chapters, the early detection of AL, is a clinical and diagnostic challenge.

This prospective observational study was therefore conducted to evaluate the diagnostic utility of inflammatory biomarkers in detecting AL early in the post-operative period following elective colectomy.

7.2 Methods

Specific methodological details were outlined in Chapter 5: Methods.

7.3 Results

A total of 405 patients were initially identified through hospital records across the four hospitals in Auckland and Christchurch over a 3-year period. At preadmission clinics, 390 patients initially agreed to participate. Sixteen patients developed AL (5.65%). A total of 283 patients had sufficient data to be included in the final analysis. Patient inclusion and exclusion details are shown in Figure 16.

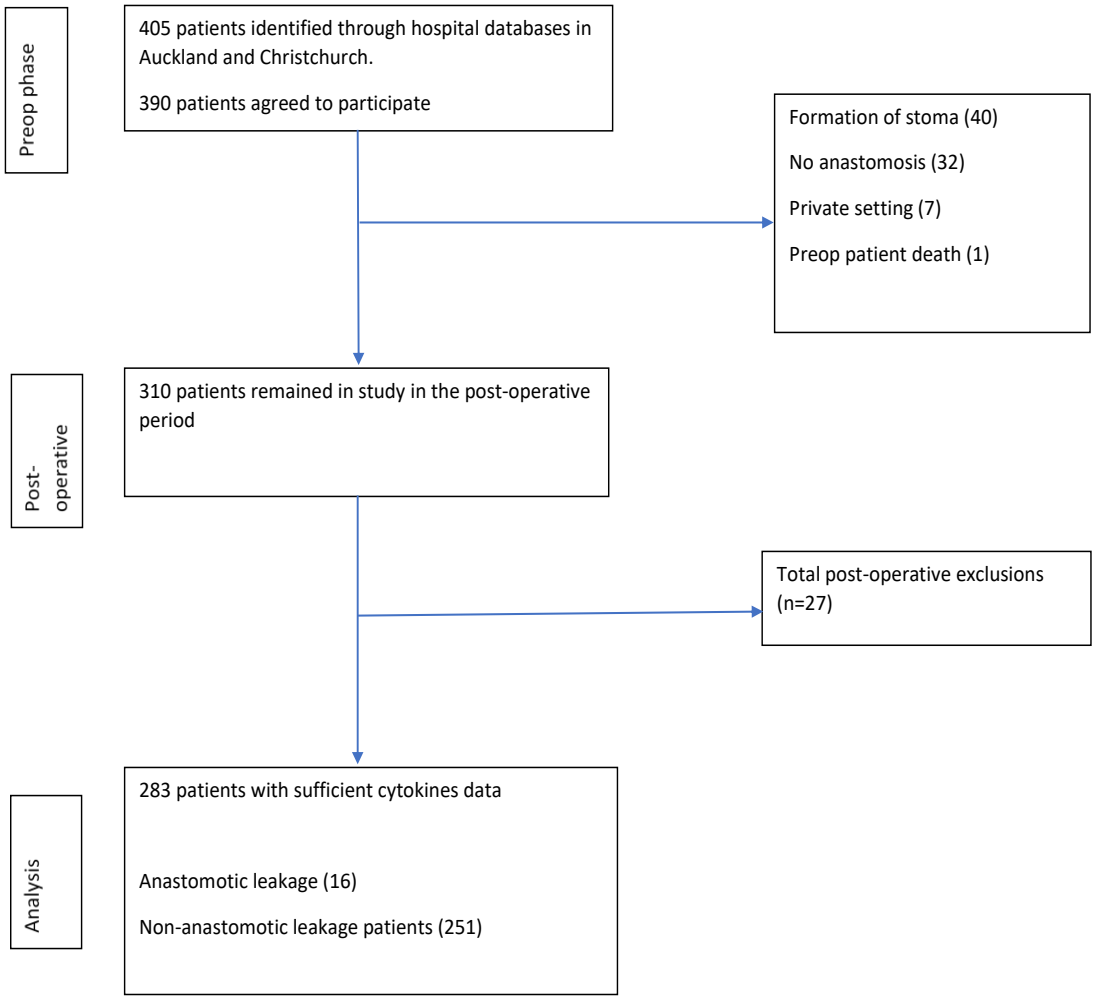

Figure 16 BALL study patient flow and recruitment

7.3.1 Baseline characteristics

Baseline demographics and medical background are shown in Table 20. Type 2 diabetes mellitus (T2DM) rates were higher in AL patients, but this result did not reach statistical significance ($p = 0.08$). Patients with AL had a significantly longer hospital day stay (13 vs. 7 days, $p < 0.005$). Other variables including operative time and type of resection were not statistically significant. The strengths of association ranged from very weak to moderate.

Table 20 Baseline demographics

Baseline demographics			
	Leak n = 16	No leak n = 267	p value $
Demographics			
Gender (male, %)	63	37	n/s
Age (mean years, SD)	68 (12)	67 (14)	n/s
BMI (mean, SD)	26 (3)	30 (19)	n/s
Previous abdominal surgery (%)	24	37	n/s
Comorbidity (%)			
Hypertension	50	55	n/s
Respiratory disease	19	19	n/s
Dyslipidaemia	31	18	n/s
Liver disease	6	1	n/s
Type 2 DM	31	15	0.08
Cardiovascular disease	25	31.5	n/s
Renal disease	0	8.8	n/s
Previous abdominal surgery	27	37	n/s
Current smoker	0	6	n/s

Following surgery	Leak	No leak	p value
Hospital length of stay	13 (7)	7.5 (4)	<0.005

n/s: not significant

$Categorical variables were compared using Pearson Chi-square.

Mann-Whit U for non-parametric variables

7.3.2 Inflammatory response in anastomotic leakage

A summary of the different inflammatory biomarker mean concentrations on each respective post-operative day (POD) are shown in Table 21. Significant results are outlined in the following sections.

Table 21 Summary of measured inflammatory biomarkers

Summary of measured inflammatory biomarkers						
	Preop	POD 1	POD 2	POD 3	POD 4	POD 5
CRP (mg/L)						
No Leak	10.7	72.5	114.3	115.7	102.5	96.7
Leak	3.4	100.2*	153.5*	177.4*	142.6	150.1*
Leucocyte ($x10^9$ /L)						
No Leak	7.6	11.3	10	8.7	7.8	7.7
Leak	7.9	11.7	11.0	8.5	7.1	8.4
Neutrophils ($x10^9$ /L)						
No Leak	5	8.9	7.7	6.5	5.6	5.4
Leak	4.9	9.4	9.1*	6.5	5.4	6.3

IL-10 (pg/mL)						
No Leak	23	26.9	22	24.8	32.8	30.5
Leak	23.6	36.2	37.8*	47.3*	96.3*	31.6
IL-6 (pg/mL)						
No Leak	10.4	43.3	30.7	25.5	23.8	28.7
Leak	9.4	83.4*	56.0*	122.8	670.9	18.5
IL-1β (pg/mL)						
No Leak	6.7	5.3	5.3	5.7	8	6.6
Leak	9.3	6.6	3.7	7.1	5.9	7
TNFα (pg/mL)						
No Leak	29	29.9	28.6	31.3	35.8	33.6
Leak	36	35	32.4	47.5*	47	39.6

* denotes a statistically significant result

7.3.2.1 C-reactive protein

There was a statistically significant difference between leak and no leak patients as determined by one-way ANOVA. Differences were found on POD 1 (F (1,185) = 5.44, p = 0.02); POD 2 F (1,198) = 3.9, p = 0.047; POD 3 F (1,193) = 6.75, p = 0.01, and POD 5 F (1,141) = 5.94, p = 0.016.

A multiple regression analysis was performed to predict POD 1 CRP from BMI, operative approach, and AL. These variables statistically significantly predicted POD 1 CRP F (3, 122) = 6.5, $p < 0.005$, $R^2 = 0.137$. The operative approach was the only variable that added statistically significantly to the prediction, p = 0.001. On POD 2, regression

analysis remained statistically significant F (3, 126) = 8.9, p < 0.005, R^2 = 0.175. However, both AL (p = 0.04), and operative approach (p < 0.005) added statistical significance to the result. On POD 3, the above variables remained statistically significant (p = 0.001) with both AL and operative contributing to the statistical significance (p = 0.02 and p = 0.06 respectively). Regression analysis did not reach statistical significance on POD 5.

Receiver operating characteristic (ROC) area under the curve (AUC) analysis was only statistically for POD 3 (0.70 p = 0.02); and POD 5 (0.69 p = 0.02). Figure 17 illustrates the ROC analysis for CRP on POD 3. A POD 3 CRP level of 106.5mg/L revealed a negative predictive value (NPV) of 97% for AL.

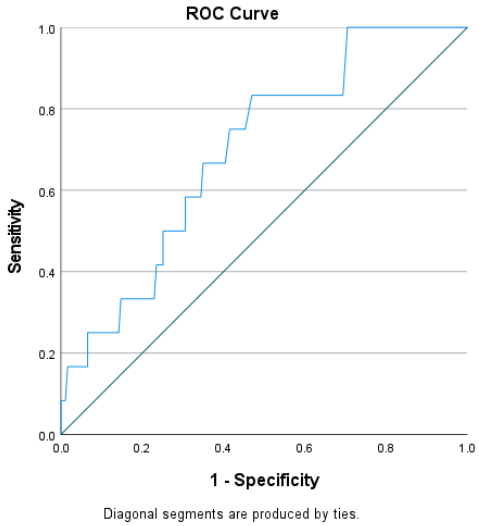

Figure 17 AUROC CRP on POD 3

7.3.2.2 Interleukin-10

IL-10 showed a statistically significant difference between leak and no leak patients as determined by ANOVA. Differences were found on POD 2 (F (1,155) = 5.52, p = 0.02); POD 3 (F (1,155) = 7.3, p = 0.008); and POD 4 (F (1,127) = 9.34, p = 0.003).

A multiple regression analysis was performed to predict IL-10 levels from BMI, operative approach, and AL, from POD 2 to POD 4. These variables statistically significantly predicted POD 2 IL-10 F (3, 196) = 7.45, $p < 0.005$, $R^2 = 0.19$. Both AL and operative approach added statistically significantly to the prediction, $p = 0.001$ and $p = 0.03$, respectively. Similarly, the same variables reached statistical significance on POD 3 (F (3, 97) = 7.45, $p = 0.001$, $R^2 = .16$). However, AL was the only statistically significant contributing variable ($p < 0.005$). On POD 4, these same variables significantly predicted IL-10 levels ($p = 0.004$). Like POD 3, only AL on POD4 contributed statistically significantly to the prediction (F (3, 77) = 4.72, $p < 0.005$, $R^2 = 0.15$). ROC analysis for IL-10 was not significant on any post-operative days.

7.3.2.3 Interleukin-6

There was a statistically significant difference between leak and no leak patients on POD 1 (F (1,154) = 6.2, $p = 0.01$) and POD 2 F (1,155) = 8.6, $p = 0.004$.

Multiple regression analysis were statistically significantly predicted on POD 1 (F (2, 153) = 6.5, $p = .002$, $R^2 = 0.1$; and POD 2 F (2, 154) = 10.2, $p < .005$, $R^2 = 0.12$). On both post-operative days, both AL ($p < 0.005$) and operative approach ($p = 0.01$) contributed significantly to its prediction.

POD 1 ROC analysis revealed a statistically significant AUC (0.68, p=0.03). A cut-off value of 10.8 pg/mL revealed a NPV of 99.1% (sensitivity 0.85, specificity 0.83) for AL. On POD 2, ROC analysis similarly showed a statistically significant AUC (0.69, $p = 0.02$).

7.3.3 Early and late anastomotic leakage

Most patients with AL were diagnosed beyond POD 6 (81%). AL patients were categorized into early or late diagnosis, with early diagnosis defined as <6 days. When comparing biomarker concentrations levels, only IL-1β had significantly elevated levels on POD 3 in early AL patients.

When grouped in AL diagnosed prior to POD 5 compared with beyond POD 5, several biomarkers were significantly elevated from POD 3 and beyond. This is likely explained by AL patients diagnosed on POD 3 and POD 4.

7.4 Discussion

This present study has shown that patients with anastomotic leak have significantly elevated inflammatory markers postoperatively. CRP, IL-10, and IL-6 were consistently elevated in patients with an anastomotic leak in the early postoperative period.

Elevated levels of inflammatory biomarkers in the early postoperative period suggests that the detection of AL can be achieved before clinical symptoms and signs become apparent. Furthermore, an exaggerated response may contribute directly to the development of AL. High levels of preoperative CRP in one study adds weight to this hypothesis. [137]

IL-10 was statistically elevated from POD2 to POD 4. Regression analysis showed AL as the only significant contributor to the elevated levels of IL-10 on these post-operative days. IL-10 is primarily an anti-inflammatory cytokine secreted by M2 Macrophages as part of the cellular immune response in the presence of commensal flora such as *Escherichia coli*.[209] The diagnostic utility of CRP reported in the literature suggests its role is useful as a negative predictor for AL.[3] Results in this study concur with this finding.

The primary limitation of this study is that surgical teams caring for enrolled patients were not blinded to the results of CRP, leucocytes, and neutrophil count. Elevated levels resulted in a more diligent search for the unpredicted rise of above inflammatory biomarkers. These results are discussed further in the next chapter.

Chapter 8

DISCUSSION

8 DISCUSSION

The preceding chapters discussed each literature review and the prospective study separately. An integrated discussion is required to summarize and appraise overall literature and study findings, discuss the limitations, and the clinical implications of this work.

8.1 Overview of results

Chapter one introduced the topic of anastomotic leakage and the important role inflammatory biomarkers have in facilitating early diagnosis. Chapter two illustrated an overview of the normal physiological response following major colorectal surgery. It showed that in published randomized controlled trials, the physiological inflammatory response is influenced by a plethora of patient and operative variables. Several inflammatory biomarkers were identified such as C-reactive protein and interleukin-6. The literature review also identified significant heterogeneity in these published studies.

Chapter three evaluated clinical parameters in patients with anastomotic leakage. It showed that none of the reported clinical signs and symptoms were consistently abnormal leading up to the diagnosis of AL. Chapter four critically appraised inflammatory biomarkers currently used to diagnose AL. It showed that, despite considerable study heterogeneity, several biomarkers sampled from the systemic circulation and intra-peritoneal environment are elevated before the diagnosis of AL. Systemic inflammatory markers that reflect the intra-peritoneal environment are more suitable given that the clinical utility of intraperitoneal perianastomotic drains is controversial.

Findings from the preceding chapters aided in the development of the biomarker and anastomotic leakage (BALL) study, outlined in Chapter Five. The study was designed to reduce the study limitations identified in Chapter four.

The BALL study results were described in Chapter six and Chapter seven. Chapter six evaluated the physiological inflammatory response following an elective colectomy without anastomotic leak. It showed that there is a significant rise in inflammatory biomarkers levels in patients with a high BMI or who had undergone an open colectomy. Chapter seven analysed the diagnostic utility of inflammatory biomarkers in the context of AL. It showed a moderate diagnostic value with AUROC of 0.7.

8.2 Appraisal of literature findings

The inflammatory response to colorectal surgery and its complications outlined throughout the thesis is a complex and dynamic process. It is designed to facilitate healing following surgery. As outlined in Chapter 1, there is a complex milieu of proteins at the site of injury to facilitate this process that are altered in the context of an anastomotic leak. Chapter 2 described the multitude of proteins and cells at the peri-anastomotic site following a colectomy with anastomosis. The inflammatory response is altered by the surgical access wound. Systemic levels of IL-6, as part of the humoral response, are significantly reduced from as early as 2 hours following a laparoscopic colectomy compared to an equivalent open colectomy. The reduced inflammatory response translates to better overall clinical outcomes, but no difference in AL rates.

In interpreting these data many investigators have simply attributed these differences in outcomes to the smaller access incisions. However, smaller access skin incisions, in isolation, may not equate to a reduced inflammatory response. Other perioperative

factors, evidenced by enhanced recovery after surgery protocols, may play a more significant role in reducing the inflammatory response. These improvements, however, do *not* translate to improved anastomotic leakage rates as shown in Chapter 4.

Chapter 3 outlined the clinical difficulty in clinically detecting anastomotic leakage early in the early postoperative period as AL can mimic other less serious complications. As AL progresses to sepsis, the body exhibits a typical systemic inflammatory response syndrome response with tachycardia, hypotension, and tachypnoea. The intra-abdominal nature of the complication further obscures the diagnosis from direct observation.

Two studies discussed in Chapter 3 found that a high preoperative diastolic pressure of >90mmHg is associated with the development of anastomotic leakage. Furthermore, an intraoperative reduction of >40% in diastolic pressures was also significantly associated with the development of leakage. These patients likely have a higher susceptibility to hypoperfusion and ischaemia at the site of anastomosis from chronic or acute systemic vasoconstriction, respectively. Some perioperative analgesic regimens such as regional anaesthesia cause significant reductions in perioperative blood pressure. Studies evaluating these have shown a reduction in tissue perfusion from reduced IMA flow associated with a decreased systemic mean arterial pressure. However this does not increase the risk of AL.[210-212] Studies that compared an ERAS environment to a non-ERAS environment showed a significant improvement in the measured inflammatory response as shown in Chapter 2. This reduction translated to overall better clinical outcomes for the patient. However, as seen in Chapter 4, AL rates did not change despite this improvement. This suggests that local processes that result in AL are likely driving the exaggerated systemic response.

Chapter 4 identified a variety of intra-peritoneal and systemic biomarkers that are significantly elevated in the presence of AL. Chapter 5 incorporated findings from the preceding chapters, which assisted in the design of the BALL study. In addition to routine biomarkers such as CRP, leucocytes and neutrophil count, four cytokines were selected based on their pro-inflammatory or anti-inflammatory properties – IL-6, a well-known potent pro-inflammatory cytokine that directly controls the release of CRP into the systemic circulation; IL-10 an anti-inflammatory cytokine released in response either to an exaggerated inflammatory response or presence of a pathogen; IL-1β, a potent pro-inflammatory cytokine that is produced by endothelial cells in response to endotoxins and micro-organisms; and TNFα, a cytokine that enhances inflammatory cell recruitment to the site of injury. Though limited, these inflammatory biomarkers covered the different phases of inflammation outlined in Chapter 1. The time-points specifically targeted early postoperative period before discharge, or prior to overwhelming sepsis.

However, not all the perceived limitations of reported previous studies could be addressed by the BALL study. The progress made by this study, however, could allow future studies to advance our understanding of anastomotic leakage. One significant, but unavoidable, limitation was the lack of blinding the surgical teams. Incidental rises in CRP or leucocytes prompted a vigorous search for underlying pathology.

8.3 Appraisal of BALL study findings

Following surgery, the peri-anastomotic environment is awash with both proinflammatory and anti-inflammatory biomarkers as part of the inflammatory and healing process. The composition of this usually follows a predictable course over

several weeks. In the context of AL this inflammatory environment is altered, and likely before any clinical features become apparent.

In an altered inflammatory response, a combination of localised infection from enteric bacteria, with an impaired macrophage phagocytic function, may result in a prolonged pro-inflammatory phase. This local change is reflected in the systemic circulation, seen in the BALL study results. A combination of both patient and surgery factors contribute to the initial inflammatory deviation at the anastomotic site.

These include the operative approach and body mass index (BMI). An open approach resulted in a higher inflammatory response; a finding consistent with the present literature outlined in Chapter 2. A bigger incision, or greater surgical trauma, leads to upregulation and recruitment of PMN leucocytes and macrophages at the site of injury. Despite the higher absolute number of recruited leucocytes, under normal healing circumstances, inflammatory cells are programmed to switch to the anti-inflammatory phase and start remodelling, as described in Chapter 1.

This difference in operative approach was especially evident on POD 1 and POD 2 and has the potential to mask the overall inflammatory response driven by AL among laparoscopy patients. At later post-operative days, differences in inflammatory biomarkers between the two operative approaches seemed to level out as seen in Chapter 7. In patients with AL however, CRP and other inflammatory biomarkers remained consistently higher at later postoperative days.

Obese patients were found to have a higher inflammatory response following surgery compared with non-obese patients. Multiple regression analysis further demonstrated a higher-than-normal inflammatory response regardless of operative approach on POD 4 in obese compared to non-obese patients ($p<0.05$). Recent experimental work has

demonstrated that obesity impacts several facets of the immune response. In lean mice, M2 macrophages predominate and enhance the anabolic actions of insulin via anti-inflammatory cytokines. In contrast, obese adipose tissue is largely infiltrated with M1 macrophages, which promote insulin resistance via secretion of pro-inflammatory cytokines. Further, innate, and adaptive immune cells normally reside in adipose tissue. Thus, adipose tissue is not only a site for storage of fats or secretion of adipokines, but also a tertiary lymphoid organ for macrophage-mediated antigen presentation and lymphocyte activation. [213, 214]

A multiple regression analysis was performed to predict POD 4 CRP from BMI and surgical approach. These variables statistically significantly predicted POD 4 CRP albeit with a moderate model fit. Only the BMI, added statistically significantly to the prediction.

The above variables therefore need to be considered when analysing the postoperative inflammatory response. After adjusting for open approach, only AL was a significant contributor to elevated levels of IL-10 from POD 2. Another study discussed in Chapter 2 showed elevated intra-peritoneal levels of IL-10, but to the author's knowledge, no other published study so far has shown significant levels of IL-10 in the systemic circulation in AL.

8.4 Altered inflammatory response

As discussed in Chapter 1, an altered inflammatory response provides the most fitting explanation for the pathophysiology of anastomotic leakage. AL develops when there is a direct *communication*, *infection*, or *healing disturbance*. [33]. It is likely that there is a degree of leakage with every anastomosis formed that inevitably results in some

contamination by enteric bacteria. The significance and size of this *direct communication* in the early postoperative period is probably related to varying degrees of technical failure whether due to a problem with the anastomotic technique or the adequacy of the blood flow to the anastomotic site. Thus, *infection* at the local anastomotic site is inevitable from enteric bacteria during the operation. How the body reacts to this infection and how well the body can repair this leakage appears to influence the clinical significance of the leak.

Healing disturbances occur in high-risk patients, such as diabetics and the immunocompromised, that result in impaired healing at the anastomotic site. Under normal physiological conditions, anastomotic healing begins with the inflammation phase with clearance of debris and phagocytosis by M1 macrophages and PMN leucocytes. In these patients, the phagocytotic ability may be reduced, resulting in an accumulation of necrotic and apoptotic cells at the anastomotic site. This can further prolong the inflammatory phase, and delay the anti-inflammatory release from M2 macrophages, seen in murine studies. [80] Other healing disturbances such as hypoxia from poor anastomotic vascularity, result in an increase in proinflammatory cytokines, which may further differentiate native cells to M1 phenotype.[81]

Instead of controlling the acute phase of infection, a prolonged M1 response (pro-inflammatory) can be detrimental.[58] In prolonged inflammatory responses, experimental studies show that M1 type macrophages upregulate the expression of nitric oxide (NO), which in addition to its defence role, plays a significant role in collagen deposition. [59, 60] Excessive NO however, results in impaired wound healing by breaking down the collagen framework at the anastomotic site. [61]

Results from the BALL study showed significantly elevated levels of IL-6, C-reactive protein, and IL-10 with AL. The theorised prolonged inflammatory response in patients with AL is consistent with the significant levels of IL-6 and CRP. The early high levels of IL-10 in the context of AL, however, has not been well documented in the literature. As an anti-inflammatory cytokine released following the initial inflammatory phase, its role is to facilitate the *proliferation* phase of healing, such as promoting fibroblastic activity as described in Chapter 1. In Chapter 2, drain peritoneal IL-10 in one study in the first 12 hours after surgery was significantly elevated in patients with AL.

The persistent elevation of IL-10 is likely to be a direct and compensatory anti-inflammatory reaction to the altered pro-inflammatory response. The anti-inflammatory response seems to match the initial pro-inflammatory response. A severe injury such as surgery will result in a greater response to reduce the un-intended effects of tissue damage from the inflammatory response. This is evident in significant IL-10 levels in an uncomplicated open approach compared with laparoscopy, seen in Chapter 6. Even adjusting for the open approach, IL-10 levels in AL remained significantly elevated on POD 3 and POD 4.

Another explanation for this persistent rise of IL-10 in AL, is related to timing. If released *too late* after a major injury (such as surgery), tissue damage will occur at the site of surgery from excessive inflammation. If released too soon, and the inflammatory response is reduced to a degree whereby local apoptotic and bacterial contamination from surgery are not appropriately cleared to enable healing. The release of anti-inflammatory mediators following injury is an innate programmed ability.[215] It isn't well understood, however experimental studies point to numerous receptors and mediators that enable internal signalling for this to occur.[216, 217]

Given the exaggerated inflammatory response observed in the preceding chapters, it is likely that IL-10 is released in reaction to the prolonged pro-inflammatory response. Thus, the development of AL is likely a result of a persistently altered postoperative inflammatory response that culminates in an impaired anastomosis that results in a clinically significant leak.

8.5 Limitations and implications for future research

Several recurring study limitations were identified in the systematic reviews. The definition of AL was heterogenous with studies using one or a combination of clinical, radiological, and operative findings. There has been no agreed upon definition specifically for colonic anastomotic leakage. This is paramount as the definition for AL determines the incidence. To address this limitation, the BALL study was prospective and utilized a standardised definition of AL.

Blinding of surgical teams is another significant limitation. Surgeons and their respective clinical teams for each enrolled patient were not blinded to routine biomarker results. Specifically, these were CRP, leucocyte, and neutrophil count. Rises in these biomarkers can lead to a more vigorous search for the underling pathology. Blinding in this context may not be possible in clinical studies as biomarkers are utilized daily in clinical decision making.

Other limitations include equipment and inter-hospital variations of CRP, WCC, neutrophils and albumin levels need to be considered. Although small variations can occur, hospitals employed similar equipment for measuring CRP and FBC as part of routine clinical practice. For the cytokine analysis, personnel variations for

measurements also need to be considered. Though the R^2, used for measuring technical accuracy, from the BALL study was consistently above 0.96 for everyone involved.

Occasionally, the physical presence of the BALL study PhD candidate around hospital wards and operating theatres, resulted in additional biomarker levels requested by the attending surgical team. This Hawthorne effect is difficult to measure but may have affected results. These included extra routine blood tests, longer than necessary hospital day stay for clinical observation, and additional imaging for incidental rises in routine inflammatory biomarkers.

In an ideal world, the author would have wanted to measure multiple other promising inflammatory biomarkers identified in Chapter 4. These include fatty acid binding protein, other cytokines, and microbiological markers. However, financial constraints limited the study to the 4 cytokines chosen together with CRP, WCC and neutrophil count. At the onset of BALL study recruitment, surgical recovery scores were also collected. However, due to mainly logistical challenges to coordinate all 4 hospitals follow up for up 30 days, this was abandoned part way through the first year of recruitment. Although not a primary focus of this thesis, attaining data on recovery for patients with AL and/or raised inflammatory biomarkers may be of interest in future studies.

To reduce study heterogeneity, future studies need to incorporate an agreed upon definition for AL. Blinding may not be possible for routine everyday biomarkers, but it is possible for novel biomarkers. Identifying real time release of anti-inflammatory and pro-inflammatory biomarkers at the injury site would shed further light into our understanding of AL.

8.6 Clinical implications

Findings of the BALL study suggest that the diagnostic utility of systemically measured inflammatory biomarkers is a useful detector of, although not specific to, clinically significant AL. This is because the measured inflammatory biomarkers are primarily an objective measurement of an overall inflammatory response rather than specific to AL. Their clinical utility may therefore be best suited as negative predictors for AL in the early postoperative period.

Only IL-10 showed significant elevation in the context of AL after adjusting for other variables. As discussed above, IL-10 release is likely a response to the altered inflammatory response following surgery.

Chapter 9

CONCLUSION

9 CONCLUSION

This thesis has explored the role of biomarkers, released as part of the inflammatory response following elective colectomy, in the early detection of anastomotic leakage.

Anastomotic leakage is a devastating complication associated with significant morbidity and mortality. Its early diagnosis is required to avoid impaired short- and long-term survival. Early and timely clinical diagnosis however remains a significant challenge. Clinical parameters in patients with anastomotic leakage are variable and remain unreliable in its early detection or prediction. Analysing biomarkers following colorectal surgery is a promising way to detect anastomotic leakage before it manifests clinically.

It is likely that there is a degree of leakage with every anastomosis formed that results in bacterial contamination. The significance and size of this direct *communication* in the early postoperative period is probably related to varying degrees of technical failure whether due to a problem with the anastomotic technique or the adequacy of the blood flow to the anastomotic site. Infection at the local anastomotic site is inevitable from enteric bacteria during the operation. How the body reacts to this infection and the subsequent inflammatory response and how well the body is able to repair this leakage appears to influence the clinical significance of the leak. This response is influenced by several predisposing factors including smoking and the use of immunosuppressants amongst others.

While a marked inflammatory response occurs with an anastomotic leak as shown in this thesis, this response is non-specific and is similar to that observed with a few other complications following colectomy. The absence of an inflammatory response is a

reliable negative predictor for anastomotic leak, however. Thus, in clinical practice, the role of currently available biomarkers is limited to facilitation of early discharge.

Prospective studies need to utilize an agreed upon definition for anastomotic leakage, follow blinding protocols where possible, and limit investigator study visibility. Understanding the pathophysiology has significant clinical implications. A search for more specific biomarkers for anastomotic leak is necessary and will require further large-scale prospective studies.

Appendix 1: Participant information sheet

THE UNIVERSITY OF AUCKLAND
FACULTY OF MEDICAL AND HEALTH SCIENCES

South Auckland Clinical School, PO Box 93311, Otahuhu, Auckland, NZ

COUNTIES MANUKAU HEALTH

Defining a biomarker profile for anastomotic leakage following colon surgery – Participant Information Sheet

Principal Investigator: Professor Andrew G Hill, Department of Surgery, Middlemore Hospital, Otahuhu, Auckland
Phone: (09) 276 0044 ext 8424, Email: a.hill@auckland.ac.nz

You are invited to take part in a study predicting anastomotic leakage following colon surgery. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you would like to take participate in the study. It sets out the purpose of the study, what your participation would involve, what the risks may be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. Please make sure you have read and understood all the pages.

Study Background

Surgery for colorectal cancer involves two (2) main parts:

1) Removing the affected bowel segment and;
2) Reconnecting the remaining bowel ends.

This bowel connection is known as an anastomosis. One of the serious complications after surgery is failure of the anastomosis (anastomotic leakage or bowel leakage). When this occurs, bowel contents spill into the abdominal cavity. This often results in the patient becoming very unwell requiring urgent treatment.

The dilemma we face as clinicians is predicting bowel leakage before the patient becomes critically unwell. Blood tests have been shown to play a role in predicting and identifying diseases that occur in the body. Therefore, we aim to identify a blood test that will predict bowel leakage before the patient becomes unwell.

About the study

Over a period of 24 months, we are planning to invite 600 patients who are undergoing colon surgery to take part in this study. Patients undergoing surgery routinely have blood tests for monitoring purposes.

If you agree, we wish to collect about 8-12mL extra blood each time you have your routine blood tests and this will occur at six points throughout your care; once before surgery and for the first five days after surgery.

Once we have adequate blood samples, we will analyse the blood to determine a suitable test that allows us to predict bowel leakage. This study will not benefit you directly, but if successful and we find a suitable blood test, it will allow us to provide better effective treatment for future patients that develop bowel leakage.

Participation in the study

Before and after surgery, you will have the same routine care as all other patients, and your participation in this study will not affect the standard of care you receive in any way.

We will collect this blood at the same time as your routine blood tests and therefore you will not have extra blood tests. The blood samples will then be sent to a laboratory for analysis.

We will also ask you to complete a set of questionnaires with the assistance of researchers. They will ask about pain, energy levels and bowel symptoms. These questionnaires will be completed at six (6) different time points; once before surgery, Day 3 after surgery, Day 7 after surgery, Day 14 and Day 30. Each 'Day' questionnaire should take only a few minutes to complete.

If you do agree to participate in the study but later change your mind mid-way through, you can withdraw your data completely or leave it for the purpose of the study. If you withdraw from the study and want your data removed, your collected data will be removed from the dataset at the end of the study. Your results will also be excluded from the study.

Once you have been discharged from hospital, we will contact you once a week for one month to follow up on how you are doing at home. We will be asking questions around your recovery and any unscheduled doctor visits.

At the end of the study

After analysing your blood samples, any leftover samples will be destroyed in accordance to laboratory protocols of disposing human blood specimen. The data collected for the study will be complex and may take many months to analyse. For this reason, any collected data will be stored for up to ten (10) years.

If requested on the consent form, any unused blood will be returned to the participant at the end of the study.
Your consent form will be stored for at least ten (10) years in a secure location separate from the collected data and blood specimens, and destroyed once all analysis completed.

Risks and Benefits

There are no direct risks or benefits for participating in the study.

Confidentiality

No material which could personally identify you will be used in any reports on this study. Your hospital records are confidential. Your name or any other personally identifying information will not be used in reports or publications resulting from this study. The information about your medical history and investigations required to interpret the research results will be identified using a special code (only accessed by investigators) to ensure your confidentiality. This code is a six figure code consisting of three letters and three numbers (for e.g. ABC123). Study data will be stored on secure files for at least ten years and then appropriately discarded.

Further Information and Results

The final results of the research will not be known until December 2018. At the conclusion of the study, results will be made available to those who have requested this on the consent form. However, if you are not sure about whether you have requested the study results or you would like further information about the study, please feel free to contact Dr Bruce Su'a, Research Fellow, Middlemore Hospital (Phone 021 068 9983, Email bruce.sua@middlemore.co.nz).

Project funding

This study has received seed funding from the Colorectal Surgical Society of Australia and New Zealand Foundation Pty Ltd. Any future funds received will be for implementation and successful completion of the project.

Advocacy and Support

For any queries regarding ethical concerns you may contact the Chair, The University of Auckland Human Participants Ethics Committee, The University of Auckland,

Research Office, Private Bag 92019, Auckland 1142. Telephone 09 373-7599 extn. 87830/83761. Email: humanethics@auckland.ac.nz.

To talk to someone who is not involved with the study, you can contact an independent health and disability advocate on: Phone: 0800 555 050, Fax: 0800 2 SUPPORT (0800 2787 7678), Email: advocacy@hdc.org.nz

To talk to a Maori cultural support person here at Middlemore Hospital and Manukau Surgery Centre, you can contact Karla Rika-Heke; email Karla.Rika-Heke@middlemore.co.nz.

Approval

This study has been approved by the Health and Disability Ethics Committee (HDEC), on the 3rd November 2014. Ethics ref: 14/NTB/173

Appendix 2 Consent form

Consent Form	

If you need an INTERPRETER, please tell us.

Please tick to indicate you consent to the following

I have read or have had read to me in my first language, and I understand the Participant Information Sheet.	Yes ☐	No ☐
I have been given sufficient time to consider whether or not to participate in this study.	Yes ☐	No ☐
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	Yes ☐	No ☐
I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	Yes ☐	No ☐
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	Yes ☐	No ☐

I consent to the research staff collecting and processing my information, including information about my health.	Yes ☐	No ☐
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes ☐	No ☐
If I decide to withdraw from the study, I consent for the information collected about me up to the point when I withdraw to be removed from the study data.	Yes ☐	No ☐
I agree to an approved auditor appointed by the New Zealand Health and Disability Ethic Committees, or any relevant regulatory authority or their approved representative reviewing my relevant medical records for the sole purpose of checking the accuracy of the information recorded for the study.	Yes ☐	No ☐
I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	Yes ☐	No ☐
I understand the compensation provisions in case of injury during the study.	Yes ☐	No ☐
I consent to the researchers taking a specimen of my blood for the purposes of this study	Yes ☐	No ☐
I know who to contact if I have any questions about the study in general.	Yes ☐	No ☐
I understand my responsibilities as a study participant.	Yes ☐	No ☐

I consent to blood samples being destroyed at the end of the study	Yes ☐	No ☐
I wish to have left over blood samples returned to me after analysis	Yes ☐	No ☐
I wish to receive a copy of the results from the study.	Yes ☐	No ☐

Declaration by participant:

I hereby consent to take part in this study.

Participant's full name:

Signature: Date:

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant and have answered the participant's questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher's name:

Signature: Date:

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