

**STUDIES ON MECHANISMS OF
ENTEROCUTANEOUS FISTULA HEALING**

By

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DECLARATION OF ORIGINALITY

I declare that the following research thesis and studies are my own original work. This includes review of the literature, data collection, statistical analysis, meta-analysis, laboratory study design, specimen collection, transport of tissue, processing, flow cytometry and immunological analysis.

Surgical procedures and colonoscopies were performed by clinical staff at St. Mark's Hospital and Academic Institute.

Immunological assistance and analysis was provided by staff at the Antigen Presentation Research Group, St. Mark's Hospital, Imperial College London.

All work reported in this thesis has been appropriately referenced. The thesis has not been previously submitted to this or any other University for a degree, and does not incorporate any material already submitted for a degree.

Finally, any errors or mistakes in this thesis are my responsibility alone.

Goher Rahbour

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ABSTRACT

This thesis reviewed and investigated several aspects of the management of enterocutaneous fistula (ECF).

The best management of ECF, particularly complex fistulas, is in specialist units where there is a multidisciplinary structured approach. Surgical management of ECF at St. Mark's National Intestinal Failure Unit is safe and improving over time. The highest incidence of ECF development occurs following abdominal surgery particularly in the presence of Crohn's disease. The audit confirmed this in our unit.

A retrospective audit of our unit revealed the majority of patients underwent definitive surgery. There were improved overall healing rates following both surgery and conservative management in the current cohort, with also improvement in the 30 day post fistula resection mortality compared with a previous series at our unit.

Multivariate analysis revealed co-morbidity, source of referral and aetiology had significant associations with ECF healing.

This thesis has included the first study and design of a scoring system for predicting ECF healing. The scoring systems devised were both validated. They can provide further information to aid clinical management and decision making for the multidisciplinary team and predict the possible long term outcome for patients at external institutions.

Meta-analyses of randomised control trials has revealed somatostatin and octreotide increase the likelihood of fistula closure, reducing the time to fistula closure, and neither has an effect on mortality.

Immunological studies revealed an increased on-going production of cytokines, in particular TNF- α , in non-IBD ECF compared with control terminal ileum tissue. The data may provide evidence for the potential use of anti-TNF- α agents in the treatment of non-IBD ECF.

A future pilot study has been designed with the aim to identify a potential novel therapy for patients with persistent enterocutaneous fistula not associated with inflammatory bowel disease. Any positive findings would equate to a major medical advance with a new use for anti-TNF- α agents.

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LIST OF ABBREVIATIONS

ACPGBI	Association of Coloproctology of Great Britain and Ireland
AGA	American Gastroenterology Association
AIDS	Acquired Immunodeficiency Syndrome
APACHE	Acute Physiology and Chronic Health Evaluation
APRG	Antigen Presentation Research Group
ARP	Anorectal physiology
ASA	American Society of Anaesthesiologists
AZA	Azathioprine
BCG	Bacillus Calmette–Guérin
BDRF	Bowel Disease Research Foundation
BMI	Body mass index
BP	Blood Pressure
CI	Confidence Interval
CINAHL	Cumulative Index to Nursing and Allied Health Literature
CLA	Cutaneous leukocyte antigen
CRP	C-reactive protein
CST	Cytometer Setup and Tracking
CT	Computer tomography
CXR	Chest x-ray
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DVT	Deep venous thrombosis
EAUS	Endo anal ultrasound

ECF	Enterocutaneous fistulas
ENS	Enhanced Normalized Subtraction
ESR	Erythrocyte sedimentation rate
EUA	Examination under anaesthesia
FACS	Flo associated cell sorting
FAP	Familial Adenomatous Polyposis
FBC	Full blood count
FSC	Forward scatter
GCP	Good Clinical Practice
HBV	Hepatitis B Virus
HLA	Human leukocyte antigen
HPN	Home parenteral nutrition
HRQL	Health-related quality of life
HCA	Health Care Assistant
ICS	Intra cellular staining
IF	Intestinal Failure
IFN	Interferon
IBD	Inflammatory bowel disease
IL	Interleukin
IR	Intensity Ratio
LFT	Liver function tests
METS	Metabolic equivalent levels
MHRA	Medicines and Healthcare Products Regulatory Agency
MP	Mercaptopurine
MRI	Magnetic resonance imaging

NET	Neuro endocrine tumour
NJ	New Jersey
OGD	Oesophago gastric duodenoscopy
PBMC	Peripheral blood mononuclear cells
PCA	Patient controlled analgesia
PET	Positron emission tomography
PN	Parenteral nutrition
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses.
QOL	Quality of life
RCT	Randomised control trials
REC	Regional ethics committee
RevMan	Review Manager 5.1
RPMI	Roswell Park Memorial Institute
RR	Risk ratios
SBS	Short bowel syndrome
SD	Standard Deviation
SEM	Standard Error of the Mean
SMD	Standardised mean differences
SmPC	Summary of product characteristics
SPSS	Statistical Package for the Social Sciences
SS	Side scatter
SSTS	Somatostatin receptors
STEP	Serial transverse enteroplasty
TB	Tuberculosis
TNF- α	Tumour necrosis factor - alpha

TREAT	The Crohn's Therapy, Resource, Evaluation and Assessment Tool Registry
U&E	Urea and electrolyte
UK	United Kingdom
US	Ultrasound
USA	United States of America
VAC	Vacuum assisted closure

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CHAPTER 1

GENERAL INTRODUCTION

Enterocutaneous fistulas (ECF) are abnormal communications between the gastrointestinal tract and the skin. Although rare, they are associated with considerable morbidity and mortality. Death related to ECF remains disproportionately high when compared with other surgical conditions. Mortality rates for ECF vary from 6 - 33% (1-6).

The incidence and aetiology of fistula are highly dependent on the surgical experience and case load at particular institutions and on patient and disease related cofactors. Much of the published data relate to experience at specialised centres treating complex cases (7). St. Mark's Hospital is a national and international referral centre for intestinal and colorectal disorders. The Intestinal Failure Unit has a long standing interest in inflammatory bowel disease (IBD), and this is reflected in the high prevalence of IBD patients treated at this institution.

It is estimated that 75–85 % of ECF that form after an operation occur as a result of bowel injury or anastomotic leakage (1, 8) . Fistula formation is also associated with surgery in the presence of IBD, malignancy or with division of dense adhesions (1, 8). In the remaining 15–25 % of cases, ECF form spontaneously secondary to the underlying pathology, with the most occurring in those with Crohn's disease (1, 8).

Up to 75% of patients undergoing abdominal or pelvic radiation will suffer some degree of radiation enteritis. In most cases this manifests as either bleeding or stricture; however, a small percentage may develop fistulous complications. In this subset of patients who develop a radiation-related ECF, it may take six months or

more for the fistula to become apparent (9). The study by Lynch et al revealed ECF caused by radiotherapy in 8% of patients (10).

Fistula formation can result in a number of serious or debilitating complications, varying from disturbance of fluid and electrolyte balance to sepsis and even death. The patient will almost always suffer from severe discomfort and pain. They may also have psychological problems, including anxiety over the course of their disease, and a poor body image due to the malodorous drainage fluid. Post-operative fistula formation often results in prolonged hospitalisation, patient disability, and enormous cost. Therapy has improved over time with the introduction of parental nutrition, intensive post-operative care, and advanced surgical techniques, which has reduced mortality rates (11).

Although ECF can close spontaneously, those that remain open after two months, will often require surgical intervention as spontaneous closure is unlikely after this interval (1, 12). It should be noted that major abdominal surgery stimulates the formation of dense adhesions. This reaction is most severe between three weeks and three months after an operation, and further surgery during this time is more likely to be complicated by fistula recurrence (1, 10, 13).

The first stage in my research will be to review the management of enterocutaneous fistulas and describe the numerous challenges it can present. This includes the difficulties which can arise from a reduction in functional length of the bowel and Intestinal Failure (IF). I will review the management undertaken by the multidisciplinary approach involving physicians, dietician, pharmacists,

microbiologists, interventional radiologists, wound / stoma care therapists, nursing staff and surgeons.

I will examine the clinical practice in the management of ECF and factors associated with fistula healing at our institution, which is a National Intestinal Failure centre in England. Retrospective data will be collected and a univariate and multivariate analysis will be performed on variables deemed to affect the outcome of ECF healing. If relationships are found to exist between variables and ECF healing then a scoring system to predict spontaneous fistula healing prior to surgery will be devised. This would be an additional source for the clinician in the decision making process.

Several pharmacological treatments have been described in the management of ECF, and in particular there is varied opinion as to the use of somatostatin and octreotide. The potential benefits include reducing patient's discomfort, shortening the length of hospital stay and reducing the cost of overall treatment. Some authors argue that high cost, equivocal therapeutic effect on underlying diseases, painful injections and potential for long-term side-effects make the use of somatostatin and its analogues controversial.

My research will include reviewing the literature comparing somatostatin and somatostatin analogues with standard medical therapy or a placebo in patients with ECF and conduct a meta-analysis.

The biological agent Infliximab inhibits a range of biological activities of TNF- α . It has been approved for treating Crohn's disease and fistulising Crohn's disease. My research will aim to study and assess the inflammatory activity, with a particular emphasis on TNF- α in non-IBD ECF when compared with control small bowel tissue.

The immunological studies will include assessing dendritic cells, which are antigen presenting cells that orchestrate the immune responses and regulate the production of cytokines by immune cells including T cells. No study to date has assessed the level of TNF- α in non-IBD ECF. The study will assess if there is evidence for the potential use of anti-TNF- α agents in the treatment of non-IBD ECF which would be an alternative option to benefit an already surgically challenging group of patients.

1.1 Hypothesis:

- Multiple factors which contribute to enterocutaneous fistula healing including patient factors, the choice and type of medical and surgical treatment options selected, and the immunological status of the ECF.

1.2 Aims of Thesis:

- Evaluate current management, practice and outcome of ECF at St. Mark's Hospital and Academic Institute.
- Examine and analyse factors associated with ECF healing.
- Devise a scoring system to predict outcome of ECF healing and assess its validity.
- Perform a meta-analysis of the use of somatostatin and somatostatin analogues in the treatment of ECF.
- Assess the inflammatory activity, with a particular emphasis on TNF- α in non-Inflammatory Bowel Disease ECF.
- Explore the association between TNF- α and dendritic cells (DC) in non-Inflammatory Bowel Disease ECF.

CHAPTER 2

OVERVIEW OF THE LITERATURE:

MANAGEMENT OF ENTEROCUTANEOUS FISTULA

2.1 ABSTRACT

Background

Enterocutaneous fistulas (ECF) can present many challenges. They may result in reduction of functional length of the bowel and Intestinal Failure (IF). Management is best undertaken by a multidisciplinary unit involving physicians, interventional radiologists, dieticians, pharmacists, microbiologists, wound / stoma care therapists, nursing staff and surgeons. Optimal surgical treatment has not been well documented in these patients.

Methods

An overview of the literature has been conducted of the English language literature in the Pub Med, Medline, EMBASE, CINAHL and Cochrane databases using the search terms: Enterocutaneous Fistula, Short Bowel Syndrome, Intestinal Failure and Intestinal Transplantation.

Results

Operative intervention should be delayed until sepsis has been controlled, nutrition optimised, and adhesions matured. Full radiological bowel mapping should be obtained prior to surgery.

If the surgery results in short bowel syndrome (SBS) techniques to slow intestinal transit or increase functional bowel length currently have a limited role in the adult population. Intestinal transplantation has improved the outcome in selected patients with SBS.

Conclusions

Units are required to offer a multidisciplinary structured approach to the management of patients with ECF and intestinal failure from high output fistula or short bowel syndrome.

2.2 INTRODUCTION

By definition, a fistula is an abnormal, epithelialised connection between two body structures. The word '*fistula*' is of Latin origin, and can mean a pipe or tube. An enterocutaneous fistula is an epithelialised connection between the gastrointestinal tract and the skin. This can be a devastating condition usually associated with abdominal surgery. ECF have been described dating back many centuries. The 18th century surgeon, Lorenz Heister, eloquently described his observations on the development of fistula after abdominal trauma in which he described "the lips of the intestines so wounded, would sometimes quite unexpectedly adhere to the wound of the abdomen (14)."

We conducted an overview of the English language literature in the Pub Med, Medline, EMBASE, CINAHL and Cochrane databases using the search terms: Enterocutaneous Fistula, Short Bowel Syndrome, Intestinal Failure and Intestinal Transplantation.

2.3 Aetiology

The incidence of ECF is highly dependent on the surgical experience and case load at particular institutions and on patient and disease related co-factors. Moreover, much of the published data relate to experience at specialised centres treating complex cases in particularly unstable patients (7) .

The incidence of ECF after surgery for Crohn's disease is approximately 13% with lower rates for other forms of abdominal surgery (15). Hollington *et al* performed an 11-year retrospective review of ECF managed at St. Mark's Hospital between January 1992 and December 2002. This study included 277 patients with a median age of 48

years. Most ECF occurred secondary to abdominal surgery (235 patients, 84.8%). Of these, 111 (47.2%) had inflammatory bowel disease (76 Crohn's disease and 35 ulcerative colitis) and 6% had been treated with radiotherapy. Of all patients, more than half (146, 57.7%) had inflammatory bowel disease (111 Crohn's, 35 ulcerative colitis) (4).

A low spontaneous healing rate was observed in 55 patients (19.9%). Healing rate after definitive fistula surgery was 82.0%. Definitive fistula resection resulted in a mortality rate of 3.0%. The overall fistula-related mortality rate was 10.8%. The spontaneous closure rate was lower than in many other reported series, perhaps reflecting the complex group of patients, many of whom were referred from other centres after failure of fistula healing. In addition, the St. Mark's Intestinal Failure Unit has had a long standing interest in inflammatory bowel disease, reflected in the relatively high prevalence of Crohn's disease in this cohort (4).

It is estimated that 75–85 per cent of ECF form after surgery as a result of bowel injury (inadvertent enterotomy) and / or anastomotic leakage (8, 16). Fistula formation is more commonly associated with surgery in the presence of malignancy or inflammatory bowel disease, and with attempted division of dense adhesions (8, 16).

In the remaining 15–25% of instances, ECF form spontaneously secondary to underlying pathology. Inflammatory bowel disease, in particular Crohn's disease, is the commonest cause of spontaneous enterocutaneous fistulation in the developed world (8, 16), other causes include radiation enteritis, diverticular disease,

malignancy, intra-abdominal sepsis and trauma (8, 16-19). The study by Lynch et al revealed ECF caused by radiotherapy in 8% of patients (10).

Decompressive surgery for abdominal compartment syndrome or laparostomy represents an additional risk for the development of ECF. Having the underlying gastrointestinal tract exposed to the atmosphere, has additional risk of ECF development as a result of bowel wall oedema, serosal injury and frequent manipulation during temporary abdominal closure dressing changes (20).

Symptoms can range from a minimal discharge from a low output fistula or recurrent abscess to multiple high output bowel loop fistulas with associated short bowel syndrome (SBS), sepsis and dehydration. Biochemical complications can arise in patients with high output ECF including severe hypokalaemia, hypocalcaemia, hypomagnesaemia and hyponatremia requiring urgent correction and resuscitation. For critically ill septic patients early goal directed management should be commenced (21).

The patient will almost always suffer from severe discomfort and pain. They may also have psychological problems, including anxiety over the course of their disease, and a poor body image due to the malodorous drainage fluid. Though not commonly addressed, persistent odour from the ECF can be a tremendous source of anxiety and social concern for these patients (22). Post-operative ECF formation often results in prolonged hospitalisation, patient disability, and enormous cost (20). In a critically ill patient with an ECF, costs can average approximately \$10,000 per day(22).

Health-related quality of life (HRQL) is lower in patients treated for enterocutaneous fistula than in matched controls, particularly in those with concurrent medical

illnesses. Patients treated successfully have normal independence in daily functioning (23).

Therapy has improved over time with the introduction of parenteral nutrition, interventional radiology, intensive care, and advanced surgical techniques and this has reduced mortality rates (4, 24). However, the number of patients suffering from gastrointestinal ECF has not declined substantially. This can be explained by the fact that with improved care, more complex surgery is being performed on patients with more advanced or complicated disease that are generally at higher risk. Therefore ECF remain an important complication following gastrointestinal surgery (11).

Death related to ECF remains disproportionately high compared with that associated with other surgical conditions, as ECF are often associated with sepsis. Studies reported over the past 30 years have shown mortality rates of 6–33 per cent in the most recent case series (2, 4, 5, 16, 25). Many of the patients who survive can go on to develop SBS resulting in significant overlap between the two conditions. Given their complex nature, severe cases should be managed in specialist units.

2.4.2 Management

The management of ECF can present a paradigm of multidisciplinary care. In those patients who fail to heal spontaneously surgical treatment is generally one of the last of many steps in the treatment of this condition (26). A structured approach to the management of ECF has been shown to improve outcomes. Owing to the heterogeneity of this condition, randomised placebo controlled trials have not been performed and most recommendations are based on expert opinions (27). This section will concentrate on the management of the most severe cases.

2.4.3 Medical management

The medical management of persistent ECF falls into four main categories - specialised nutritional support, control of sepsis, judicious use of pharmacological agents and wound / stoma care.

2.4.3.1 Specialised nutritional support

Laparotomy should be deferred until there is re-epithelisation of the abdominal wall and attainment of adequate nutrition and good general medical status (4). This process can take six months or more. There is no consistent evidence to support the concept that oral nutrition needs to be withdrawn in the acute phase of an enterocutaneous fistula. Healing is not enhanced and nutrition and patient moral may suffer. Encouraging enteral feeding may also decrease parenteral requirements and the risk of associated liver disease (28).

It has been suggested that total parenteral nutrition (TPN) has influenced fistula closure rates and mortality positively, although this has not been borne out by historical studies (16). The concept of 'bowel rest' in this setting is based on the observation that gastrointestinal secretions drop by 30–50 per cent in patients receiving PN and that this might aid fistula closure. However, although this may improve fluid and electrolyte balance, published data to suggest an improvement in spontaneous fistula closure does not exist, as randomised trials investigating outcomes in patients kept 'nil by mouth' versus PN have not been performed (16).

PN also has the additional risks of complications associated with central venous line placement. These include pneumothorax, vascular injury and venous thrombosis. PN may result in re-feeding syndrome characterised by electrolyte disturbance from the

rapid repletion of elements into depleted cells. This can result in hypokalaemia, hypophosphatemia and hypomagnesia as a result of glucose and amino acids rapidly moving into cells in exchange for potassium, phosphate and magnesium moving out. There is considerable data in the literature highlighting the benefit of enteral feeding maintaining gastrointestinal mucosal immunity, as well as decreasing fistula output. Reductions in intravenous fluid requirements can be aided by steps to reduce fistula output such as restricting hypo-osmolar fluids, encouraging electrolyte mixes and using anti-secretory agents and anti-motility agents (29).

During prolonged fasting the body aims to conserve muscle and protein breakdown by switching to ketone bodies derived from fatty acids as the main energy source. Many intracellular minerals become severely depleted during this period, although serum levels remain normal. Importantly, insulin secretion is suppressed in this fasted state and glucagon secretion is increased (30).

During refeeding, insulin secretion resumes in response to increased blood sugar, resulting in increased glycogen, fat and protein synthesis. This process requires phosphates, magnesium and potassium which are already depleted and the stores rapidly become used up. Formation of phosphorylated carbohydrate compounds in the liver and skeletal muscle depletes intracellular ATP and 2, 3 – diphosphoglycerate in red blood cells, leading to cellular dysfunction and inadequate oxygen delivery to the body's organs. Refeeding increases the basal metabolic rate. Intracellular movement of electrolytes occurs along with a fall in the serum electrolytes, including phosphate, potassium and magnesium. Glucose and levels of the vitamin B1 and thiamine may also fall. Cardiac arrhythmias are the most common cause of death from refeeding syndrome, with other significant risks including confusion, coma and

convulsions and cardiac failure. Biochemistry should be monitored regularly until it is stable (30).

Patients who have been starved for some time often experience gastrointestinal disturbance during refeeding, in particular colicky abdominal pain, reflux symptoms, nausea and early satiety. To help manage this, patients are often prescribed pro-kinetic agents such as domperidone or metoclopramide, as well as acid suppressants such as omeprazole.

The ability to discharge a patient pre-operatively on enteral or home parenteral nutrition (HPN) can allow further wound healing and increase in muscle strength and general wellbeing prior to surgery (28).

Parenteral nutrition may be avoided using enteral feeding administered into the distal bowel in a small minority of patients. It can be given either via a fistula or mucous fistula (fistuloclysis) (31). Use of enteral nutrition via fistuloclysis, if used appropriately, avoids the complications of long term PN and may provide faster fistula healing (32).

Teubner *et al* attempted fistuloclysis in 12 patients with jejunocutaneous or ileocutaneous fistulas with mucocutaneous continuity (33). Feeding was achieved by inserting a gastrostomy feeding tube into the intestine distal to the fistula. Infusion of enteral feed was increased in a stepwise manner until predicted nutritional requirements could be met by a combination of fistuloclysis and regular diet. Parenteral nutrition was replaced in 11 of the 12 patients. Nutritional status was maintained, without complication, for a median of 155 (range 19 - 422) days until

reconstructive surgery could be safely undertaken in nine patients. PN had to be recommenced in one patient (33).

2.4.3.2 Control of sepsis

Sepsis is mainly found in the acute phase of ECF and is the commonest cause of death (34). Initial measures to treat abdominal sepsis are associated with an improved outcome in patients with intestinal failure. Reber *et al* have reported that the mortality rate was increased and the spontaneous closure rate of ECF reduced in patients with uncontrolled sepsis when compared with those who had their sepsis treated within one month of diagnosis (35).

Antibiotic therapy should be complemented by identification of the site of sepsis by imaging modalities. Computed tomography (CT) should be used as the first line investigation (36). The success of percutaneous drainage of abdominal abscesses has improved in recent years (36-38). In a study by Benoist *et al* percutaneous drainage was successful in 81% of patients and predictors of failure were abscesses less than 5cm in diameter and lack of concomitant antibiotic therapy (38).

Resection of the fistulating segment with exteriorisation of the bowel ends as stomas (total gastrointestinal disconnection), proximal diversion, abscess drainage or formation of a laparostomy may occasionally be needed to control sepsis (15, 39). However, the increased morbidity and mortality associated with early re-operation may preclude such an approach and these measures should be only adopted as a last resort.

Gastrointestinal disconnection combined with tube decompression of the bowel proximal to any fistulating segments resulted in an overall 77% survival rate in 22 patients with intra-abdominal sepsis from gastric or small bowel fistulae (40).

Adkins *et al* found that patients managed with an open abdomen had a higher mortality and ECF formation rate when compared with historical case-matched controls with a closed abdomen (41). The patients with the open abdomen had a mortality rate of 25% and fistula formation rate of 14.8%.

The incidence of anastomotic leak varies depending on the level of anastomosis. Small bowel and ileocolic anastomoses have the lowest reported leak rates (1-3%). The highest rates are after coloanal anastomoses (10-20%). The risk of anastomotic leak after low anterior resection of the rectum is inversely related to the distance of the anastomosis from the anal verge (42).

The management of an anastomotic leak depends on whether the leak is “free” or “contained”. “Free” leaks or those in which faeculent fluid spreads throughout the abdominal cavity, leading to diffuse peritonitis and septic shock. Patients with a free leak should undergo emergency laparotomy with control of the anastomotic leak (takedown and creation of an end stoma +/- mucous fistula, primary repair and proximal faecal diversion, wide drainage and proximal faecal diversion) along with abdominal washout. “Contained” leaks are those in which extravasation of contrast material is limited to the peri - anastomotic space and often result in the development of an abscess. These situations are usually managed by insertion of a radiologically-guided percutaneous drainage catheter. Contained leaks managed in this manner may

heal spontaneously or may result in the formation of a controlled enterocutaneous fistula often accompanied by an anastomotic stricture. Reoperation to address an anastomotic fistula or stricture should be deferred for six months to allow for resolution of the intra-abdominal inflammatory state.

2.4.3.3 Pharmacological agents

Proton pump inhibitors including Omeprazole (Losec®) and Lanzoprazole (Zoton®) have been shown to increase water absorption. Using omeprazole 40mg twice a day in functional short bowel patients reduces fistula output (43-47).

Omeprazole is a selective and irreversible proton pump inhibitor. It suppresses stomach acid secretion by specific inhibition of the H⁺/K⁺ + ATPase system found at the secretory surface of gastric parietal cells. Because this enzyme system is regarded as the acid (proton, or H⁺) pump within the gastric mucosa, omeprazole will inhibit the final step of acid production (48).

The inhibitory effect of omeprazole occurs within one hour after oral administration. The maximum effect occurs within two hours. The duration of inhibition lasts up to 72 hours. When omeprazole is stopped, baseline stomach acid secretory activity returns after three to five days. The inhibitory effect of omeprazole on acid secretion will plateau after four days of repeated daily dosing. The absorption of omeprazole takes place in the small intestine and is usually completed within three to six hours (48)

Proton pump inhibitor administration might cause diarrhoea via several different mechanisms, including easier colonization of enter pathogenic bacteria, inadequate protein digestion in the stomach, and a possible influence on H, K-ATPase in the

colonic mucosa. In the absence of gastric acid, several kinds of bacteria, including *Clostridium difficile*, *Salmonella*, and *Campylobacter* species, were reported to easily colonize in the gut, causing enter colitis accompanied by diarrhoea or loose bowels (49).

The loss of intestinal fluid in patients with high intestinal output most likely reflects the loss of normal daily secretions produced in response to food intake. Increased duodenal volume could flush and dilute nutrients and electrolytes, thereby impairing intraluminal food assimilation and fluid and electrolyte absorption. The transient gastric hyper secretion, which occurs after massive small bowel resection, may affect the digestive and absorptive processes by inactivating pancreatic enzymes and precipitating bile salts. There are, however, no data to support the hypothesis that gastric acid hyper secretion continues for longer periods in patients with a short bowel. Patients with functional short bowel syndrome and a high faecal output do benefit from H₂ receptor antagonists and proton pump inhibitors when considering wet weight and sodium absorption (43).

Loperamide and other anti-diarrhoeal agents such as codeine phosphate can also be used to reduce fistula output. Loperamide should be administered half an hour before ingestion of food and results in slowing of intestinal transit time. It slows down the usual gut activity that is stimulated by eating. Most people find that the bowel is most active in the morning and so loperamide helps most if taken before breakfast. It is effective for eight to 12 hours. Higher doses of loperamide are needed in patients with SBS as it circulates through the entero-hepatic circulation.

St Mark's Electrolyte Mix can be provided to those patients whom become dehydrated. This drink contains a solution of salt, glucose, and sodium bicarbonate (baking soda). A useful test to identify dehydrated patients is 'random urinary sodium.'

Treatment of a high output ECF is similar to that from a high output jejunostomy. If there is marked sodium and water depletion and severe thirst, it is often necessary to establish equilibrium by giving intravenous normal saline (2–4 l/day), keeping the patient "nil by mouth", which will also demonstrate that output is driven by their oral intake. Over two to three days intravenous saline is gradually withdrawn while food and restricted oral fluids are reintroduced. Great care must be taken not to administer too much fluid, which will readily cause oedema (partly due to high circulating aldosterone levels). The aims of treatment are to maintain hydration/body weight and a daily urine volume of at least 800 ml with a sodium concentration greater than 20 mmol/l (50).

It is a common mistake for patients to be encouraged to drink oral hypotonic solutions to quench their thirst but this causes large stoma sodium losses. Treatment for high output from a jejunostomy (ileostomy or high fistula) begins with the patient restricting the total amount of oral hypotonic fluid (water, tea, coffee, fruit juices, alcohol, or dilute salt solutions) and also of hypertonic fluids (fruit juices, Coca cola, and most commercial sip feeds) to less than 500 ml daily. To make up the rest of the fluid requirement, the patient is encouraged to drink a glucose-saline replacement solution (90 mmol/l sodium or more). Many patients at home with marginally high stoma outputs (1–1.5 litres) will be helped by a combination of oral fluid restriction (less than 1 litre per day) and addition of salt to their diet. Patients with stoma losses

of less than 1200 ml daily can usually maintain sodium balance by adding extra salt to the limit of palatability at the table and when cooking. When stoma losses are in the range 1200–2000 ml, or sometimes more, it is possible for a patient to maintain sodium balance by taking a glucose-saline solution or salt capsules (50).

As the sodium content of jejunostomy (or ileostomy) effluent is relatively constant at approximately 90 mmol/l and as there is coupled absorption of sodium and glucose in the jejunum, patients are advised to sip a glucose-saline solution with a sodium concentration of at least 90 mmol/l throughout the day. The World Health Organization (WHO) cholera solution has a sodium concentration of 90 mmol/l and is commonly used (without the potassium chloride). Sodium chloride capsules (500 mg each) are effective when taken in large amounts (14/24 h) but can cause some patients to feel sick and even vomit. If an enteral feed is given, sodium chloride needs to be added to make the total sodium concentration of the feed 100 mmol/l while keeping osmolality near to 300 mosmol/kg. Mineralocorticoids (for example, 2 mg oral fludrocortisone or 2 mg intravenous d-aldosterone) or high dose hydrocortisone may reduce stoma output in patients with a retained ileum (50).

The use of somatostatin or its analogue octreotide in the treatment of intestinal fistula is controversial. Conflicting evidence of the efficacy of these agents is usually the result of heterogeneous patient populations and small patient numbers (51, 52). A review of all controlled studies evaluating the effects of somatostatin or octreotide showed that no study demonstrated a significant increase in the rate of fistula closure (53). While most studies demonstrate a reduction in fistula output in the initial few days of therapy, the cost of the treatment may not warrant their use as there is no impact on morbidity or mortality (25, 54, 55).

Crohn's patients can present with stricturing, inflammatory or fistulating disease. The medical management of patients has improved with the use of biological and immunomodulatory therapy. Tumour necrosis factor alpha (TNF- α) blocking agents such as Infliximab have been shown to be effective for the maintenance of remission and maintenance of ECF healing in patients (56).

2.4.3.4 Wound care and stoma therapy

Expert care is paramount in maintaining skin integrity in patients with ECF. The corrosive nature of the intestinal effluent may lead to painful and irritable skin destruction. The effects of continuous moisture from the ECF and small bowel effluent in direct contact with the skin results in breakdown of skin with possible resultant excoriation, erythema, severe pain and an unsightly wound with the risk of developing infection.

A specialised wound / stoma care nurse can avoid these problems with the adequate size and placement of stoma bags, or with the use of specific appliances that are designed to manage large defects (34). Skin barrier protection creams, pastes, adhesives, dressings, pouches are all available and have further advanced wound care management.

Devitalised or necrotic tissue within a wound or around the ECF should be debrided prior to dressing or pouch application; otherwise the necrotic tissue will prevent granulation tissue formation whilst also acting as a culture medium for bacteria to grow.

When the wounds are small, a simple stoma bag may be all that is necessary. Where large defects are present with large fistula outputs specialised pouches connected to a

drainage bag or even to low pressure / negative pressure wall suction may be used (57). However there is concern regarding the process of applying negative pressure in close proximity of the exposed viscera and bowel, as this may contribute to subsequent ECF formation. VAC dressings have been used extensively in the management of complex wounds due to proven enhancement in wound granulation and wound contracture (22). Concerns about negative pressure increasing fistula output seem to be overstated. Modifications to this technique have shown great promise where the VAC dressing is applied to the peri fistula wound while skin barrier wafers are used to isolate the fistula itself. The fistula is then managed with the pouch applied on top of the VAC dressing, thereby isolating fistula drainage from the remainder of the wound while taking advantage of all the benefits of a negative pressure dressing. The role that the VAC plays in this algorithm for care of these wounds remains to be seen as larger series and further experience is gathered (22).

If ECF output is high and dressings are being changed more frequently than every four hours, a pouching system should be used. Frequent dressing changes are impractical, and can also be associated with damage to the skin and healing tissue.

It is essentially important to have a multidisciplinary approach with the wound care / stoma therapist. This can contribute to improved protection for the skin, control of effluent and odour and a better quality of life and psychological wellbeing for the patient.

2.4.4 Radiology

Understanding the anatomy of the ECF optimises its evaluation and management. Imaging can confirm the anatomical site of the ECF. A proximal or distal location

will require different nutritional and fluid requirements. Imaging the bowel is essential prior to elective surgery for patients with intestinal failure. A clear picture of the status of the bowel distal to any fistulating segments is paramount in excluding distal obstruction. Anatomical details of the ECF include diameter, length and the presence of additional side branch's / tracts or associated abscesses.

Several, modes of imaging are available to obtain the necessary information. Barium follow through in combination with fistulograms have been shown to be accurate in determining length of remaining bowel (58). Fistulograms are the most rapid and direct method of detecting an ECF. They often show distal obstruction and also have the benefit of providing real time images and do not require the ingestion of contrast or intravenous injections. A drawback is that they can fail to define the presence of disease proximal to the ECF or distal if an associated abscess cavity is present.

Small bowel follow through is useful in evaluation of the distal bowel and identification of intraluminal causes of ECF, but it may fail to identify the location of the ECF. It also has the drawback of taking several hours to perform. Small bowel enterography provides greater mucosal detail than a follow through, but requires the placement of a nasogastric tube.

While these examinations are useful in determining the length of remaining bowel other examinations such as CT enteroclysis and Magnetic Resonance Imaging (MRI) enteroclysis are also very useful in examining pathological bowel and areas of on-going pathology such as areas of on-going obstruction or septic collection (59-61). They can also provide valuable information such as safe sites of entry into the abdominal cavity and the size of the abdominal wall defect.

CT scans are used with intravenous contrast to demonstrate inflammation, and oral contrast to differentiate loops of bowel from extra luminal abscesses or collections.

CT enteroclysis differs from traditional CT in the type of contrast agent used. CT enteroclysis has better sensitivity and accuracy for intraluminal findings, but traditional CT is better for visualising abscesses. CT provides the clinician with the option of percutaneous drainage of an abscess or collection under radiological guidance.

MRI is useful for identifying extra intestinal complications. MRI is better than CT to differentiate between inflammatory and fistula disease. In the postoperative patient, oedema or inflammation may be highlighted on T1-weighted images. Enhancement on T1 gadolinium images appears to be highly specific for active disease (62). MR enteroclysis has also been heralded as a superior form of investigation in these situations as it has the advantage of superior tissue characterisation and detection of intra and extra mural lesions without the use of ionising radiation (61).

Limitations of MRI include problems with motion artefact from bowel peristalsis which can degrade picture quality, the time required to obtain images, and patients experiencing claustrophobia.

Other special investigations such as conduitograms and vaginograms can be used in selected patients. Anorectal physiology and endoanal ultrasound should also be considered where loose stool or faecal incontinence is predicted as a post-operative outcome.

Endoscopy is the gold standard for detecting mucosal disease. Difficulty arises with identifying the internal opening of the ECF. Although not widely used, there are

examples in the literature of clinicians using interventions such as fibrin glue for ECF and self-expanding silicone stents as treatment options for managing colostomy related ECF (63, 64).

2.4.5 Anaesthetic considerations

Major insults such as intra-abdominal sepsis and multi organ failure, necessitating management in an intensive care unit, invariably result in a residual reduction in cardio-respiratory and renal functional reserve that could restrict the ability to recover uneventfully from further major surgery (65).

Exercise tolerance and physical fitness can be assessed in metabolic equivalent levels (METs). This is a validated method for determining functional capacity from a patient's history and will help determine the need for further specialised cardio-respiratory investigations. Peri-operative cardiac risk is increased in patients unable to achieve four 'MET' levels of exercise (66). If optimisation of physiological reserve is not achieved these patients are highly likely to develop critical illness.

Patients receiving parenteral nutrition require careful monitoring of clinical, laboratory and nutritional indices. Sodium and water retention can be a problem if malnourished patients are provided regimens in which all the non-protein energy is supplied as carbohydrate. Peripheral and central venous access can be difficult in patients with chronic intestinal failure due to thrombosis from repeated venous cannulation's and parenteral nutrition. Doppler ultrasound is useful for identifying thrombosed central veins prior to surgery (67).

Surgery for ECF is often lengthy, technically demanding and associated with large fluid shifts and a profound physiological stress response. Blood loss from collaterals

and generalised venous oozing can be significant. Cross-matched blood should always be available prior to the commencement of surgery.

Effective prophylactic prevention of deep venous thrombosis (DVT) must be employed for these patients particularly in those with a history of bowel infarction or inflammatory bowel disease.

Chronic usage of opioid and non-opioid analgesics is not uncommon in many patients with chronic ECF. It is worthwhile harnessing the skills of the hospital's pain service in the pre-operative management of such patients (68). Modern approaches to the management of pain after abdominal surgery rely on two analgesic techniques, patient controlled analgesia (PCA) using an opioid, self-administered in small doses, and epidural analgesia (69).

The aims of pain management are to achieve a level of pain relief which will allow coughing, deep breathing, mobilisation and communication with carers and facilitate rest and normal sleep patterns. Ideally analgesic regimes should take into account periods where pain intensity is increased due to therapeutic interventions for example during physiotherapy and dressing changes.

In patients having colorectal surgery oesophageal Doppler guided fluid management has been shown to reduce gastrointestinal morbidity (70). The study concluded that patients in the Doppler guided fluid group achieved significantly higher cardiac output and stroke volume measurements as well as higher oxygen delivery at the end of the operation than the control group. A better intra-operative splanchnic blood supply may have contributed to the quicker recovery of gut function and reduction in gastrointestinal complications in the Doppler guided fluid group.

Conversely, fluid overload may lead to oedema of the gut, possibly contributing to enteric nutritional intolerance and prolonged ileus. Abdominal compartment syndrome may result in respiratory and renal failure (71).

Hypothermia is defined as a core temperature of less than 36°C. If hypothermia is not managed intra-operatively, 70-80% of patients may be hypothermic on admission to the recovery ward (72). Heat loss under anaesthesia occurs as a result of impaired thermoregulatory responses, anaesthetic induced peripheral vasodilatation, infusion of cold intravenous solutions, exposure of large surface areas of the body and opening of large body cavities during surgery. The degree of heat loss is also influenced by ambient temperature and airflow in theatre and skin preparation methods.

Antibiotic prophylaxis with a single dose of cephalosporin and metronidazole is associated with reduced septic complications and should be administered at the commencement of each case (73, 74), unless otherwise recommended by the local microbiologist. In prolonged cases this should be repeated intra-operatively. The 2013 update of the World Society of Emergency Surgery (WSES) guidelines for the management of intra-abdominal infections contains evidence-based recommendations for management of patients with intra-abdominal infections (75).

Hyoscine butylbromide (Buscopan) can also be useful in reducing the volume of intestinal effluent. Although its use in enterocutaneous fistula surgery has not previously been described, Buscopan has been shown to cause intestinal paralysis in 21 seconds and result in reduced small bowel effluent (76).

2.4.6 Surgical management

Several techniques for the management of ECF have been used. Simple suture closure techniques, wedge resection and resection of the diseased bowel segment have been described. Materials which have been inserted into the fistula tract to aid healing include fibrin glue, pastes, vicryl mesh plugs and bovine small bowel tissue (63, 77).

In patients with an open abdomen, preventative techniques for ECF development include coverage of underlying viscera with omentum or other non-adherent protective barrier, avoidance of excessive resuscitation leading to bowel wall oedema, avoidance of serosal injury during dressing changes, and expedited fascial and skin closure.

2.4.6.1 Prevention of Fistula Formation

Meticulous surgical technique is imperative with attention to detail during the laparotomy to ensure that inadvertent enterotomies are avoided or identified and repaired (34). Avoiding early re-laparotomy where possible is probably the most important factor in reducing fistula formation (78). Careful attention to suture placement in laparotomy closure will also reduce the risk of iatrogenic fistula formation.

Several reports in the literature have advocated the use of vacuum assisted closure devices to aid healing of laparostomy wounds (79-84). In these series, successful closure of laparostomy wounds and enterocutaneous fistulae has been achieved with vacuum suction.

The vacuum suction wound management system can be used to isolate the ECF within the open wound, and hence allow pouching of the fistula. The vacuum sponge can also be applied directly to the ECF in an attempt to aid closure.

However, in our referral practice there have been several cases of vacuum suction devices applied to adjacent bowel leading to the formation of ECF (85). There have been several reports of ECF formation in association with the use of vacuum assisted therapy on open wounds and exposed bowel (62).

In one study with 112 patients treated with a specially created device that protected the bowel during application of the vacuum device there was 4.5% rate of fistula formation and an overall mortality rate of 25.9% (79) . The technique involved a polyethylene sheet perforated multiple times with a scalpel blade and placed over the peritoneal viscera and beneath the peritoneum of the abdominal wall. Next a moist sterile surgical towel is folded to fit the abdominal wall defect and placed over the sheet. Two 10-French drains are placed over the towel and connected to a suction device (86). Another study examined the clinical outcomes of 29 patients in whom vacuum assisted closure (VAC) was used to aid the closure of abdominal wounds (87). A high incidence (20%) of patients developed leakage of small bowel contents into the abdominal cavity because of intestinal fistulation during the VAC therapy. We are not aware of any prospective trials assessing the use of vacuum assisted therapy in the management of patients with ECF.

The use of non - absorbable mesh can also lead to fistula formation and is discussed under the section on abdominal wall closure 2.4.6.5 (88).

2.4.6.2 Prevention of short bowel syndrome

In conditions such as Crohn's disease where multiple laparotomies may be required it is important to tailor any resections to preserve as much intestinal length as possible. Where feasible, techniques such as stricturoplasty are useful in maintaining intestinal length while addressing the underlying pathology (89-92).

When normal bowel is adherent to a diseased segment of bowel, every attempt should be made to preserve the normal bowel. Use of medical therapy to control inflammation in Crohn's disease and judicious use of endoscopy to dilate strictures can also delay the need for surgery (52, 91).

Post-operative sepsis and its related complications can lead to intestinal loss and prevention of such complications is important. Pre-operative low albumin, steroid use and the presence of an abscess or fistula at the time of surgery significantly increases the risk of post-operative sepsis (15).

Colonic preservation where possible, can be an important determinant in the need for long term parenteral nutrition. Nightingale *et al* showed that patients with greater than 50cm of jejunum with an intact colon did not usually require long term parenteral nutrition – this was not the case in those with longer segments of jejunum but no colon (93).

2.4.6.3 Intestinal reconstruction

During the period of the 'frozen' abdomen, delayed surgical intervention for persistent fistula is advisable allowing for the obliterative peritonitis to subside and allowing laparotomy and adhesiolysis to be performed safely. Careful clinical

examination may indicate when the dense reaction of adhesion formation has subsided. The abdomen should be soft and non-tender. Mobility of the peritoneal contents may be appreciated on palpation of the abdomen. A good clinical indicator of the appropriate time to operate on ECF patients is the presence of non-oedematous prolapsing bowel in the fistulating segments (4, 94).

Although many ECF close spontaneously, if the ECF remains open after two months surgical intervention is likely to be needed as spontaneous closure is unlikely after this interval (16, 35). Major abdominal surgery stimulates the formation of dense adhesions, especially when complicated by intra-abdominal sepsis. This reaction is most severe between three weeks and four months after operation, and further surgery during this time is more likely to be complicated by ECF recurrence (13, 16, 95). Other authors have commented that operative repairs should be performed when spontaneous closure does not occur, but it should be delayed for at least three months (16).

2.4.6.4 General principles of surgical reconstruction

1) Plan the surgical procedure in advance, delaying reconstructive surgery until optimal nutrition status, general fitness and re-epithelialisation of the peritoneum have been achieved (95). Noting the above, re-operation should be avoided for at least four to six months after the last abdominal procedure where possible (4, 34, 95).

2) Resection and anastomosis rather than suture alone is associated with a better outcomes in enterocutaneous fistula repairs (96).

3) Any intestinal anastomosis should be clear of septic abdominal sites and fistula tracks.

4) Anastomosis should be avoided in the presence of ischaemia and in severely malnourished patients.

5) Judicious use of a covering stoma should be considered.

6) Where large volume diarrhoea is a predicted outcome or in those patients with a weak sphincter an end colonic stoma should be considered although this can be avoided in most cases.

The main advantages of restoration of intestinal continuity are increasing absorptive surface area and enhancing intestinal adaptation by enhancing the surface area exposed to the nutrient micro-environment (97). There is evidence that bringing the colon back in to circuit reduces the incidence of hepatic disease in patients on large volumes of parenteral nutrition (93).

In situations where sepsis has prevailed the rate of anastomotic dehiscence can be higher than expected (98). In patients with SBS meticulous attention to anastomotic technique is vital. It has been considered that intestinal anastomosis in the malnourished patient should not be carried out. Given that most patients with short bowel syndrome (SBS) are hypoproteinemic and one of the aims of management in these patients is to increase the surface of absorption this principle may not necessarily apply to this group.

Other studies including experimental studies have shown that factors such as serum albumin less than 3.0 g/L, steroids use, presence of peritonitis, bowel obstruction, perioperative transfusion of more than two units of packed red blood cells and chronic obstructive pulmonary disease were all significant predictive factors of anastomotic leakage (99, 100).

Lynch *et al*, in a retrospective review analysed the management of 205 patients with enterocutaneous fistula (95). The primary diagnosis was Crohn's disease in 95 patients, ulcerative colitis in 18 patients, diverticular disease in 17 patients, carcinoma in 25 patients (16 cases after radiotherapy), and mesh ventral hernia repair in 21 patients was performed. After initial management, including CT-guided drainage of an intra-abdominal abscess or defunctioning stoma in some patients, definitive intestinal reconstruction surgery was performed in 203 patients. The median time between the diagnosis of enterocutaneous fistula and the onset of a surgical procedure in the specialised unit was 5.8 months. Most of the patients (159 patients) had resection and re-anastomosis of the involved small bowel segment or ileocolic anastomosis. An average of 21% of patients developed fistula recurrence within three months and postoperative mortality was 3.5% (95). Wedge repair or over sewing the fistulous track were associated with a significant risk of fistula recurrence when compared with intestinal resection and anastomosis (32.7% vs. 18.4%, respectively) (95).

Experimental studies have shown that in ischaemic anastomoses neo-vascularisation can occur via the omentum (101). Omental wrapping has been shown to be effective in post radiotherapy injuries to the colon and the successful use of omentum has also been demonstrated in aortoenteric fistula repairs (102).

Although its use in the repair of ECF has not been described, it may be beneficial in protecting anastomoses or as a layer between any mesh and the intestines where fascial closure of the abdomen is not possible.

The results of intestinal reconstruction vary according to the techniques employed and with the end points assessed. Hollington *et al* reported an 11 year experience of surgery for ECF in 277 consecutive adult patients (4). In all situations resection with anastomosis was the choice of surgery. The fistula healing rate was 82% although in some patients more than one attempt at repair was necessary to achieve healing. This comprehensive report of surgery for ECF in adult patients and it highlights the success of simple reconstructive surgery without the use of complex adjunctive procedures.

More recently we have reported results from the same unit with an improved overall healing rate (24). 150 out of 177 patients underwent definitive surgery (84.7%) with an overall healing rate of 94.6% compared with 82% in the previous series. This is discussed in Chapter 3.

2.4.6.5 Abdominal wall reconstruction

ECF are often associated with abdominal wall dehiscence and large skin defects.

In most patients contraction of the granulation tissue over time reduces the midline defect to such an extent that primary closure of at least the skin of the abdomen is possible. Releasing incisions in the abdominal flanks can be useful in reducing tension on the abdominal wall and allowing primary closure (94).

The modified components separation technique which consists of mobilising the rectus sheath and external oblique fascia, followed by division of the internal oblique component of the anterior rectus sheath down to the arcuate line and finally suturing the medial border of the posterior sheath to the lateral border of the anterior sheath is

a well described technique of abdominal reconstruction but this can be associated with significant morbidity (98, 103-105).

Reinforcement of an abdominal wall closure can be achieved with a non-absorbable mesh. However, in situations where there is direct contact with the bowel and the mesh other reconstructive techniques should be considered as there is a high rate of enterocutaneous fistula formation in this setting (106). Non-absorbable meshes are also avoided because ECF surgery is often performed in contaminated or grossly infected wounds. There is the possibility secondary mesh infection leading to mesh removal, and hence this is avoided.

There is difference of opinion as to whether there is anything better than placing an absorbable synthetic mesh like Vicryl (Ethicon, Somerville, NJ, USA) (107).

The use of absorbable mesh such as the biologic porcine-dermis derived cross-linked Permacol™ (Tissue Science Laboratory, Covington, USA) or non-cross linked Strattice™ (Lifecell Corporation, Branchburg, NJ, USA) is safe, but have recurrence rates of 0 to 15% (107).

One study aimed to determine factors which influence the outcome of surgical technique to close ECF within the open abdomen (88). Their results revealed that re-fistulation was more common when the abdominal wall was constructed with a prosthetic mesh (7 of 29, 24.1%) than with sutures (0 of 34, 0%). The use of porcine collagen mesh was associated with a particularly high rate of re-fistulation (5 of 12, 41.7%). The authors concluded that simultaneous reconstruction of the intestinal tract and abdominal wall was associated with a high complication rate and simultaneous reconstruction of the abdominal wall with prosthetic mesh was associated with a

particularly high incidence of recurrent postoperative fistulation and should be avoided if possible.

2.5 Complications of ECF: Intestinal failure and short bowel syndrome

In the United Kingdom the estimated incidence of intestinal failure (IF) requiring treatment at a specialised unit is 5.5 per million population (26). A recent study showed an overall survival rate of 86% in patients undergoing autologous surgical reconstruction, at a median follow up period of two years, in one such specialised unit (108). Similar results have been achieved in other specialist units (26, 109).

IF has been sub-classified into three types depending upon duration and irreversibility (110). Thus:

Type 1 IF is usually considered to be of less than 28 days duration. It is typified by conditions such as postoperative ileus or small intestinal obstruction, usually requiring short term parenteral nutrition and generally resolving within 28 days.

Type 2 IF is usually considered to be of greater than 28 days duration. Typical patients might include those with complex Crohn's disease, intestinal fistulas or abdominal sepsis. These patients require much more prolonged nutritional and metabolic support pending spontaneous resolution or surgical treatment. Patients with both Type 1 and Type 2 IF would be expected to return to full enteral autonomy in time.

Type 3 IF is generally irreversible and usually occurs as a consequence of massive small bowel resection, leading to short bowel syndrome, although there is increasing recognition that severe motility problems may account for a small proportion of cases.

Patients with type 3 IF will require HPN and, in some cases, referral for intestinal transplantation may be appropriate. Significant data exist on the management of this condition in the paediatric population, but treatment options are not necessarily applicable to the adult population (111).

SBS refers to IF associated with a loss of intestinal length or competence below the amount required to maintain adequate enteral nutrition (112). Normal small bowel length ranges from approximately 300cm to 800cm and SBS usually occurs when the length of small bowel is less than 100cm with a stoma, less than 50cm with an intact colon or when the patient continues to be dependent on parenteral nutrition after three months (93, 112-114).

2.5.1 Aetiology

The causes of IF also vary widely between institutions depending on their referral patterns. Scott *et al* reviewed 300 consecutive patients admitted to a specialised unit and showed that 42% of patients had Crohn's disease and the most common complication necessitating admission was formation of enterocutaneous fistulae (26). Peptic ulcer (9%), pancreatitis (8%), mesenteric vascular disease (7%), malignancy (6%) and other miscellaneous causes (28%) accounted for the remainder of admissions.

Intestinal failure is rare, occurring in 2 – 6.8 per 1 000 000 children in developed countries. Each year in Australia, there is expected to be approximately 40 new patients with intestinal failure. The individual health care burden of intestinal failure, in terms of both social and economic costs, is enormous. Children with intestinal failure usually spend the first six months to two years of life in hospital, and the

average cost per child with intestinal failure has been calculated as US \$1 619 851 over a 5-year period (115).

The aetiology of SBS is related to a loss of functional intestinal length usually as a result of multiple intestinal resections or a massive intestinal resection (112, 116). Multiple intestinal resections are most commonly associated with Crohn's disease whereas massive intestinal resections are associated with catastrophic vascular events such as those following venous or arterial infarction, trauma, resections following a volvulus, or desmoid disease.

In a European multi-centre study, the indications for PN in a group of patients with SBS were cancer (39%), Crohn's (19%), vascular diseases (15%), radiation enteritis (7%) and AIDS (2%) (117). Post-operative causes such as adhesions, volvulus and ischemia accounted for 25% of all patients with SBS in an American institution (118).

The United States Food and Drugs Administration approved recombinant Growth Hormone for patients with short bowel syndrome based on the recent results of a double-blind randomised clinical trial (119). Further studies have shown that low dose growth hormone has successfully reduced the PN requirements for those with SBS (119-121). However, experience of its use remains limited and long term outcome evaluation is required prior to its recommendation.

2.5.2 Concurrent Cholecystectomy

The risk of gall stone formation in patients with SBS is estimated at 44% (122, 123). This rate is not reduced by the presence of an intact colon (123). An intestinal length of less than 120cm, Crohn's disease, long term PN and the absence of an ileocaecal valve are significant risk factors for gall stone formation (124). The risk of

inflammatory complications of cholelithiasis is greater than 50% in patients with SBS (125). Given the potentially severe nature of these complications serious consideration should be given to performing prophylactic cholecystectomy at the time of any definitive surgery (126).

2.5.3 Adjunctive surgery

A variety of techniques have been described to optimise the function of the remaining intestine (104). However they are of limited efficacy as the overall surface area of the bowel cannot be increased. Their use has not been extensively evaluated in adults.

Sudan et al (108) reported their experience in the management of 50 patients (20 adults, mean age 42 years) in a multidisciplinary intestinal failure unit. The surgical procedures included intestinal reconstruction (n=5), repair of enterocutaneous fistulas (n=5), resection of bowel segments (n=7), stricturoplasty (n=2), Bianchi tapering and lengthening (n=20), and STEP procedure (n=8). Sixty five per cent of patients were completely weaned off PN at follow-up.

2.5.4 Reversed small bowel segments

A short segment of distal small bowel is prepared and disconnected from the remaining bowel. It is then reversed, and re-anastomosed, with its blood supply intact (127). The aim of this procedure is to increase transit times. The ideal length of the reversed intestinal segment is about 10 cm in the adult population (128). While the short term results of this procedure may be promising the long term results are limited and the clinical indications restricted (34).

Segmental reversal of the small bowel (SRSB) is proposed in patients with short-bowel syndrome (SBS) as a rehabilitative therapy. A retrospective study

consecutive patients who had an SRSB between 1985 and 2010. The authors concluded that SRSB allows a gain in macronutrient absorption, which is associated with a lower HPN dependence. They also recommend that SRSB should be integrated in intestinal rehabilitative adult programs (129).

2.5.5 Colonic interposition

Several cases of colonic interposition have been reported with the use of segments of at least 20 cm in length (130). There is evidence that the adaptive histological changes in the interposed colon are not just an experimental finding (130, 131). This technique has not been studied extensively in the adult population.

2.5.6 Neo valves

The creation of ileocaecal neovalves was described some time ago for the purpose of delaying intestinal transit time (104, 132). There are several types of neovalves described in the literature (132-134). While some experimental models have shown a reduction in intestinal transit time, creation of these valves requires the use of part of the length of small bowel, with possible resultant complications such as pseudo-obstruction and secondary enterocolitis (135, 136).

2.5.7 Bowel “lengthening” procedures

Bianchi described a unique technique to lengthen small bowel in the paediatric population (137). The technique consists of dividing dilated bowel longitudinally in the midline along the anti-mesenteric edge and then the creation of two vascularized hemi loops anastomosed in tandem. It has been suggested that its main effect is to increase the transit time rather than mucosal absorption.

Most reports of this technique are in paediatric series. In a recent analysis most studies had small numbers of patients with an overall survival rate ranging between 30 and 100%. Between 28 and 100% of patients were able to come off PN (104). The morbidity is the main drawback of this technique. Anastomotic leakage in the long sutures lines and the need for further surgery due to pseudo-obstruction are common problems. The heterogeneity of the patients and technique in the published series make it difficult to achieve further definite conclusions (96, 104, 108).

The serial transverse enteroplasty (STEP) technique involves sequential linear stapling of the distal small bowel from alternating directions, perpendicular to the long axis of the intestine (108, 138). The STEP creates a zigzag-shaped bowel that is greater in length without putting the mesenteric blood supply at risk. In a cohort of five infant patients there were no perioperative complications at 26 months follow up. Again this procedure has not been extensively investigated in adults (139).

More recently a retrospective review of a single centre series of intestinal lengthening over 15-year period in patients ≥ 18 years old has been performed (140). Overall of 17 patients who underwent Bianchi or serial transverse enteroplasty (STEP), ten (59%) patients achieved enteral autonomy and were off PN. Of seven patients who were on PN, three patients showed significant improvement in enteral caloric intake. All except one showed significant improvement in symptoms of bacterial overgrowth. The authors concluded that bowel lengthening is technically feasible and effectively leads to weaning from PN in more than half of the adult patients and that at present lengthening procedures may be an underutilized treatment for adults with short bowel syndrome.

2.5.8 Future techniques

The use of electrical pacing, mechanical tissue expansion and stem cells, have been described and are still the subject of on-going research (141-143). In one study, two patients with low-output enterocutaneous fistulas after surgery were treated with electrical nerve stimulation (ENS). Fistula output diminished rapidly in both cases, and the closure of the track was achieved after several sessions of ENS. The authors suggest that the procedure is simple and safe and is suggested as an option for the treatment of low-output enterocutaneous fistulas (144).

Volpe et al showed that mesenchymal stromal cells from adipose tissue attached to suture material enhance the closure of enterocutaneous fistulas in a rat model. This is done through maintaining viability and proliferative capacity. The authors suggest this approach may be a new and effective tool for ECF treatment (145).

2.5.9 Intestinal Transplantation

Intestinal transplantation is an alternative to long term parenteral nutrition offering restoration of normal physiology and gut function. The first small intestinal transplantation was undertaken in Minnesota in 1967. The first intestinal transplantation undertaken in the UK was performed at Addenbrooke's Hospital in Cambridge in 1991. St James's Hospital in Leeds also undertook several intestinal transplants in the mid 1990's. The results of intestinal transplantation have greatly improved over the last 15 years. The main area of improvement has been the first two years after the transplantation. This seems to be largely due to the use of better anti-rejection drugs (146).

Transplantation of the intestine offers a special challenge as not only is the intestine highly immunogenic but it also presents a significant infective risk if acute rejection episodes do occur due to bacterial translocation occurring across the damaged mucosal barrier. This dual problem of immunogenicity and infective risk resulted in failure of early attempts at transplantation (147).

Following the advent of calcineurin inhibitor based immunosuppression with cyclosporine, renewed interest in intestinal grafting was eventually rewarded with successful combined liver / intestine grafting (148, 149). However successful widespread application of intestinal grafting was not really possible until the introduction of the more potent and easily administered agent tacrolimus (150).

Acute rejection of the intestine is now quite uncommon and has been replaced by sepsis as the major post-operative complication and cause of death. Survival seems now to be more related to careful preoperative preparation and scrupulous post-operative management of patients (146).

Long term PN can be associated with fatal complications such as irreversible liver failure, multiple line infections and thrombosis of central veins. These complications, along with the possible requirement for extensive evisceration to remove desmoid tumours, are the indications for intestinal transplantation in patients with SBS (151, 152).

It is important to consider the pros and cons of transplantation for each patient on an individual basis. Patients who are doing well on PN have a very good life expectancy and can live with a very good quality of life.

Survival following intestinal transplantation is not as good, although patients are usually free from PN and for some this can improve their quality of life (QOL), those patients who are doing well on PN will not notice a great deal of improvement in QOL, they might swap PN for other problems associated with the transplantation procedure and their life expectancy is not nearly as good. Therefore, intestinal transplantation should only be used for patients who are not doing well on PN.

On the other hand if put forward too late when they have multiple complications and are in poor condition they are less likely to survive the procedure. The timing of the transplantation is therefore critical and this needs to be considered along with a host of factors which can vary widely between patients.

Therefore in general, intestinal transplantation is indicated in patients with intestinal failure who are failing on PN (146).

The following list of indications has been approved by the American Gastroenterology Association (AGA):

1. Impending or overt liver failure due to PN induced liver injury
2. Thrombosis of two or more major central veins
3. Frequent central venous catheter infection & sepsis
4. Frequent severe dehydration despite IV fluid supplement in addition to PN
5. Quality of life

Transplantation does not put patients back to normal and does give them on-going problems to deal with including taking lifelong medication, being more prone to infections, the need for regular visits to hospital for check-ups, often a permanent stoma (ileostomy) is required and obtaining employment is usually much more

difficult after a transplantation. Furthermore, patients on PN who have not had any major complications are likely to live a lot longer than if they had transplantation. A general comparative survival would be 90% survival at 10 years on PN compared to 45% 10 year survival after transplantation (146).

There are three types of intestinal transplantation: (i) isolated small intestine transplantation, (ii) combined liver and small intestine transplantation, and (iii) multivisceral (stomach, duodenum, pancreas, intestine and liver) (153, 154). The intestinal transplant registry (last updated in 2005) documents 65 centres worldwide performing 1292 intestinal transplants in 1210 patients with around 140 intestinal grafts of all kinds being performed annually (155).

The ratio of children to adults is approximately 60:40 due to the incidence of intestinal failure in children following on from developmental disorders such as intestinal atresia, Hirschprung's, microvillus inclusion disease or bowel loss due to neonatal problems such as gastroschisis, volvulus or necrotizing enterocolitis. The difficulty of maintaining small children on parenteral nutrition is compounded by their apparent increased sensitivity to parenteral nutrition related liver disease resulting in 50.4% of paediatric grafts being combined small bowel liver grafts as compared with 19.4% in adults.

Of the 1210 patients recorded in the registry there are 658 current survivors, the longest now 17 years post-transplant with a functioning graft. Analysis of the two centres performing adult intestinal transplantation in England and Wales showed that, of 36 patients assessed for transplantation, 14 had surgery (156). Of these patients the

one year graft and patient survival was 50% while the 3 and 5 year survival was 36% and 21% respectively (156).

Graft and patient survival has improved with increasing experience of transplant teams (156, 157) and increases with centre volume (>10 cases) and have reached approximately 50% at 5 years (155). Patients with isolated small intestinal transplants tend to have a better overall graft survival than those with liver and small bowel transplants or multivisceral transplants in some adult series (151, 156-158) but overall survival in the registry is similar for all groups, perhaps reflecting the inclusion in the data of some exceptionally good results in combined liver/small bowel transplantation in children (159).

Careful pre-operative, post-operative and long term management of intestinal transplant patients by an experienced team remains the mainstay of long term graft survival (160).

Multiorgan donation itself does not compromise the outcome of individual organ transplants. Dissection of abdominal organs for transplantation is best performed after cold perfusion. Abdominal organs should be removed rapidly, en bloc, and separated during back-table dissection in the cold, particularly if pancreas or intestine is included. Perfusion itself should be carried out after single cannulation of the aorta with an increased pressure (146, 160).

The surgery can be very challenging in the face of a contracted abdomen, following multiple previous abdominal operations. The challenge of finding adequate space to implant the new organ or organs has led to much surgical innovation with the use of

tissue expanders pre transplant and reduced size en bloc combined liver/small bowel grafts (161).

The Miami group have included composite grafts of abdominal wall in the transplant procedure to allow abdominal closure following implantation of the grafted organs (162, 163). Improvements in immunosuppression, particularly antibody induction protocols and the use of tacrolimus have led to an improvement in overall graft survival (153). Immunosuppression related Post Transplant Lymphoproliferative Disorders (PTLD) remains a major problem and a significant cause of death following intestinal transplantation but adopting a long term anti-viral regime to minimise cytomegalovirus and Epstein-Barr virus associated lymphoma has contributed to improved survival in these patients (153, 157).

Early diagnosis and treatment of rejection is important in avoiding rejection related sepsis and this can be achieved by frequent endoscopies in the immediate post-operative period through a temporary loop ileostomy (153).

Nutrition after small bowel transplantation is aimed at early enteral feeding with the use of non-elementary formulae resulting in discontinuation of PN within 4-6 weeks of transplantation (153, 164).

2.6 CONCLUSIONS

With specialised medical, radiological and surgical management the prognosis of patients with enterocutaneous fistula has improved (165, 166). There are significant psychological, physiological and financial consequences following the development of an ECF, and the most effective means of treatment is prevention with meticulous surgical technique.

Any definitive surgical interventions should be delayed until there is nutritional optimisation, eradication of sepsis, maturation of adhesions and until full bowel mapping has been performed. A period of home leave prior to surgery may be both mentally and physically beneficial, rather than an extended period of inpatient stay.

Techniques to slow intestinal transit or to increase functional bowel length have a very limited place in adult patients with short bowel syndrome. They may only serve to increase the risks of already lengthy and technically challenging surgical procedures.

The survival of patients on long term PN has been estimated at 88% and 78% at three and five years respectively (166). The addition of intestinal transplantation to the armamentarium of treatment modalities has further enhanced the survival prospects of those with SBS.

CHAPTER 3

EXPERIENCE OF ENTEROCUTANEOUS FISTULA WITH UNIVARIATE AND MULTIVARIATE ANALYSIS EXAMINING FACTORS ASSOCIATED WITH HEALING LEADING TO DEVELOPMENT OF A VALIDATED SCORING SYSTEM

3.1 ABSTRACT

Background

The management of enterocutaneous fistulas is complex and challenging. We have examined factors associated with fistula healing at a National Intestinal Failure (IF) centre in England, and have devised the first scoring system to predict spontaneous fistula healing prior to surgery.

Methods

A retrospective audit of 177 patients with mean age of 48.7 years (median 49) treated over seven years (January 2003 to December 2009) has been completed. Results have been compared to a previously reported series from this unit. Stata statistical programme was used. ECF healing was measured on a binary scale. Logistic regression analysis was performed.

To assess the relationship with ECF healing a univariate analysis was performed. Univariate analysis was performed on variables: age, co-morbidity, referral source, referral time, aetiology, fistula origin, fistula complexity, fistula output, presence of laparostomy on admission, albumin at first presentation, number of previous

operations, time of surgery and bowel defunctioning at ECF surgery. Odds ratio was used to reflect the odds of healing. Multivariate analysis of the variables upon the outcomes was examined.

Results

There were 149 patients who underwent definitive surgery (84.7%). The overall healing rate following surgery in the current series was 94.6% compared with 82% in the previous series. Median delay from previous surgery to current operation was one year compared with eight months. The 30 day post fistula resection mortality was 0% compared with 3.5% in the previous series. Twenty eight patients underwent medical management alone with an overall healing rate of 46.4% compared to 19.9% in the previous series.

Multivariate analysis revealed co-morbidity ($p = 0.02$), source of referral ($p = 0.01$) and aetiology ($p = 0.006$) had significant associations with ECF healing. Regression coefficients were used to devise a scoring system. The predicted probability of healing was calculated and compared with those observed. This revealed an extremely good agreement between the predicted and observed percentage of ECF that healed within each category at our centre.

This scoring system and another devised to be used by an external centre were applied to a prospective cohort of patients (85 patients) managed at our centre (Jan 2010 to December 2011). The scoring systems were validated as both revealed good agreement between the predicted and observed percentage of ECF that healed within each category. Almost all patients with a score of zero and one healed, whilst those with highest scores healed least.

Conclusion

Surgical management of ECF is safe and improving. The most common aetiology for ECF formation is surgery performed in patients with a background of inflammatory bowel disease. Fistula healing is affected by aetiology, co-morbidity, and source of referral. The scoring system has potential to predict ECF healing and can be a useful decision making tool. Recommendation is for the scoring system to be used by other centres in this regard.

3.2 INTRODUCTION

Enterocutaneous fistulas are abnormal communications between the gastrointestinal tract and the skin. The treatment of ECF is complex and challenging. It is estimated that 75–85 per cent occur as a consequence of bowel injury during surgery (inadvertent enterotomy) or anastomotic leakage (8, 16). Our study examines healing and mortality in ECF patients. We have also evaluated the current management practice and outcome over a seven year period at a National Intestinal Failure (IF) centre in England. Results have been compared to a previously reported series from this unit (4).

3.3 AIMS

The primary aim of this study was to examine factors associated with fistula healing. The secondary aim of the study was to devise a scoring system to predict spontaneous fistula healing prior to surgery.

3.4 METHOD

A structured approach to the management of ECF is used in our institution. Management of persistent ECF includes control of sepsis, specialised nutritional support, wound care, mobilisation and mapping of the bowel anatomy. Judicious use of pharmacological agents including the use of loperamide, codeine phosphate and high dose proton pump inhibitors and St. Mark's Electrolyte Mix. This is a glucose-electrolyte solution.

Definitive surgery with ECF resection is deferred until there is re-epithelisation of the abdominal wall and attainment of an adequate nutritional and general medical status.

Adequate nutritional status is when the patient is in an anabolic state (evidenced by gaining weight, muscle mass and muscle strength). General medical status relates to stability of the underlying conditions as well as correction of any electrolyte and metabolic derangements. This process can take over six months.

Vacuum assisted pumps are avoided in our unit. The use of non-absorbable meshes is also avoided because ECF surgery is often performed in contaminated or grossly infected wounds. When required an absorbable synthetic mesh such as Vicryl (Ethicon, Somerville, NJ, USA) or the biological non-cross linked Strattice™ (Lifecell Corporation, Branchburg, NJ, USA) are used. A stapled and oversewn or hand sewn, interrupted anastomosis is performed with 4-0 Ethibond (Ethicon, USA).

Various techniques have been described in reconstruction plastic surgery flaps for ECF. Sakurabaa et al describes an enterocutaneous fistula which developed between the small intestine and the sacral skin. The fistula was resected, and the resulting defect was successfully reconstructed with a superior gluteal artery perforator flap (167).

Reconstructive surgical options for ECF include primary closure, components separation and the use of local, regional or free flaps with or without prosthetic mesh. *Ali et al* report a case of an abdominal enterocutaneous fistula secondary to a locally invasive sigmoid carcinoma, which was reconstructed with a pedicled antero-lateral thigh perforator (ALT) flap (168).

From January 2003 to December 2009, 235 patients were coded by medical records as having ECF at St. Mark's Hospital and North West London Hospitals NHS Trust, UK. Hospital notes and clinical details were obtained from medical records, the

intestinal failure database, and from the use of the hospital computer clinical information system.

Thirty two patients were excluded as they had incorrectly been coded for ECF. These included entero enteric: eleven, entero vesical: four, entero vaginal: seven, perianal: six, or no fistula: four.

Overall, 203 patients were managed with ECF at our hospital. Case notes were not located for 26 patients and hence 177 patients (87.3%) were available for review. Results have been compared to a previously reported series from this unit of an 11 year experience of 277 patients (4).

Statistical analysis was undertaken using the Stata software programme. There was one main outcome of the study, that being fistula healing, which was measured on a binary (yes/no) scale. The secondary objectives of the analysis were to examine factors associated with fistula healing. As a result of the binary nature of the outcome, all analysis was performed using logistic regression.

The separate effect of each explanatory variable upon the outcome was examined individually in a univariate analysis. Univariate analysis was performed on variables: age, co-morbidity, source of referral, time to referral, fistula aetiology, fistula origin, fistula complexity, fistula output, presence of laparostomy on admission, albumin at first presentation, number of previous operations, time of surgery and bowel defunctioning at fistula surgery.

Subsequently the joint effect of the explanatory variables upon the outcomes was examined in a multivariate analysis. Only factors which showed some evidence of an effect upon each outcome ($p < 0.2$) from the univariate analyses were considered for

the multivariate analysis. A different p value could have been reasonably chosen, however this value captured all variables that may have had even a very slight effect on outcome.

A backwards selection procedure was performed to retain only those variables that were found to significantly influence the outcome. This involved removing non-significant variables, one at a time, until only significant variables were left.

BMI and weight were not included in any of the analyses, as data were only available for a subset of the total number of patients.

The next stage in the analysis used a multivariate analysis results to generate a scoring system for predicting fistula healing. This was done using the final logistic regression model from the multivariate analysis. The score attributed to each variable was based on the size of the regression coefficients. Once the scoring system was set up, the performance of the scoring system was evaluated by comparing the predicted healing rate with that observed from the data.

This internal scoring system and another devised to be used by an external centres were applied to a prospective cohort of patients (85 patients) managed at our centre (Jan 2010 to December 2011), to validate the scoring system.

3.5 RESULTS

3.5.1 Aetiology of ECF

Of the 177 patients, 87 (49.1%) were male and 90 (50.9%) female. The 177 patients had a median age of 49 (range 9-84) years, with mean of 48.7 years.

There were 65 (36.7%) patients who had no co-morbidity. Twenty eight (15.8%) had minor co-morbidity and 84 (47.5%) had a major co-morbidity. Minor co-morbidities category included patients with mild systemic disease (ASA 2) and major co-morbidity included patients with severe systemic disease or worse (ASA $\geq$ 3).

Forty seven (26.9%) ECF occurred at our Trust. This was a reduction from 36.8% from the previous study (4).

External referrals accounted for 130 patients (73.1%).

The time elapsed between initial fistulation and referral to St. Mark's Hospital was a median of four months, with mean of 9.1 months (range 0.25 – 96 months).

Fistula resulted from surgery in 165 (93.2%) of the 177 patients (Table 1). Of these, 75 (45.4%) had inflammatory bowel disease (59 Crohn's disease and 16 ulcerative colitis). One patient had previous radiotherapy. Twenty two patients (13.3%) developed fistula following prior surgery for intra-abdominal malignancy. Diverticular disease contributed to 12 patients (7.2%) developing fistula. Fifty five patients (33.3%) developed a fistula due to a range of other causes following surgery including trauma, self-harm injuries, gynaecological surgery, ischaemic bowel surgery and iatrogenic injuries.

Of the 177 patients, 12 (6.8%) developed a fistula in the absence of prior surgery. None of these patients had a prior ultrasound (US) or computer tomography (CT) guided radiological drainage procedure which could have contributed to fistulation. Ten of these patients who fistulated had Crohn's disease. One patient had advanced rectal sigmoid cancer invading the bladder and with metastatic spread to the groin.

The groin initially drained as an abscess and then an ECF was identified. One patient developed spontaneous fistulation with a background of diverticular disease.

Eighty five (48.6%) of all 177 patients who developed a fistula had inflammatory bowel disease. Of these 69 (39.0%) had Crohn's disease and 16 (9.0%) had ulcerative colitis.

Table 3.1: Aetiology of ECF

	Post-operative	Spontaneous	Total
Crohn's disease	59	10	69
Ulcerative colitis	16	0	16
Radiotherapy	1	0	1
Intra-abdominal malignancy (including FAP)	22	1	23
Diverticular disease	12	1	13
Other*	55	0	55
Total	165 (93.2%)	12 (6.8%)	177

* Includes ECF secondary to trauma, self-harm, iatrogenic injuries, ischaemic bowel surgery, gynaecological surgery or no associated condition.

FAP – Familial Adenomatous Polyposis

3.5.2 Fistula characteristics

The ileum was the most common anatomical site of fistulation. The stomach was involved in five patients, duodenum in three, jejunum in 23, ileum 124 and colon in 42. No site was recorded in three patients. Patients with oesophageal, biliary, pancreatic, and perianal fistulas were excluded from this study.

The majority of patients (149/177) had only one anatomical site of fistulation involved. Twenty two patients had two sites of fistulation and three patients had three sites. Data were not available for three cases.

ECF were classified as simple or complex. Simple ECF involved a single tract from skin to bowel. Complex fistula involved multiple loops of bowel or had the presence of an internal abscess cavity. Complex fistulas were identified radiologically or intraoperatively. Forty one patients had a simple fistula. The majority, 134 patients, had complex fistula. Complexity was not known in two patients.

Fistula output was classified as high if the average output at presentation was greater than 500 ml in a 24 hour period. High fistula output was present in 77 patients. The majority presented with a low fistula output; 99 patients. The fistula output was not recorded in one instance.

Nineteen patients had a laparostomy associated with the ECF at the time of presentation to St. Mark's Hospital.

3.5.3 Radiological / Endoscopic Investigations

Radiological or endoscopic investigations were performed to map the bowel anatomy in 170 patients (Table 2). Seven patients had investigations performed at the referring hospital and did not require further investigations at St. Mark's Hospital. More than one investigation was undertaken in 88 patients to adequately assess and identify the bowel anatomy. As the bowel was discontinuous in most situations more than one radiological procedure needs to be carried out to map the entire bowel prior to definitive surgery. In situations where there was intra-abdominal sepsis multiple

investigations were sometimes required to assess the progress of measures to control or alleviate the sepsis.

Small bowel follow through was the most common investigation (63 patients), followed by CT (61 patients) and fistulography (36 patients). Retrograde studies of the distal bowel using a gastrograffin or barium enema were performed in 30 patients to ascertain whether any distal obstruction was present. Twenty one patients had magnetic resonance imaging scan (MRI) to aid characterisation of the fistula tract especially in relation to the abdominal wall. US were performed in 17 patients. During the course of their illness two patients had a positron emission tomography (PET) scan to determine the presence and severity of malignancy. Interventional radiologically guided drainage procedures were performed in four patients; two with CT and two with US. None of these four patients went on to develop spontaneous fistulation.

Thirty patients underwent lower gastrointestinal tract endoscopic procedures (colonoscopy; 21, flexible sigmoidoscopy; 9). Examination under anaesthesia (EUA), anorectal physiology (ARP) and endo anal ultrasound (EAUS) were performed in ten patients prior to surgery to ascertain bowel continence.

Table 3.2: Radiological / Endoscopic investigations performed at St. Mark's

Radiological / Endoscopic Investigation	Number
Small bowel follow through	63
CT	61
Fistulogram	36
Retrograde study of the distal bowel	30
MRI	21
US	17
Pouchogram	2
PET	2
Colonoscopy	21
Flexible sigmoidoscopy	9
Ileoscopy	2
Upper Gastrointestinal endoscopy	2
EUA	8
ARP	1
EAUS	1

3.5.4 Nutritional support

Fifty nine patients (33.3%) were either receiving parenteral nutrition (PN) or commenced on PN at first presentation. Following either definitive surgery or conservative management at St. Mark's Hospital this was reduced to 21 patients (11.9% requiring PN at discharge or last follow up including patients requiring home parenteral nutrition).

All but one patient had blood tests taken for albumin level at first presentation as part of their initial assessment. Of the 176 patients, the median was 32 with a mean level of 31.9 (range 17-49). Both of these were slightly lower than the normal laboratory range at St. Mark's Hospital of 35-50 g/l.

Body mass index (BMI) was calculated and recorded at the first consultation in 47 patients (26.6%) whilst only the weight was recorded in 53 of the remaining patients. The median BMI was 20.3 with a mean of 22.4 with (range 14.6 – 47.6). The median weight was 60 Kg with a mean 63.3 Kg (range 20 – 118Kg).

3.5.5 Surgical and Conservative Management

One hundred and forty nine patients (84.2%) required definitive fistula surgery and 28 patients (15.8%) were managed conservatively.

The median number of surgical operations performed at the referring hospital was 3 with a mean of 3.1 (range 0-13). The median number of operations performed at St. Mark's Hospital prior to definitive fistula surgery was 2 with a mean of 2.4 (range 0-9).

Conservative treatment was indicated if a patient was deemed to be not fit to undergo major ECF resective surgery or due to bowel anatomy. Those patients who declined surgery or who were referred for nutritional management were also managed conservatively.

Of the 28 patients treated conservatively seven underwent minor surgery to control sepsis or close a wound. Eleven patients were not suitable for surgical intervention and five patients declined to have surgery.

The median time to definitive fistula surgery at St. Mark's Hospital after a previous major surgery was 12 months with a mean of 25.9 months (range 1-170). There was an increased median time from that described in the previous study (4).

The bowel was defunctioned in 49 (32.9%) patients who underwent definitive fistula surgery by formation of a stoma brought out proximal to the anastomosis. This was an increase from 38 (22.7%) patients in the previous study (4).

Thirty six patients (20.3%) had the presence of an abdominal wall hernia as well as the co-existing fistula. At the time of definitive fistula surgery the abdominal wall closure and hernia repair was aided with the use of a mesh in 26 patients (17.4%). A Vicryl mesh (Ethicon) was used in the majority of patients; 24. A prolene mesh (Ethicon, Somerville, New Jersey, USA) and Gore-Tex Dual mesh (WL Gore and Associates, Flagstaff, AZ, USA) were used in the other cases.

3.5.6 Follow up

Patients were followed up for a median of 22.8 months (range 1-88 months).

3.5.7 Fistula Healing

Of the 149 patients who underwent definitive surgery, re-fistulation occurred in 21 patients (14.1%). Figure 1 displays the ECF healing outcomes of all patients.

Nine patients were managed conservatively after re-fistulation and of these four fistulas healed with the remaining five staying unhealed. Two patients who re-fistulated were treated conservatively with the use of Infliximab. Of these one patient's fistula healed whilst the others still persisted. Ten patients underwent repeat definitive surgery, and following resection a further eight fistula healed. Two continued to discharge effluent. Overall of the patients who underwent definitive fistula surgery eight fistulae did not heal. The final fistula healing rate after definitive surgery was 94.6% (141 of 149) compared with 82% in the previous series (4).

Of the 28 patients managed initially with conservative treatment 11 ECF healed (39.2%). From this group of 11 fistulas there were two patients with re-fistulation.

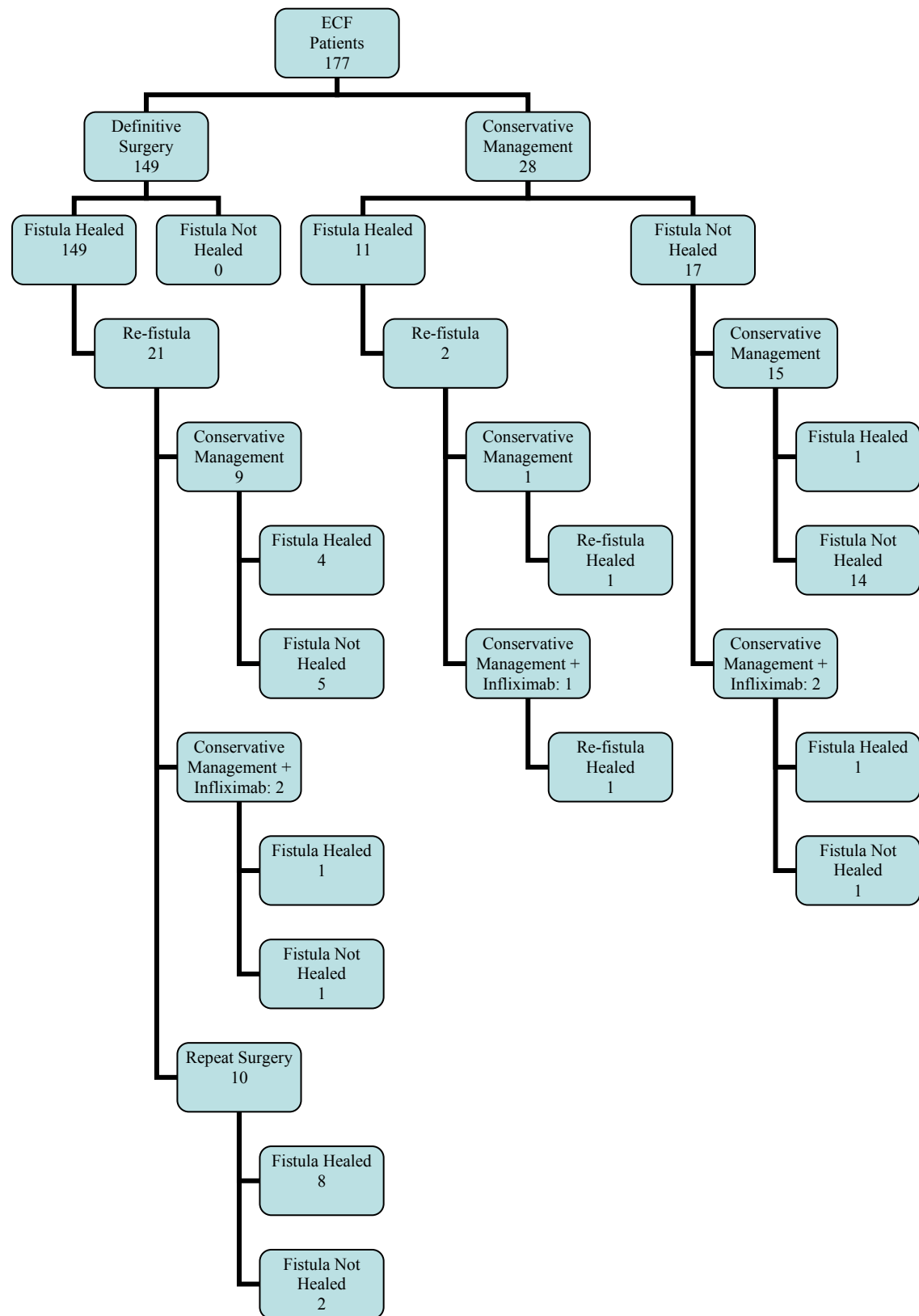
One patient was managed with further conservative treatment and the other with addition of Infliximab. Both of these ECF healed.

Seventeen out of the twenty eight managed conservatively did not heal (60.7%).

Fifteen patients of this group underwent further conservative treatment with only one fistula healing. Two patients underwent further conservative treatment with the addition of Infliximab in one case. Of these, one patient's fistula healed. Overall following conservative management 13 fistula healed of the 28 (46.4%) in comparison to 19.9% in the previous study (4).

From those managed conservatively (28 patients), 10 out of 17 (58.8%) patients with low fistula output had spontaneous closure in comparison with only two out of 11 (18.2%) with high fistula output. The Fisher's exact test gives a borderline statistical significance ($p = 0.05$) difference in healing between the two ECF output groups.

Figure 3.1: Fistula Healing



3.5.8 Deaths

The 30 day post fistula resection mortality was 0% in this current series compared with 3.5% in the previous series (4).

There were two late deaths. The first patient was a 71 year old lady who was transferred to St. Mark's Hospital with albumin at first consultation of 17. She had undergone six operations at the referring hospital. Colonic biopsies revealed histology with a picture consistent with ischemic colitis. She underwent a laparotomy and excision of an enterocutaneous fistula 17 months following initial presentation. At surgery she had multiple enterocutaneous fistula with abscesses noted in her peritoneal cavity and also a vesico-colonic fistula. In addition to extensive adhesiolysis she had fistulating disease excised along with the excision of a sigmoid abscess. She had a small bowel reconstruction of her abdominal wall with Vicryl onlay mesh. Her subsequent recovery was marred by problems of mobility. She was transferred to her local hospital where she continued to have abnormal liver function tests despite no biliary obstruction. Parenteral nutrition was considered a possibility. Histology from the resected specimens revealed non-specific inflammatory changes with no diagnostic features of Crohn's or Behcet's disease. Six months following return to the referring hospital she developed biliary sepsis and multi organ failure.

The second patient was a 79 year old male with a history of high grade non-Hodgkin's lymphoma. Radiological imaging gave impression of a complex enterocutaneous fistula secondary to non-Hodgkin's small bowel lymphoma. Due to significant co-morbidities including cardiac impairment with a past history of coronary artery bypass graft, it was decided that the patient was not suitable for

surgical intervention. His enterocutaneous fistula was managed conservatively. He underwent eight cycles of chemotherapy followed by a period of palliative care passing away at 15 months into follow up.

3.5.9 Statistical Analysis

The univariate analysis is shown in Table 3.3. The first and second column highlights each of the variables analysed with their respective sub-categories. The third column provides details on the number and percentage of subjects that were healed in each group. Also reported are odds ratios, which reflect the odds of fistula healing in each category relative to a baseline category. A value of greater than one implies that fistula healing is more common for that category than in the baseline category, whilst a value less than one implies that fistula healing is less common in that category than in the baseline category.

The results indicated that only comorbidity was strongly associated with the occurrence of ECF healing. Patients with either a minor or major co-morbidity were less likely to heal relative to patients with no co-morbidity. The odds of healing were more than five times lower (or less than 20% as large) for patients with a co-morbidity relative to patients with no co-morbidity.

There was evidence of a difference between patients referred from internal and external sources, with this result of statistical significance ($p = 0.05$). Patients from external sources were less likely to heal, with the odds of healing only around a quarter of the size of the odds for patients referred from an internal source.

Table 3.3: Univariate Analysis

Variable	Group	Healed Number (%)	Odds Ratio (95% CI)	P-value
Age	≤ 40	48/51 (94%)	1	0.08
	40 - 60	75/86 (87%)	0.43 (0.11, 1.61)	
	> 60	31/40 (78%)	0.22 (0.05, 0.86)	
Co-morbidity	None	63/65 (97%)	1	0.02
	Minor	24/28 (86%)	0.19 (0.03, 1.10)	
	Major	67/84 (80%)	0.13 (0.03, 0.56)	
Source of referral	Internal	46/47 (98%)	1	0.05
	External	108/130 (83%)	0.22 (0.05, 0.99)	
¹ Time to referral	≤ 4 months	48/54 (89%)	1	0.66 (*)
	> 4 months	43/50 (86%)	0.77 (0.24, 2.46)	
	Internal referral	46/47 (98%)	2.88 (0.55, 15.0)	
Aetiology	Post-op - IBD	62/74 (84%)	1	0.06
	Post-op - Other	78 /84 (93%)	2.52 (0.89, 7.08)	
	Other ^(**)	14/19 (74%)	0.54 (0.16, 1.79)	
² Origin of fistula ^(***)	Gastric - Jejunal	24/25 (96%)	1	0.15
	Ileal	112/129 (87%)	0.27 (0.03, 2.16)	
	Colonic	15/20 (75%)	0.13 (0.01, 1.18)	
³ Fistula complexity	Simple	36/41 (88%)	1	0.84
	Complex	116/134 (87%)	0.90 (0.31, 2.58)	
⁴ Fistula output	Low	64/77 (83%)	1	0.19
	High	89/99 (90%)	1.81 (0.75, 4.37)	
Laparostomy at presentation	No	137/158 (87%)	1	0.74
	Yes	17/19 (89%)	1.30 (0.28, 6.06)	
⁵ Albumin at presentation	≤ 25	43/46 (93%)	1	0.14
	> 25	110/130 (85%)	0.38 (0.11, 1.36)	
⁶ Number of	≤ 2	53/62 (85%)	1	

previous operations	3 +	55/65 (85%)	0.93 (0.35, 2.48)	0.89
⁷ Time of definitive surgery	≤ 12 months	70/72 (97%)	1	
	> 12 months	61/65 (94%)	0.44 (0.08, 2.46)	0.35
⁸ Bowel defunctioned	No	95/100 (95%)	1	
	Yes	46/49 (94%)	0.77 (0.18, 3.35)	0.72

(*) P-value for comparison of ≤ 4 months and > 4 months only.

(**) Other category contains patients who developed spontaneous fistula.

(***) There were too few patient numbers in the gastric and duodenal group to have their own group.

For the purpose of analysis these two groups were combined with the jejunal group.

¹Time to referral; data not available for 26 patients.

²Origin of fistula; data not available for 3 patients.

³Fistula complexity; data not available for 2 patients.

⁴Fistula output; data not available for 1 patient.

⁵Albumin; data not available for 1 patient.

⁶Number of previous operations; data not available for 50 patients.

⁷Time to definitive surgery; data only available in 137 out of the 149 patients.

⁸Bowel defunctioned; in 49 of the 149 patients undergoing surgery.

There was also evidence that aetiology affected healing, although this result was not statistically significant ($p = 0.06$). Patients in the 'post-operative other (non – IBD)' category were the most likely to heal. Those with an 'other' (patients who developed spontaneous fistula) aetiology were least likely to heal. There was also slight evidence that age was associated with healing, with older patients less likely to heal.

Univariate analysis from the prior study at our unit had identified a significant difference in fistula healing, between an albumin of ≤ 25 and > 25 (169). However using this cut off for albumin, there was no evidence of an effect from our univariate analyses. After adjusting for the other variables, this variable was statistically significant in the multivariate analysis ($p = 0.05$). Patients with an albumin level above 25 were less likely to have healed. The odds of healing for this group were only a quarter of the size of that for patients with lower albumin levels. The results for albumin were clinically not justifiable.

The effect of albumin was re-examined by changing the cut-off from all points ranging from 15 to 40. This made little difference to the results, and it was still significant in the opposite direction to that which you might interpret in a multivariable analysis. With these findings the analysis was repeated without incorporating albumin.

The results indicated that after adjusting for the other explanatory variables, age, co-morbidity, source of referral and aetiology all had some significant association with fistula healing. After adjusting for these four variables, there was no longer any significant effect of age upon fistula healing.

Table 3.4: Multivariate Analysis

Variable	Group	Odds Ratio (95% CI)	P-value
Co-morbidity	None	1	0.02
	Minor	0.11 (0.02, 0.72)	
	Major	0.10 (0.02, 0.49)	
Source of referral	Internal	1	0.01
	External	0.14 (0.03, 0.67)	
Aetiology	Post-op - IBD	1	0.006
	Post-op - Other	3.78 (1.26, 11.4)	
	Other	0.35 (0.08, 1.47)	

The final results of the multivariate analysis model is summarised in Table 3.4. As in the univariate analyses, patients with co-morbidity (minor or major) had a much reduced chance of healing. Those referred from external sources were also less likely to heal. Those ECF with 'other' aetiology (spontaneous fistulation primarily in the presence of IBD) were least likely to heal.

The next stage in the analysis used the multivariate analysis results to generate a scoring system for predicting fistula healing. This was done using the final logistic regression model from the multivariate analysis. Instead of expressing the size of effects in the form of odds ratios, the size of effects was expressed in the form of regression coefficients. The score attributed to each variable was based on the size of the regression coefficients.

These are listed in Table 3.5, along with the points that have been attributed to each factor. The order of the categories for aetiology was moved, so that all regression coefficients were in the same direction.

Table 3.5: Regression coefficients applied

Variable	Group	Regression Coefficient	Points
Constant	-	6.3	-
Co-morbidity	None	0	0
	Minor	-2.2	2
	Major	-2.3	2
Source of referral	Internal	0	0
	External	-2.0	2
Aetiology	Post-op - Other	0	0
	Post-op - IBD	-1.3	1
	Other	-2.4	2

The points were attributed based on each point equated to a regression coefficient of approximately - 1.2. This would indicate that two points equated to a coefficient of -2.4. Based on this formula the predicted regression equation can be written as follows: $\text{logit}(p) = \log(p/(1-p)) = 6.3 - 1.2 \times \text{Number of points}$, where p is the probability of fistula healing.

From this equation, the predicted probability (or percentage) of fistula healing could be calculated. A summary of these predicted patients is shown in the Table 3.6.

Table 3.6: Predicted probability of ECF healing

Points	% chance of healing
0	99.8%
1	99.4%
2	98.0%
3	93.7%
4	81.8%
5	57.4%
6	28.9%

Once the scoring system was set up, the performance of the system was evaluated by comparing the predicted healing rate with that observed from our data of the seven year experience involving 177 patients.

There were small numbers of patients with low and high scores. As a result, for an evaluation, these groups of patients were combined into a smaller number of groups.

Table 3.7 shows the predicted percentage of subjects who would be expected to heal in each group based on the earlier equation, and also the observed number and percentage that were actually healed in each group.

Table 3.7: Predicted versus Observed ECF healing – Retrospective Cohort (Scoring System for St. Mark’s Hospital)

Points	Number patients	Predicted healing (%)	Observed healing Number (%)
0 – 1	16	99%	16 (100%)
2	36	98%	35 (97%)
3	32	94%	31 (97%)
4	57	82%	59 (88%)
5 – 6	36	53%	13 (61%)

Table 3.7 (Scoring System for St. Mark’s Hospital) suggests an extremely good agreement between the observed and predicted percentage of patients that are healed within each category. Within each category the percentage numbers are very similar. This suggests that the model performs well for this dataset.

It should be noted, that it is not surprising that the model performed well for this dataset. This is because the model has been both developed and tested on the same set of data. In such an instance, this sort of result might be expected. The key test of the scoring system was to apply this model on an additional data source.

3.5.10 Scoring System devised for use by an ‘External Hospital’ - (Scoring System for External Centres)

Further revision of the multivariate analysis and scoring system was performed. Source of referral was removed from the multivariate analysis to make the system suitable for use by any external institution (Table 3.8). The statistical methods used were equivalent to those described previously.

Table 3.8: Multivariate analysis with source of referral removed

Variable	Group	Odds Ratio (95% CI)	P-value
Co-morbidity	None	1	0.02
	Minor	0.14 (0.02, 0.85)	
	Major	0.10 (0.02, 0.49)	
Aetiology	Post-op - IBD	1	0.03
	Post-op - Other	2.54 (0.88, 7.31)	
	Other	0.36 (0.09, 1.35)	

The results indicate that after adjusting for the other explanatory variables, co-morbidity ($p = 0.02$) and aetiology ($p = 0.03$) both had a significant association with fistula healing.

Points were again attributed based on each point equating to a regression coefficient of approximately -1.0. So this would indicate that one point equated to a coefficient of -1.0, and two points equated to a coefficient of -2.0 (Table 3.9).

Table 3.9: Regression coefficients with source of referral removed

Variable	Group	Regression Coefficient	Points
Constant	-	4.4	-
Co-morbidity	None	0	0
	Minor	-2.0	2
	Major	-2.3	2
Aetiology	Post-op - Other	0	0
	Post-op - IBD	-0.9	1
	Other	-2.0	2

Based on this formula the predicted regression equation can be written as follows:

$\text{logit}(p) = \log(p/(1-p)) = 4.4 - 1.0 \times \text{Number of points}$, where p is the probability of fistula healing.

From this equation, the predicted probability (or percentage) of healing was calculated. A summary of these predicted percentages is shown in Table 3.10.

Table 3.10: Predicted probability of ECF healing

Points	% chance of healing
0	98.8%
1	96.8%
2	91.7%
3	80.2%
4	59.9%

Predicted occurrences of healing were compared with those observed from the data.

There were only nine patients with a score of four. This was a fairly small number to assess their performance. As a result, for an evaluation, patients with a score of four were combined with those with a score of three.

Table 3.11 (Scoring System for External Centres) shows the predicted percentage of subjects that would be expected to be healed in each group based on the earlier equation, and also the observed number and percentage that were actually healed in each group.

Table 3.11: Predicted versus Observed ECF Healing – Retrospective Cohort (Scoring System for External Centres)

Points	Number patients	Predicted healing (%)	Observed healing Number (%)
0	30	99%	29 (97%)
1	25	97%	25 (100%)
2	64	92%	58 (91%)
3 or 4	58	77%	42 (72%)

Table 3.11 also suggests a fairly good agreement between the observed and predicted percentage of patients that are healed within each category. Almost all patients with a score of zero and one healed, whilst the observed healing of those with the highest points score (three or four) was the lowest. Generally these results suggest that the model performs well for this dataset.

3.5.11 Validation of scoring systems

As describe above, the key test of both scoring systems Tables 3.7 and 3.11, was to apply the models on an additional data source. The initial intention was to obtain data from an external source.

Unfortunately this was not possible due to inadequate patient numbers at external hospitals, limited access to patient records and reluctance by some surgeons to contribute patients with poor outcomes.

I therefore collected data prospectively from January 2010 to December 2011 (two year period) at St. Mark's Hospital to validate the scoring system. Continuous data were collected from the endpoint of the previous seven year experience cohort.

Overall, 85 patients were managed with ECF over the two year period. Of the 85 patients, 42 (49.4%) were male and 43 (50.6%) female. The median age was 52 years (range 21-80), with a mean of 52.8 years.

Nineteen (22.4%) had no co-morbidity. Twelve (14.1%) had minor co-morbidity - mild systemic disease (ASA 2). Fifty four (63.5%) had a major co-morbidity - severe systemic disease or worse (ASA ≥ 3).

Nine (10.6%) of the ECF occurred within the Trust. External referrals accounted for the majority of patients: 76 patients (89.4%).

Fistula resulted from surgery in 81 (95.3%) of the 85 patients (Table 3.12). Of these, 24 had inflammatory bowel disease (18 Crohn's disease and six ulcerative colitis). No patient had previous radiotherapy leading to ECF formation. Fourteen patients developed a fistula following prior surgery for intra-abdominal malignancy. Diverticular disease contributed to ten patients developing a fistula. Thirty three patients developed fistulas due to a range of other causes.

Of the 85 patients, 69 ECF healed. Seventy five patients underwent definitive surgery. There were twelve re-fistulae following surgery and from these, a further two ECF healed following redo definitive surgery. The overall ECF healing rate following surgery was 87% (65/75 patients).

Of the ten patients who underwent conservative management, four ECF healed (40%).

Table 3.12: Aetiology of ECF Jan 2010 – Dec 2011

	Post-operative	Spontaneous	Total
Crohn's disease	18	4	22
Ulcerative colitis	6	0	6
Radiotherapy	0	0	0
Intra-abdominal malignancy	14	0	14
Diverticular disease	10	0	10
Other	33	0	33
Total	81(95.3%)	4 (4.7 %)	85

The 'Scoring System for St. Mark's Hospital (Table 3.7) was applied to this new 85 two year prospective cohort of patients. The results are displayed in Table 3.13.

The treatment protocol during both the seven year period and two year prospective cohort remained essentially the same. Table 3.12 displays the aetiology of the ECF patients in the two year prospective group (January 2010 – December 2011).

The increase in the number of patients was due to a steady increase in the number of referrals to both the surgical and medical teams as well as to the appointment of a second Consultant General and Intestinal Failure Surgeon at the beginning of January 2010.

Table 3.13: Predicted versus Observed ECF healing (Jan 2010 – Dec 2011). Prospective Validation Cohort. (Scoring System for St. Mark's Hospital)

Points	Number patients	Predicted healing (%) (As Table 3.7)	Observed healing Number (%)
0 – 1	1	99%	1 (100%)
2	17	98%	16 (94%)
3	6	94%	5 (83%)
4	43	82%	35 (81%)
5 – 6	18	53%	12 (67%)

This showed a good agreement between the predicted and observed percentage of patients that are healed within each category with a downward trend displayed for the highest scores.

The final stage was to apply the ‘Scoring System for External Centres’ (Table 3.11) to this two year cohort of patients. Although these patients attended St. Mark’s Hospital, we only scored the patients based on co-morbidity and aetiology and excluded the referral type (Internal / External). Hence this could be used and appropriately applied by any external institution. The results are shown in Table 3.14.

**Table 3.14: Predicted versus Observed ECF Healing.
Prospective Validation Cohort. (Scoring System for External Centres)**

Points	Number patients	Predicted healing (%) (As Table 3.11)	Observed healing Number (%)
0	14	99%	13 (93%)
1	9	97%	8 (89%)
2	39	92%	32 (82%)
3 or 4	23	77%	16 (70%)

Table 3.14 also suggests a fairly good agreement between the predicted and observed percentage of patients that are healed within each category, with trends in a similar direction. Almost all patients with a score of zero and one healed, whilst the observed healing of those with the highest points score (three or four) was the lowest. Generally these results suggest that the model performs well.

3.6 DISCUSSION

This is the first study that has led to the development of a scoring system to predict ECF healing. Predictive factors for mortality could not be meaningfully analysed as there were no mortalities.

Univariate and multivariate analysis has identified that fistula healing is affected by co-morbidity, source of referral and aetiology.

There was extremely good agreement between the predicted and observed percentages of patients who healed within each category.

The time to definitive fistula surgery at St. Mark's Hospital after previous major surgery was a median of 12 months. This was an increased median time delay from eight months in the previous series (4). Delaying surgery may be one factor which has led to the improved overall fistula healing rate after definitive surgery; 94.6% compared with 82%, and 30 day post fistula resection mortality; 0% compared with 3.5% (4).

Delayed surgical intervention for persistent fistula is advisable allowing for the obliterative peritonitis to subside and allowing laparotomy and adhesiolysis to be performed safely. Operability of the peritoneal contents can usually be assessed on palpation of the abdomen. A further clinical indicator for the appropriate time to operate on ECF patients is the presence of non-oedematous prolapsing bowel in the fistulating segments (4, 94).

There was evidence of a difference between patients referred from internal and external sources ($p = 0.05$). Patients from external sources were less likely to heal.

This may be due to a greater number of operations being performed at external institutions prior to the decision to refer to specialist care. Our results did not show a large difference in the median number of surgical operations prior to definitive fistula surgery performed at the referring hospital compared to St. Mark's Hospital (3 versus 2). However there was a notable difference in the range (range 0-13 versus 0-9).

Most of the patients presented with a complex fistula, however only 33.3% received parenteral nutrition. Patients with adequate nutritional requirements were investigated and followed up through the outpatient setting. Identification of a complex fistula was at either following radiological investigation or during surgery.

Previous studies have described factors which may contribute to ECF healing (16, 170, 171). Kuvshinoff *et al.* studied whether there were any laboratory or other features that would enable prediction of spontaneous closure in patients with gastrointestinal cutaneous fistulas (170). Seventy nine patients with 116 fistulas were reviewed. The presence of local sepsis, systemic sepsis, remote sepsis, the number of fistulas, fistula output, and the number of blood transfusions were not predictive of spontaneous closure, whereas serum transferrin was. Serum transferrin, retinol-binding protein, and thyroxin-binding prealbumin were predictive of mortality. These laboratory investigations are not carried out routinely in hospitals United Kingdom and would therefore not be of practical use in a scoring system. They were hence not analysed.

Somatostatin and its analogues are sometimes used in the management of ECF to aid a reduction in fistula output. They are not licensed for use in the UK, although octreotide can be used at the physician's discretion. Recent meta-analysis has shown

that both somatostatin and its analogues increase the likelihood of ECF closure and both are beneficial in reducing the time to ECF closure. Neither had an effect on mortality. Results of the meta-analysis suggest somatostatin is better than its analogues in relation to the number of ECF closed and time to closure (171).

Lloyd *et al.* found there that is no evidence to suggest that restriction of enteral intake and 'bowel rest' results in increased rates of fistula closure and it may increase the incidence of complications. Enteral nutrition should be used if possible, although high-output small bowel fistulas usually require supplemental parenteral nutrition (16). The practice at St. Mark's Hospital is to allow enteral nutrition for all patients if possible, with supplemental intravenous fluids or parenteral nutrition where necessary.

Harvey *et al.* identified albumin as a factor that might predict sepsis and mortality in patients referred for metabolic and nutritional therapy (172). More recently a multivariate analysis by Lu *et al.* showed that serum albumin levels and the level of fistula output can be used as important prognostic predictors of ECF healing in patients having PN following surgery for gastrointestinal malignancies (173). Neither of these factors was found to be predictive in our unit. With the delay to definitive surgery, the albumin can be corrected and with optimisation of fluid management, high outputs can be managed.

Datta *et al.* noted that factors which influence spontaneous closure of ECF include; long fistulous tract, intestinal continuity, absence of distal obstruction, no sepsis, low fistula output (< 500ml/24hr), no malnutrition, no anastomotic leakage and absence of

inflammatory bowel disease (174). Our study revealed a statistically significant ($p = 0.05$) difference in spontaneous healing between the two output groups.

Infliximab is indicated for use to treat patients with ECF in Crohn's disease, and its use has also shown benefit in patients without inflammatory bowel disease (175).

Infliximab is now an established treatment for ECF in Crohn's disease. Three Crohn's patients from our initial cohort were treated with Infliximab with healing in two. There is one small study showing benefit in ECF patients without inflammatory bowel disease (175).

Some studies have reviewed data and identified factors predictive of healing and mortality in patients managed medically alone or in those who required definitive surgical intervention. (2, 169, 176-178).

Altmore *et al.* developed a prognostic index to classify post-operative ECF patients according to risk of death (176). Following logistic regression analysis of risk factors the prognostic index comprised the Acute Physiology and Chronic Health Evaluation (APACHE II) and serum albumin concentration. This severity score and mortality estimation tool comprises physiological measurements available within intensive care units. The majority of our patients were transferred directly to the ward, and therefore this index was not assessed in this study.

Campos *et al.* was the first to perform a multivariate analysis to determine the relative importance of factors associated with patients with gastrointestinal fistulas (2). One hundred and eighty eight patients were prospectively analysed over a ten year period. Unlike our data, this sample of patients included both biliary and pancreatic fistulas. Variables significant for spontaneous closure were; cause of the fistula, fistula output,

institutional origin of the patient and occurrence of complications. Variables significant for death were fistula output and the presence of complications.

Mawdsley *et al.* analysed predictive factors for healing in patients with ECF in a cohort of 277 patients (169). In patients who were treated operatively, successful ECF closure related to only one factor; the complexity of the ECF. In patients managed conservatively, a decreased likelihood of ECF closure was associated with high fistula output, comorbidity and being referred from an external institution.

Martinez *et al.* reviewed patients with post-operative ECF over a ten year period (178). A similar number of patients, 174, were treated as at our institution. Although the most frequent site of origin of the ECF was the small bowel, there were a greater number of patients with jejunal ECF as opposed to ileal ECF in our study. This may be because in our unit, surgery primarily involved the ileum rather than the jejunum. ECF closure was achieved in 84 per cent of patients who underwent an operation in this study. Multivariate analysis showed that high outputs, jejunal fistulas, multiple fistulas, and sepsis were independent adverse factors related to non-spontaneous closure, need for operative treatment, and death.

A more recent study by Martinez *et al.* assessed factors predictive of recurrence and mortality after surgical repair of ECF in 71 patients over a five year period (177). Univariate analysis revealed non-colonic ECF origin, high output, and non-resective surgical options as risk factors for recurrence, with the latter two remaining significant after multivariate analysis. Univariate analysis revealed several significant risk factors for mortality, but after multivariate analysis there were two remaining significant factors; serum albumin < 3 g/dl and age $\geq$ 55 years.

Our study of 177 patients had a similar demographic profile as compared to the previous study at St. Mark's Hospital (4). There was a wide range (0.25 - 96 months) in time elapsed after initial fistulation before referral to St. Mark' Hospital. By far the greatest aetiology for ECF formation was surgery performed in patients with a background of inflammatory bowel disease, and more specifically Crohn's disease. Spontaneous fistulation was also most commonly present in patients with a background of Crohn's disease.

The data included a wide variety of sites and number of ECF in relation to the gastrointestinal tract, but the most frequent site for fistulation occurred in the ileum. The majority of ECF were complex in nature.

Fistula output has been classified differently by various authors but with our classification of 500ml in a 24 hour period, there was a slightly greater proportion of patients presenting with low output fistula (56%) than high output fistula.

A wide range of radiological investigations were used to assess the bowel anatomy, with small bowel follow through and CT scan being the most frequently used. We believe a greater number of interventional radiological procedures and conduitograms were performed than were identified. This was due to lack of accessibility to early records of radiological procedures on the hospital computer records programme as well as poor documentation in hospital notes.

The data obtained revealed a lack of recording of BMI with only 26.6% of patients having this documented. After identifying and presenting these results, there has been a change in the hospitals practice in an attempt to improve recording of BMI and for it to make a positive impact on nutritional management of patients.

The results for albumin were clinically not justifiable. This can be explained in that following the previous study at our unit which showed a better chance of fistula healing with an albumin of > 25 (169), a change in practice was undertaken at St. Mark's Hospital, with a decision to operate on patients with an albumin as close to normal as possible. This is evident as the vast majority of patients in our data set had albumin levels greater than 25. This gave a greater opportunity of failed fistula healing in patients to be within the albumin > 25 group than ≤ 25 , and this was borne out in the results shown.

There was a greater range of number of operations performed at the referring hospital (0-13) than were performed at St. Mark's Hospital (0-9) prior to decision for definitive surgery.

Compared to the previous study at our institution (4), this study has revealed an improved fistula healing rate following both definitive surgery (94.6% versus 82%) and an improved fistula healing rate following conservative management (46.4% versus 19.9%). There was also an improved 30 day fistula resection mortality rate (0% versus 3.5%).

An important factor contributing to the improved outcome may be highlighted by the increase in median delay time to surgery to twelve months from eight months in the previous study (4). This is in keeping with the literature in that delaying reconstructive surgery until optimal nutrition status; general fitness and re-epithelialisation of the peritoneum have been achieved. Re-operation should be avoided for at least four to six months after the last abdominal procedure where ever possible (4, 34, 95).

Other factors leading to an improved outcome may include reduction in line infections and relate to an improved cohesion of our dedicated multidisciplinary team with increasing experience, insistence that the patient is discharged home prior to surgery wherever possible and improved medical management of Crohn's disease with immunosuppressive and biological drugs.

In cases showing no healing, strategies include optimising patients for surgery with treatment of sepsis, nutrition, mapping of the bowel anatomy and planned surgery after a period of at least six months after the last abdominal surgery.

There is a wide variety of ECF patients attending St. Mark's Hospital. This can range from elective outpatient clinic attendance to admission to the Intestinal Failure Unit, with several months of inpatient stay whilst being managed conservatively or requiring surgical intervention after the prolonged period of time.

Our analysis and scoring system had an aim of informing the patient on admission with a probability percentage chance of their ECF healing. This would be without foretelling whether these patients ECF would require medical management to aid closure, or would require surgical intervention in the future. With this in mind, our analysis of the 177 patients combined all data of patients treated both medically alone and those who underwent definitive surgery. This technique was again used on the prospective cohort of 85 patients. Predictive factors for mortality were not analysed as a result of the low mortality rate.

Univariate and multivariate analysis has identified that fistula healing is affected by co-morbidity, source of referral and aetiology.

There was an extremely good agreement between the predicted and observed percentages of patients that healed within each category (Table 3.7).

Both of the scoring systems devised to be used at either St. Mark's Hospital (Table 3.7) or external institutions (Table 3.11) have been validated on our prospective cohort of 85 patients (Tables 3.13 & 3.14).

Table 3.9 can be used by external centres to apply points to patient at the time of first presentation with an ECF. Table 3.11 can then be used to identify predicted healing rates for the patient.

Adequate data was not available regarding time to closure and hence this could not be analysed. This would be useful for any future study.

The course of Crohn's fistulae and ECF associated with UC are different. To classify them in one group as has been done in this study is an over simplification. Thirteen variables were assessed in the univariate analysis. Aetiology was sub divided into three further categories, and IBD was taken to include both Crohn's and UC. The total number of patients with UC related fistula was low in this series and it was difficult to independently assess this. It can also be argued that the UC related fistula are more related to surgical misadventure or misdiagnosis of Crohn's disease which may not always be apparent immediately. Assessing their individual impact on clinical outcome would be useful for any future study as well as the potential impact on the scoring system.

This study is the first to design a scoring system for ECF healing. Future work intended is to contact units both nationally and internationally to apply the scoring

system. This will allow assessment of its effectiveness and modifications to take place.

3.7 CONCLUSION

The scoring system is intended to be applied on all patients at their first presentation with an ECF. This is prior to knowing if the patient will require medical treatment alone or progress to requiring surgery.

The scoring systems can provide further information to aid clinical management and decision making for the multidisciplinary team and predict the possible long term outcome for patients at external institutions.

CHAPTER 4

A META-ANALYSIS OF OUTCOMES FOLLOWING USE OF SOMATOSTATIN AND ITS ANALOGUES FOR THE MANAGEMENT OF ENTEROCUTANEOUS FISUTLAS

4.1 ABSTRACT

Background

Several randomised control trials (RCT) have compared somatostatin and its analogues versus a control group in patients with enterocutaneous fistulas. This study meta-analyses the literature and establishes whether they have a beneficial effect on ECF closure.

Methods

We searched MEDLINE, EMBASE, CINAHL, Cochrane and PubMed databases according to PRISMA guidelines. 79 articles were screened. Nine RCT met the inclusion criteria. Statistical analyses were performed using Review Manager 5.1.

Results

Somatostatin analogues versus control

Number of fistula closed: A significant number of ECF closed in the somatostatin analogue group compared to control group, $p = 0.002$.

Time to closure: ECF closed significantly faster with the use of somatostatin analogues compared to controls, $p < 0.0001$.

Mortality: No significant difference between somatostatin analogues and controls, $p = 0.68$.

Somatostatin versus control

Number of fistula closed: A significant number of ECF closed with somatostatin as compared to control, $p = 0.04$.

Time to closure: ECF closed significantly faster with somatostatin than controls, $p < 0.00001$.

Mortality: No significant difference between somatostatin and controls, $p = 0.63$.

Conclusion

Somatostatin and octreotide increase the likelihood of fistula closure. Both are beneficial in reducing the time to fistula closure. Neither has an effect on mortality. The risk ratio for somatostatin was higher than the risk ratio for analogues. This may suggest that somatostatin could be better than analogues in relation to the number of fistulas closed and time to closure. Further studies are required to corroborate these apparent findings.

4.2 INTRODUCTION

Somatostatin was discovered in 1972 (179) and is a naturally occurring peptide hormone. It has an inhibitory effect on gastrointestinal secretion (180). Somatostatin has been found in the pancreas, stomach, intestinal mucosa and mesenteric neurones. It is used in the management of upper gastrointestinal haemorrhage, secretory diarrhoea, dumping syndrome and peptide-secreting neuro endocrine tumours because of its anti-secretory action (181).

As the plasma half-life is 1–2 minutes it must be administered by continuous intravenous infusion. Somatostatin reduces the secretion of a range of gastrointestinal hormones, including gastrin and cholecystokinin, which in turn reduce gastric and pancreatic secretions. In addition, somatostatin reduces splanchnic blood flow, reduces the rate of gastric emptying and inhibits gallbladder contraction.

As a result of the short half-life, a number of synthetic analogues have been developed, and of these octreotide is used most commonly in the treatment of gastrointestinal fistula (16).

Its significantly longer half-life allows it to be given by intermittent subcutaneous injection (usually three times daily). Despite the similarities, the receptor binding properties of somatostatin and octreotide are not identical and their actions may not be equivalent (16).

Octreotide is licensed for use in the UK but not for use in the management of ECF. If used in treating ECF, then the responsibility lies with the treating clinical physician. The cost of octreotide is £16.90p for 250 mcg (£33.80p for 500 mcg – maximum daily dose).

Somatostatin costs over three times that of octreotide at £56.60p for 250 mcg. It is not licensed for use in the UK for any medical condition. If required for use at the physician's discretion, it is imported from the Netherlands.

Enterocutaneous fistulas are abnormal communications between the gastrointestinal tract and the skin. Although rare, they are associated with considerable morbidity and mortality. Death related to ECF remains disproportionately high when compared with other surgical conditions. Mortality rates for ECF have been reported to range from 6 - 33% (1-5).

Fistula formation can result in a number of serious or debilitating complications, varying from disturbance of fluid and electrolyte balance to sepsis and even death. The patient will almost always suffer from severe discomfort and pain. They may also have psychological problems which include; anxiety over the course of their disease, and a poor body image due to the malodorous drainage fluid (182). Post-operative fistula formation often results in prolonged hospitalisation, patient disability, and enormous cost.

Both somatostatin and octreotide have been described as effective treatments for ECF in adults, with few short-term side effects (diarrhoea, flushing and abdominal pain). Accelerating spontaneous ECF closure reduces the period of parenteral nutrition requirement as well as the morbidity inherent to long-lasting conservative treatment. There is also the potential benefit of reducing patient's discomfort, shortening the length of hospital stay and of cutting the cost of ECF treatment. Some authors argue that high cost, equivocal therapeutic effect on underlying diseases, painful injections

and potential for long-term side-effects make the use of somatostatin and its analogues controversial (5).

Although there are data suggesting reduced time to fistula closure, there is little evidence of the increased probability of spontaneous closure (1).

There have been several randomised control trials (RCT) and studies (182-193) comparing somatostatin and somatostatin analogues versus standard medical therapy or a placebo (control group) in patients with ECF.

4.3 AIM

The objective of this study is to undertake a meta-analysis of the published literature to assess the efficacy of somatostatin and somatostatin analogues in the treatment of ECF.

4.4 METHODS

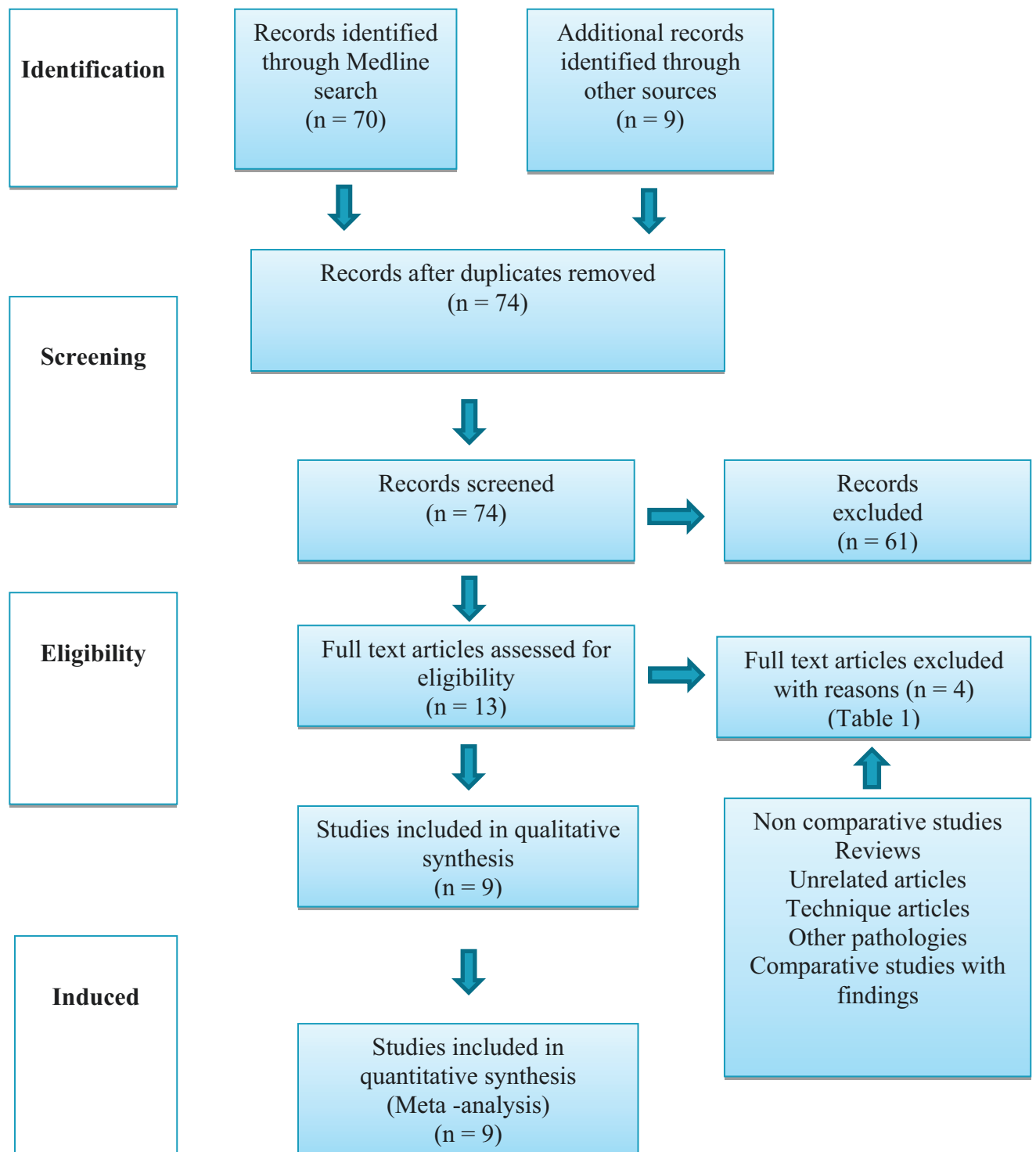
All comparative studies investigating the use of somatostatin and somatostatin analogues in the management of ECF were identified. Articles dated between January 1987 and March 2011. We searched the MEDLINE, EMBASE and CINAHL available through the National Library of Health website, the Cochrane library and PubMed databases available online.

The text words “somatostatin”, “octreotide” and “analogues” were used in combination with the medical subject headings “enterocutaneous” and “fistula.” Irrelevant articles and reviews evident from the titles and abstracts were excluded. Relevant articles referenced in these publications were obtained and the "related

article" function was used to widen the results. No language restriction was applied and trials on patients of any age or sex were included (Table 4.1).

All abstracts, comparative studies, non-randomised trials and citations were searched comprehensively. A flow chart of the literature search according to PRISMA guidelines (194) is shown in Figure 4.1. Seventy nine articles were screened for relevance. Sixty one articles were not suitable for analysis as they were non-comparative studies, reviews, unrelated articles or articles on other pathologies. On further scrutiny nine randomised controlled trials were found to have useful data for this meta-analysis.

Figure 4.1: Flow chart of the literature search according to PRISMA guidelines



Each article was critically reviewed by two researchers using a double extraction method for eligibility in our review. This was performed independently and any

conflict resolved prior to final analysis. A third researcher confirmed the data extraction. Outcome measures were chosen based upon whether they were common to the included articles.

Statistical analyses were performed using Review Manager 5.1 (RevMan; Cochrane Collaboration, Copenhagen) (195).

A value of $p < 0.05$ was chosen as the significance level for outcome measures. For continuous data (time to closure of fistula), the Inverse-Variance method was used for the combination of standardised mean differences (SMD). Binary data (number of fistula closed and mortality) were summarised as risk ratios (RR) and combined using the Mantel-Haenszel method. In each case a heterogeneity test was carried out to see whether the fixed effects model was appropriate. In a sensitivity analysis, 1 was added to each cell frequency for trials in which no event occurred, according to the method recommended by Deeks et al (196). Where standard deviations were not reported these were estimated either from ranges or P-values. Forest plots were used for the graphical display.

Table 4.1 - Inclusion Criteria

- | |
|---|
| <ul style="list-style-type: none">• All studies comparing somatostatin and somatostatin analogues with placebo or standard medical therapy• Trials on patients of any age or sex• Trials in all languages |
|---|

4.5 RESULTS

Thirteen full text articles were retrieved from the electronic databases and assessed for eligibility. Four trials (183, 188, 191, 197) were excluded and reasons are explained in Table 4.2.

Table 4.2: Excluded Trials

Trial	Reason for exclusion
Perez et al 1994 (183)	Somatostatin used in 19 patients compared with 50 historical cases. Non-randomised clinical trial.
Sitges-Serra et al 1993 (191)	Series of 27 patients treated with octreotide after having received parenteral nutrition for a mean of 25 days. Non-randomised clinical trial.
Kusuhara et al 1992 (197)	Reduction of the effluent volume in high-output ileostomy patients by a somatostatin analogue octreotide. Not related to enterocutaneous fistula.
Nubiola-Calonge et al 1987 (188)	Blind evaluation crossover trial. Patients randomised to 2 days of octreotide followed by 2 days on placebo or vice versa, after which all patients were treated with octreotide. Inadequate length of study arms prior to randomisation.

Nine randomised controlled trials comparing somatostatin and somatostatin analogues for use in the management of ECF were included in our study according to our inclusion criteria.

Characteristics of trials comparing somatostatin analogues (octreotide and lanreotide) versus control or placebo are given in Table 4.3.

Table 4.3: Characteristics of trials: Somatostatin analogues (octreotide and lanreotide) versus control

Trial	Year	Type	N	Type of RCT	CON Protocol	OCT protocol (LAN if stated)	Population Characteristics
Gayral et al	2009	CON	53	P	30mg placebo i.m at first visit	30mg lanreotide i.m (prolonged release) at first visit. Subsequent treatment dependent on response of at least 50% reduction in fistula vol.	Male 32 (60.4%), female 21 (39.6%). Mean age (SD) 59 (14.4). Pancreatic 35 (66%), duodenum 12 (22.6%), small bowel 6 (11.3%). Mean fistula vol. (SD) / 24hr: 333.4 (225.0)
		LAN	54				Male 27 (50%), female 27 (50%). Mean age (SD): 54.8 (15.6). Pancreatic 36 (66.7%), duodenum 6 (11.1%), small bowel 12 (22.2%). Mean fistula vol. (SD) / 24hr: 340.4 (200.0)
Leondros et al	2004	CON	15	P	Standard medical therapy	100 µg s.c tds (Until fistula closure)	Male/female 10/5. Mean age (SD) 72 (13). Enteric 14, pancreatic 1. Low output 10, high output (>500 ml / 24hr) 5
		OCT	17				Male/female 9/8. Mean age 70 (SD 11.1). Enteric 12, pancreatic 5. Low output 8, high output 9
		SMT	19				Male/female 12/7. Mean age 61.5 (SD 16.9), p < 0.05. Enteric 12, pancreatic 7. Low output 9, high output 10

Jamil et al	2004	CON	17	P	Standard medical therapy	Sandostatin (octreotide) 100 µg s.c tds (Until fistula closure)	55% male, age range 14-68, mean 39 yrs. 45% female, age range 19-56, mean 34 yrs. ECF: 52% ileum, 18% jejunum, 12% colon, 9% biliary tree / pancreas, 6% duodenum, 3% appendix. Low output ECF (< 200ml / 24 hr.) 24.24 % in control, 18.18% octreotide group.
		OCT	16				
Hernandez -Aranda et al	1996	CON	45	P	Standard medical therapy	100 µg s.c tds (Until fistula closure)	Male/female 27/26 at start. Mean age 51 +/- 17 yrs. Oesophagus 9, gastric 7, duodenal 9, intestinal 28. ECF < 500 ml /24 hrs. = 6, > 500ml / 24 hrs. = 47
		OCT	40				Male/female 28/18 at start. Mean age 49 +/- 22 yrs. Oesophagus 2, gastric 3, duodenal 13, intestinal 28. ECF < 500 ml / 24hrs = 9, > 500 ml / 24 hrs. = 37
Sancho et al	1995	CON	17	P	Standard medical therapy + Placebo: saline	100 µg tds s.c for 20 days	Male/female 8/9. Age 65 yrs. (58-73). Stomach 1, pancreas 2, duodenum 5, jejunum 4, ileum 5
		OCT	14				Male/female 11/3. Age 64 yrs. (58-70). Pancreas 3, duodenum 6, jejunum 1, ileum 4
Scott et al	1993	CON	8	P	12 days placebo: acetate buffered saline s.c	100 µg tds s.c for 12 days	Male/female 2/6. Mean age 66.7 (range 33-78). Duodenal 4, small bowel 4
		OCT	11				Male/female 5/6. Mean age 57.5 yrs. (range 22-67). 2 gastric, 2 pancreatic, 7 small bowel

SMT = Somatostatin, OCT = Octreotide, LAN = Lanreotide, CON = Control

P = Prospective, R = Retrospective

Characteristics of trials comparing somatostatin versus control or placebo are given in

Table 4.4.

Table 4.4: Characteristics of trials: Somatostatin versus control

Trial	Yr	Type	N	Type of RCT	CON Protocol	SMT Protocol	Population Characteristics
Leondros et al	2004	CON	15	P	Standard medical therapy	Continuous intravenous infusion at 6000 IU/day (Until fistula closure)	Male/Female 10/5. Mean age (SD) 72(13). Enteric 14, pancreatic 1. Low output 10, high output (> 500 ml / 24 hr.) 5
		SMT	19				Male/female 12/7. Mean age (SD) 61.5 (16.9), p<0.05. Enteric 12, pancreatic 7. Low output 9, high output 10
Isenmann et al	1994	CON	20	P	Standard medical therapy	Continuous intravenous infusion at 250 µg/hr. (Until fistula closure)	Mean age 59.9. Pancreas 9, duodenum 9, bile duct 2. Output average: 373.3 ml / 24 hr.
		SMT	25				Mean age 56. Pancreas 11, duodenum 12, bile duct 2. Output average: 303.8 ml / 24 hr.
Torres et al	1992	CON	20	P	Standard medical therapy	TPN for 48-72 hrs. followed by SMT continuous iv infusion at 250µg/hr. for 20 days. Infusion stopped if fistula un-productive for 48 hrs.	Male/female 10/10. Mean age 49.5 (37-78). Fistula output: 202.5 ml /24 hr. Pancreas 3, duodenum 2, jejunum 4, ileum 9, ileocolic 2
		SMT	20				Male/female 16/4. Mean age 61.9 (36-74) p < 0.05. Fistula output: 369.7 ml / 24 hr., p < 0.05. Pancreas 4, duodenum 3, jejunum 3, ileum 9, ileocolic 1
Spiliotis et al	1990	CON	30	P versus R group	Standard medical therapy	Continuous intravenous infusion at 250µg/hr.	Male/female 18/12. Mean age 63.5 (range 22-84 yrs.). Gastro duodenal 10, small bowel 9, biliary 4, pancreatic 7

		SMT	18			(Until fistula closure)	Male/female 10/8. Mean age 59.3 (range 26-74). Gastro duodenal 6, small bowel 6, biliary 2, pancreatic 4
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Leondros et al (187) compared three groups in the study; somatostatin, octreotide and control. Characteristics and outcomes of this study were separated into both of the relevant tables accordingly.

There were 141 patients in the somatostatin analogue group and 147 in the control or placebo group. When comparing somatostatin with control or placebo, there were 82 and 85 patients in each group respectively.

The methodological quality of included trials is explained comprehensively in Table 4.5 (198, 199) and Table 4.6 (200).

**Table 4.5: Modified Quality Score for Randomised Controlled Trials
(Jaddad et al & Chalmers et al)**

Quality variables	Gayral et al	Leondros et al	Jamil et al	Hernandez- Aranda et al	Sancho et al	Scott et al	Isenmann et al	Torres et al
Was the study described as randomized such as using the words randomly, random, and randomization? [0,1]	1	1	1	1	1	1	1	1
Was randomization described and appropriate? [-1,0,1]	1	1	1	1	1	1	1	1
Was the study described as double blind? [0,1]	1	0	0	0	1	1	0	0
Was method of blinding appropriate? [-1,0,1]	1	0	0	0	1	1	0	1
Was there a description of withdrawals and dropouts? [0,1]	1	1	1	1	1	1	1	1
Inclusion Criteria	1	1	1	1	1	1	1	1
Exclusion Criteria	1	0	1	1	1	1	1	1
Study Period Given	1	1		1	1	1	1	1
Appropriate statistical analysis	1	1	1	1	1	1	1	1
Hard End Points	1	1	1	1	1	1	1	1
Sample size calculation	1	0	0	0	1	0	0	0
Baseline comparable	1	1	1	1	1	1	1	1
Any missing post op data	1	1	1	1	1	1	1	1
Allocation concealment	1	0	0	0	1	1	0	0
Analysis by intention to treat	1	1	1	1	1	1	0	0
Score	15	10	10	11	15	14	10	11
Score Max 15. Poor = -1-5 Fair = 6-10 Good = 11-15								

Table 4.6: Methodological qualities of retrospective studies included in the trial. Adapted from the Scottish Intercollegiate Guidelines Network and Rangel et al.

Quality variables	Spiliotis et al
Inclusion Criteria	1
Exclusion Criteria	0
Demographics comparable	1
Can the number of participating centres be determined	1
Can the number of clinicians who participated be determined	0
Can the reader determine where the authors are on the learning curve for the reported investigative procedure	1
Is the technique adequately described	1
Is there any way that they have tried to standardise the technique	1
Is the age and range given for patients	1
Do authors address whether there is any missing data	1
Were patients in each group treated along similar timelines	1
Dropout rates stated	1
Outcomes clearly defined	1
Blind Comparators	0
Analysis by intention to treat	1
Score	12
Total 15 Less than 6 – Poor quality. 6-10 – Fair Quality. 11 or more – Good quality	

The outcome variables extracted are shown in Table 4.7 and 4.8.

Table 4.7: Outcomes: Somatostatin analogues (octreotide and lanreotide) versus control

Study	Year	Type	N	Closure n	Time to closure (days)	Mortality rate (%)
Gayral et al	2009	Control	53	20	Median 26	0 (0)
		Lanreotide	54	35 (p = 0.006)	Median 17 (assume p= 0.06)	0 (0)
Leondros et al	2004	Control	15	4	Median 18 (SD 5.3)	2 (13.3) p > 0.05
		Octreotide	17	11	Median 16.5 (SD 16.6)	0 (0)
Jamil et al	2004	Control	17	14	Mean 17.7	2 (12) p > 0.05
		Octreotide	16	15	Mean 14 (assume p = 0.06)	3 (19)
Hernandez-Aranda et al	1996	Control	45	30	27	14 (31%)
		Octreotide	40	30	18 (p = 0.002)	10 (25%) p > 0.05
Sancho et al	1995	Control	17	6	Mean 12 (SD 7)	2 (12%)
		Octreotide	14	8	Mean 7 (SD 3) p=0.16	2 (14%)
Scott et al	1993	Control	8	3		
		Octreotide	11	1 (p > 0.05)		

Table 4.8: Outcomes: Somatostatin versus control

Study	Year	Type	N	Closure n	Time to closure (days)	Mortality rate (%)
Leondros et al	2004	Control	15	4	Median 18 (SD 5.3)	2 (13.3) $p > 0.05$
		Somatostatin	19	16 ($p = 0.007$)	Median 10.5 (SD 7.5)	0 (0)
Isenmann et al	1994	Control	20	3 / 16	19	
		Somatostatin	25	14 / 18	13	
Torres et al	1992	Control	20	3 / 16	19	0 (0)
		Somatostatin	20	17 / 20	13 (assume $p = 0.06$)	0 (0)
Spiliotis et al	1990	Control	30	20	27.4	3 (10)
		Somatostatin	18	14 ($p > 0.05$)	18.2 ($p < 0.01$)	1 (5.5)

4.5.1 Somatostatin analogues versus control

4.5.1.1 Number of fistula closed

Six studies (182, 184, 186, 187, 189, 190) contributed to a summative outcome. There was no significant heterogeneity among trials ($Q = 9.38$, $df = 5$ ($p = 0.09$); $I^2 = 47\%$). The number of ECF closed was significantly greater in the somatostatin analogue group ($n = 100/152$) compared to the control group ($n = 77/155$) [fixed effects model: $RR = 1.36$, 95% CI (1.12, 1.63), $z = 3.17$, $p = 0.002$; figure 4.2].

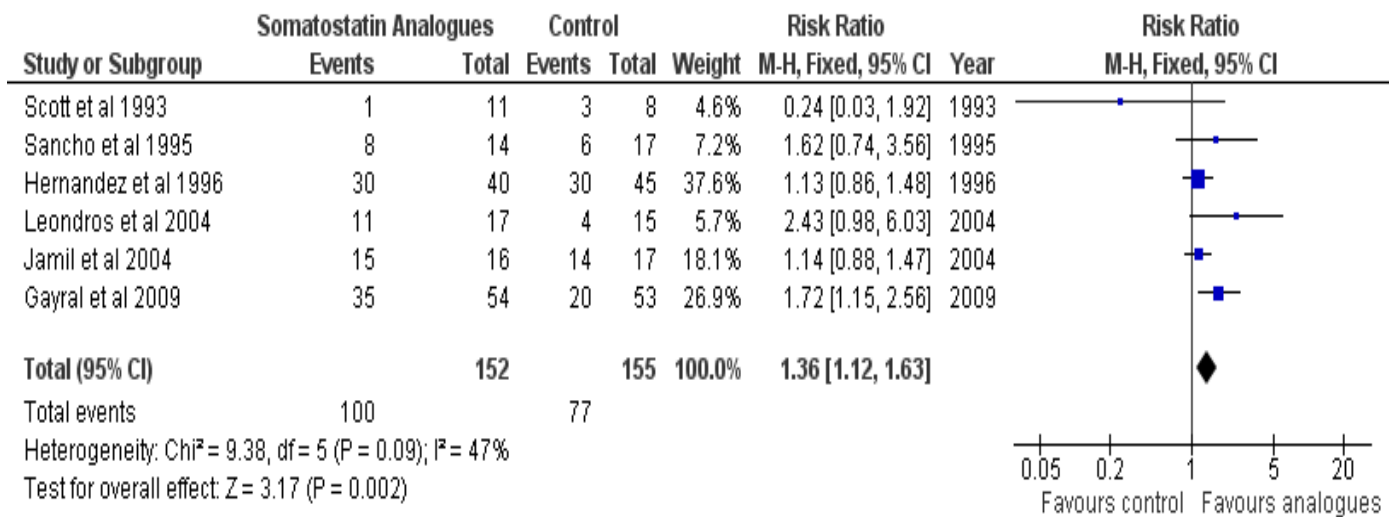


Figure 4.2: Forrest Plot of Somatostatin analogues versus control -Number of fistula closed

4.5.1.2 Time to closure

Five trials (182, 184, 186, 187, 189) reported on time to closure. There was no significant heterogeneity among the trials ($Q = 3.52$, $df = 4$ ($p = 0.47$); $I^2 = 0\%$). ECF were significantly more likely to close faster with somatostatin analogues ($n = 141$) compared with the control ($n = 147$) group [fixed effects model: $SMD = -0.51$, 95% CI (-0.75, -0.28), $z = 4.27$, $p < 0.0001$; figure 4.3]. Of these, only one study (184) showed a significant difference in a faster time to closure of fistula with the use of octreotide ($p = 0.002$).

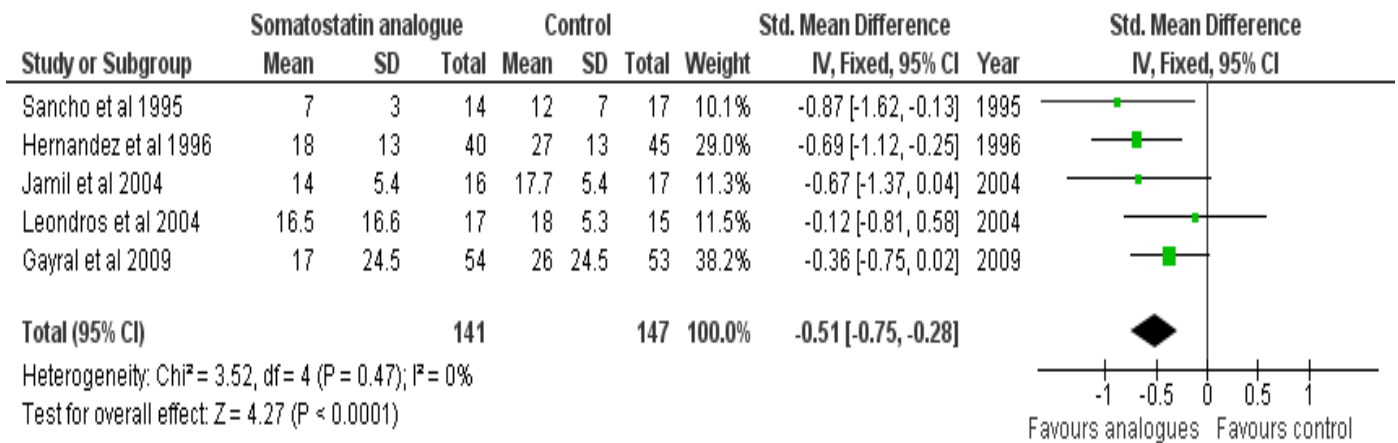


Figure 4.3: Forrest Plot of Somatostatin analogues versus control - Time to closure

4.5.1.3 Mortality

Five studies (182, 184, 186, 187, 189) reported on mortality. There was no significant heterogeneity among the trials ($Q = 1.03$, $df = 4$ ($p = 0.90$); $I^2 = 0\%$). No significant difference was highlighted between analogues ($n = 17/141$) and controls ($n = 21/147$) [fixed effects model: $RR = 0.89$, 95% CI (0.50, 1.56), $z = 0.41$, $p = 0.68$; figure 4.4].

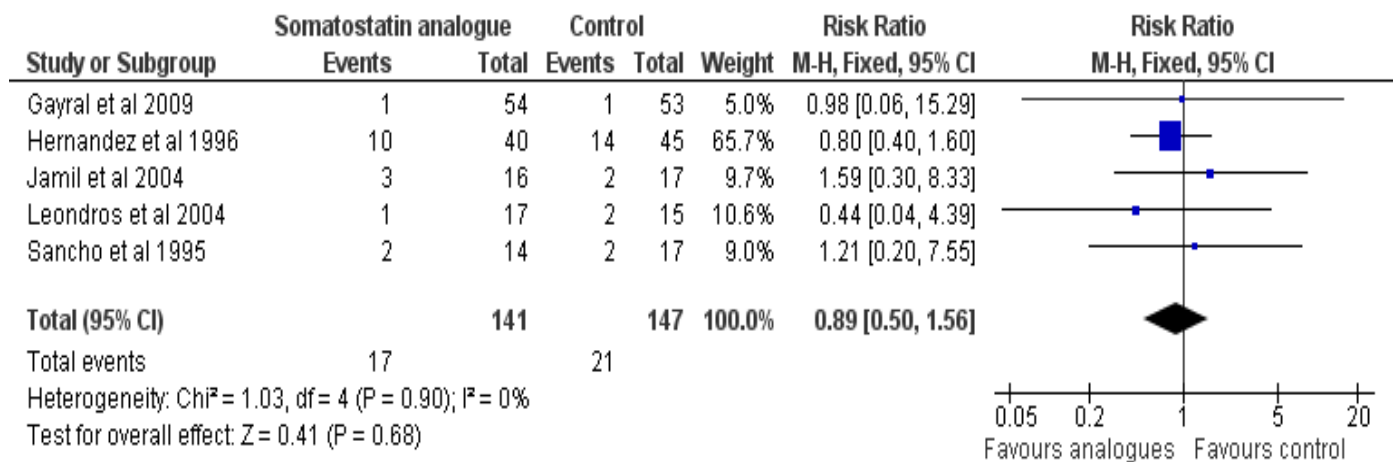


Figure 4.4: Forrest Plot of Somatostatin analogues versus control - Mortality

4.5.2 Somatostatin versus control

4.5.2.1 Number of fistula closed

Four studies (185, 187, 192, 193) contributed to a summative outcome. There was significant heterogeneity among trials ($\text{Tau}^2 = 0.84$; $Q = 19.07$, $df = 3$ ($p = 0.0003$); $I^2 = 84\%$); therefore the fixed effects model was inappropriate. There was significant difference with greater number of ECF closed with the use of somatostatin ($n = 61/82$) as compared to control group ($n = 30/85$) [random effects model: $RR = 2.79$, 95% CI (1.03, 7.56), $z = 2.02$, $p = 0.04$; figure 4.5].

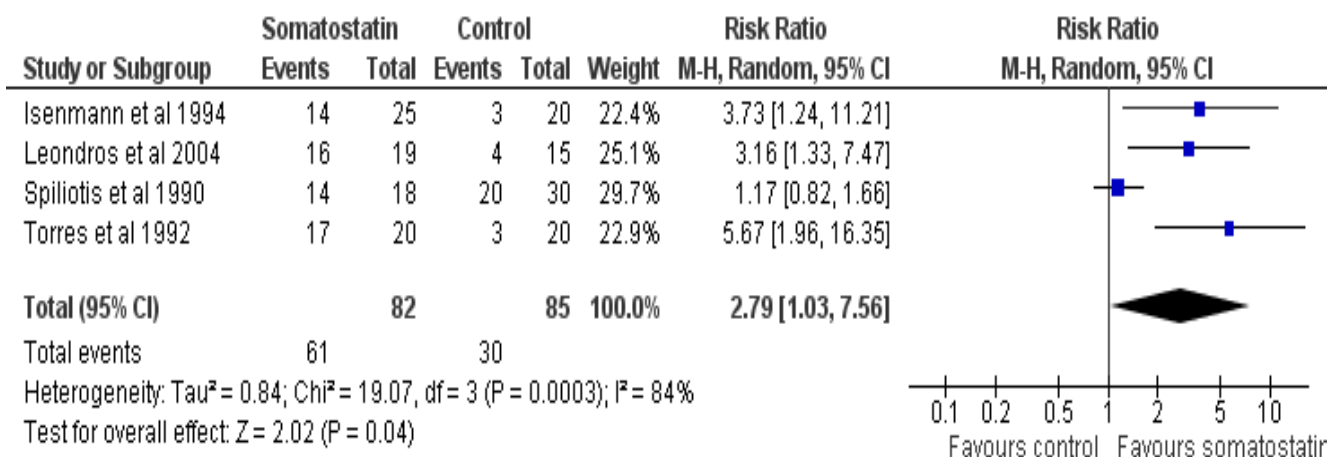


Figure 4.5: Forrest Plot of Somatostatin versus control - Number of fistula closed

4.5.2.2 Time to closure

Four trials (185, 187, 192, 193) reported on time to closure. There was no significant heterogeneity among the trials ($Q = 1.06$, $df = 3$ ($p = 0.79$); $I^2 = 0\%$). There was a significant difference, with ECF more likely to close faster with somatostatin ($n = 82$) than with controls ($n = 85$) [fixed effects model: $SMD = -0.79$, 95% CI (-1.11, -0.47), $z = 4.86$, $p < 0.00001$; figure 4.6].

A significant benefit for the use of somatostatin was seen in one study (192) ($p < 0.01$).

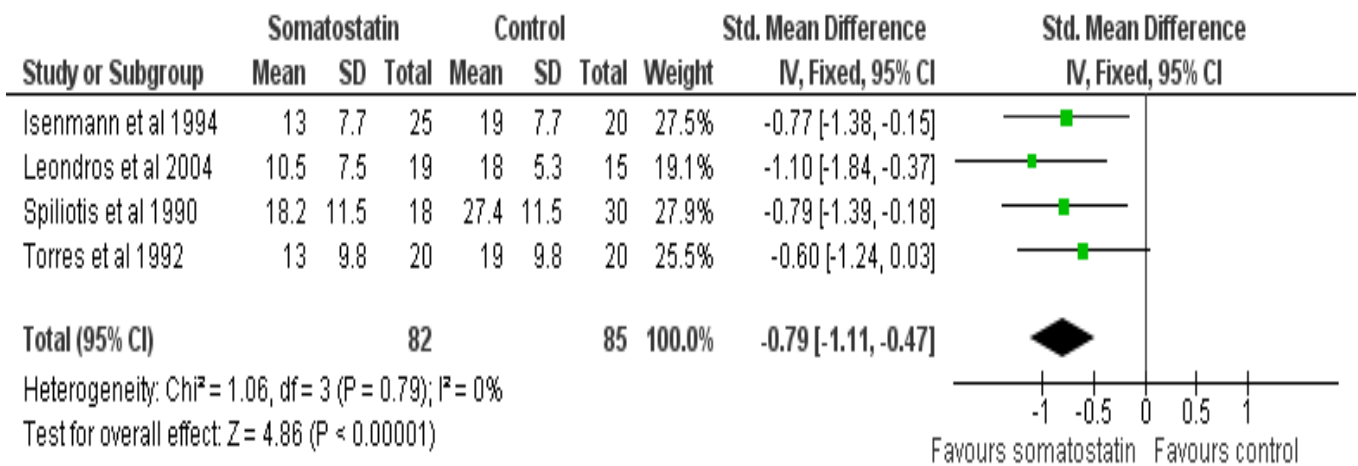


Figure 4.6: Forrest Plot of Somatostatin versus control - Time to closure

4.5.2.3 Mortality

Four studies (185, 187, 192, 193) reported on mortality. There was no significant heterogeneity among the trials ($Q = 0.12$, $df = 3$ ($p = 0.99$); $I^2 = 0\%$). No significant difference was highlighted between somatostatin ($n = 4/82$) and controls ($n = 6/85$) [fixed effects model: $RR = 0.73$, 95% CI (0.21, 2.59), $z = 0.48$, $p = 0.63$; figure 4.7].

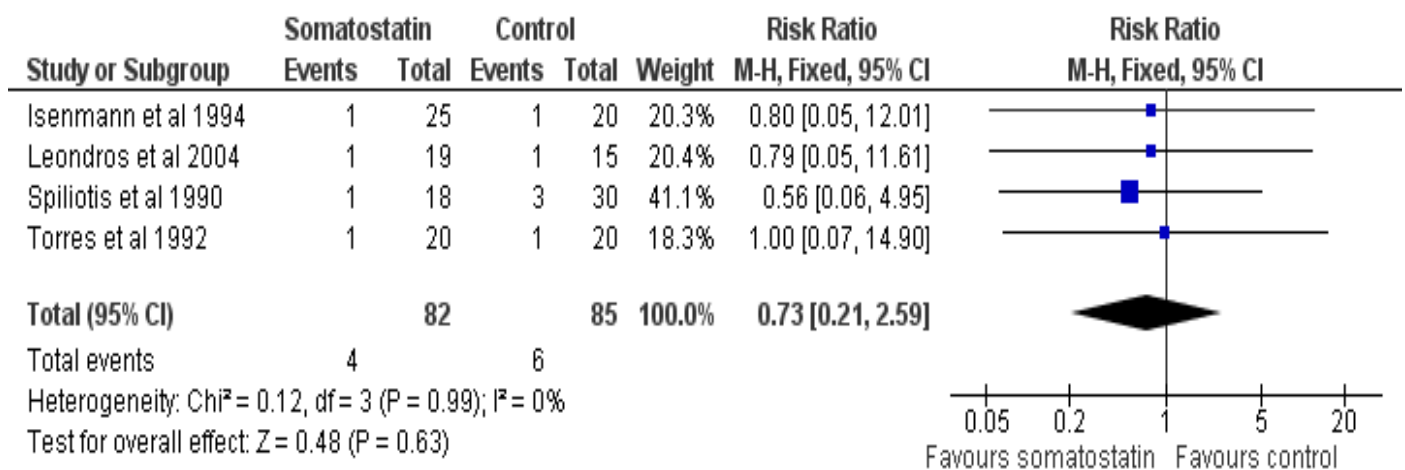


Figure 4.7: Forrest Plot of Somatostatin versus control - Mortality

4.5.3 Fistula Output

Of the nine randomised controlled trials, fistula output reduction was not recorded in three trials (184, 185, 187), Torres et al (201) recorded a significant reduction in number of days ($p < 0.05$ and $p < 0.01$ respectively) in the somatostatin group when compared to the control group for a 50% or 75% reduction in fistula output volume respectively.

Spiliotis et al (201) records that the somatostatin group had a 50% reduction in fistula volume in three days, in comparison to one week in the control group. This is commented to be a significant difference. There was also a significant difference to time to closure ($p < 0.01$), but no significant difference in number of fistula closed ($p > 0.05$).

Jamil et al (186) recorded time for fistulous secretions to cease at 7, 14, 21 and 28 days when comparing octreotide to control group. No significant difference was noted in reduction of secretions and also there was no significant difference in time to fistula closure ($p = 0.06$).

Sancho et al (189) recorded no significant difference in fistula output at 24, 48 or 72 hours between octreotide and control group, and also there was no significant difference in time to fistula closure ($p = 0.16$).

Scott et al (190) recorded fistula losses during 12 days of placebo and octreotide injections. They noted no advantage in reduction in fistula losses or increased rate of spontaneous fistula closure in the patients that received octreotide. There was also no significant difference in number of fistula closed ($p > 0.05$).

Gayral et al (182) showed a significant ($p = 0.005$) mean reduction in fistula output at 72 hours of 45.1% in the lanreotide group versus 8.9% in the control group. Time to closure did not have a significant difference ($p = 0.06$), however there was a significant difference in the number of fistula closed ($p = 0.006$).

4.6 DISCUSSION

Somatostatin receptors (sst) comprise a family of five heptahelical membrane proteins encoded by five related genes that map to separate chromosomes (6). The clinically used somatostatin analogues, octreotide and lanreotide, act primarily by only binding to somatostatin receptor 2 (sst2) (202), and this may contribute to a comparatively less effective outcome.

Octreotide has a much longer half-life (approximately 90 minutes, compared to 1-2 minutes for somatostatin). Since it is absorbed poorly from the gut, it is administered parenterally (subcutaneously, intramuscularly, or intravenously). The pharmacokinetic and pharmacodynamics differences between somatostatin and its analogues require continuous infusion with somatostatin and the parenteral route for analogues.

In this meta-analysis we have evaluated the effect of somatostatin and its analogues as an effective treatment for fistula closure. Somatostatin was shown to be associated with an increased number of fistulas closed and a reduced time to closure when compared with analogues.

All trials comparing somatostatin with control, favoured somatostatin. All but one trial (190) comparing somatostatin analogues with control, favoured somatostatin analogues.

There was adequate data on three outcomes; fistula closure, time to closure and mortality. Data of these are shown in Table 4.7 and 4.8. Meta-analysis was performed on these outcomes.

In comparing somatostatin analogues with control there was a significant difference in only one study (182) for fistula closure. Gayral et al (182) recorded a significant difference ($p = 0.006$) with the use of the long acting analogue, lanreotide in fistula closure versus control.

The meta-analysis reveals a significant difference ($p = 0.002$) in the number of fistula closed in the somatostatin analogue group compared to the control group; figure 4.2.

Scott et al (190) was the only trial where the number of fistula closed was better in the control group rather than the somatostatin analogue group. This is highlighted in the Forest Plot figure 4.2. This anomalous result may be explained by the reduced duration of use of octreotide (12 days) when compared with other studies using somatostatin analogues.

This was the first prospective double blind trial of octreotide therapy in postoperative enterocutaneous fistula, and the authors would have only been aware of the previous mean therapy time to fistula closure rates reported to be 4.5 days (188).

Most of the studies use octreotide three times per day as a subcutaneous injection (184, 186, 187, 189, 190) but a continuous infusion may be more effective as demonstrated in the study using lanreotide (half-life: 2 hours) (182, 203) where a significant increased rate of fistula closure was observed. Further research with continuous infusion of somatostatin analogues may be useful to evaluate this.

There was also one study (187) which had a significant benefit ($p = 0.007$) in the number of fistula closed with the use of somatostatin as opposed to control. The meta-analysis reveals a significant difference with a greater number of fistulas closed with the use of somatostatin as compared to the control group $p = 0.04$; figure 4.5.

Somatostatin analogues are often thought to be effective at a clinical level as fistula output will often decrease as they are introduced. This study has not been able to analyse the effect on fistula output due to the variation in reporting this between the studies. There was wide variability in the methodology to record fistula output. Pooling of data was not possible and hence a summative comparison was excluded from the meta-analysis.

It can be noted from the trials where fistula output was recorded that both trials using somatostatin (192, 193) showed significant benefit in fistula output reduction. However we are unable to elucidate if this has an effect on closure rates.

The three trials using octreotide (186, 189, 190) showed no significant benefit in fistula output reduction as well as closure rates. In contrast the long acting analogue, lanreotide, had both significant benefit in fistula output reduction and number of fistula closed.

Some authors have reported that enteral elemental diets may reduce fistula output as much as parenteral nutrition (1, 204) without the associated complications of central line sepsis, thrombosis or pneumothorax and hence adherence to a short bowel regime is the most effective way of decreasing fistula output. This is also relevant when considering the use of somatostatin as it is believed to induce intestinal atrophy.

Common adverse effects of treatment with somatostatin analogues include nausea, abdominal cramps, loose stools, mild steatorrhea (presumably resulting from transient inhibition of pancreatic exocrine secretion and malabsorption of fat) and flatulence. These symptoms start within hours of the first subcutaneous injection, are dose-dependent, and usually subside spontaneously within the first few weeks of treatment (205).

In particular there is the risk of developing gallstones in patients undergoing therapy with somatostatin analogues. Rates approaching 50 % have been reported when used in treating patients with metastatic gut neuro endocrine tumours (NETs) or malignant islet cell tumours (206).

Data on complications including catheter, abdominal, urinary sepsis, pneumothorax, pneumonia, wound or skin problem were limited.

Torres et al (193) reported a greater complication rate within the somatostatin study group (68.75 %, 11/16 patients) than the control group (35 %, 7/20 patients). This was a significant difference. Isenmann et al (185) and Leondros et al (187) noted no significant difference in complications between the study groups. Jamil et al (186) had a 50 % less complication rate in the somatostatin group when compared to the control group.

There was a wide variability in population characteristics in all the studies analysed.

Torres et al (193) had a significant difference in the mean ages between the comparison groups as well as fistula output prior to the commencement of the trial. Leondros et al (187) showed a significant difference in mean age within the somatostatin group as opposed to the octreotide and control groups.

There was wide variability in the location of fistula as well as criteria used to define low or high output fistula in all studies (Table 4.3 and 4.4). All studies except one (190) stated time to closure.

In five (184, 186, 187, 189, 190) out of the six randomised control trials which compared octreotide to standard medical therapy a dose of 100 micrograms subcutaneous injection three times per day was used, for a range of durations. In comparing somatostatin with control, three of the four studies (185, 187, 192) used a continuous somatostatin intravenous infusion dose of 250 micrograms per hour, for varying durations. Reviewing 'time to closure' for each of these studies, the current literature supports the use of either octreotide 100 micrograms subcutaneous injection three times daily or somatostatin intravenous infusion dose of 250 micrograms per hour for a minimum period of two weeks.

Sancho et al (189) was the only trial to comment on when treatment should be commenced in relation to the duration of an enterocutaneous fistula. Their inclusion criteria included all patients with postoperative enterocutaneous fistula of less than eight days duration with a daily output greater than 50ml.

Our meta-analysis is limited due to several studies having small numbers of patients recruited. There is further limitation due to the wide variability in study designs; which has been reflected in patient characteristics and study protocol. There is also a lack of clear definitions relating to recording of study outcomes: fistula closure, time of fistula closure, fistula output reduction, complication rate and mortality.

There was no significant heterogeneity except for when comparing somatostatin versus control for number of fistulas closed. This may relate to Spiliotis et al (192) having higher number of patients in the control group than any of the other studies.

The risk ratio for somatostatin was higher than the risk ratio for somatostatin analogues. This may suggest that somatostatin could be better than analogues in relation to the number of fistulas closed and time to closure. This finding may be compounded by limitations such as different treatment protocols (Table 4.3 and 4.4), patient selection, population characteristics and methodology used to determine the actual time of closure of fistula for each trial. Further studies are warranted directly comparing both these agents to establish whether there is a significant difference between them.

4.7 CONCLUSION

Our meta-analysis suggests that somatostatin and octreotide increase the likelihood of fistula closure. Both are beneficial in reducing the time to fistula closure. Neither has a significant effect on mortality when compared with standard medical treatment. The best results were following the use of somatostatin or long acting somatostatin analogue, lanreotide.

The risk ratio for somatostatin was higher than the risk ratio for analogues. This may suggest that somatostatin is more effective than analogues in relation to the number of fistulas closed and time to closure. Further studies are required comparing these two agents to corroborate these findings.

Recommendation is for a large randomised control trial with adequate patient numbers or a multi-centre trial with well-defined criteria and agreed parameters

between the participating centres. If a clear option is identified this would benefit an already surgically challenging group of patients associated with a high morbidity and mortality where it is deemed standard medical treatment has failed.

CHAPTER 5

PROSPECTIVE STUDY OF IMMUNOLOGICAL FACTORS IN NON-INFLAMMATORY BOWEL DISEASE ENTEROCUTANEOUS FISTULAS

5.1 ABSTRACT

Aim

Tumour necrosis factor alpha (TNF- α) is a cytokine elevated in inflammatory bowel disease enterocutaneous fistula (IBD ECF). Dendritic cells are antigen presenting cells that orchestrate the immune responses and regulate the production of cytokines by immune cells including T cells. No study to date has assessed the level of TNF- α or the presence of dendritic cells in non-IBD ECF. The aim of this study was to assess the inflammatory activity, with a particular emphasis on TNF- α in non-IBD ECF when compared with control small bowel tissue.

Methods

Tissue biopsies were obtained from ECF at operation from non-IBD patients and from terminal ileum in normal colonoscopy control patients. After overnight culture, accumulation of intracellular TNF- α was measured by flow cytometry in cells treated with monensin to assess the on-going cytokine production. Data were acquired using FACS Canto II. Unpaired Student's t-test was used to compare variables between groups and $p < 0.05$ was regarded as significant.

Results

The on-going production of TNF- α from dendritic cells ($p = 0.0007$), putative monocyte and B cell populations ($p = 0.04$) and CD3+ T cells ($p = 0.04$) was significantly higher in non-IBD ECF tissue than that from control tissue.

Conclusions

This study reveals results which may provide evidence for the potential use of anti-TNF- α agents in the treatment of non-IBD ECF. Recommendations are for a pilot study to assess whether this can be an alternative option to benefit an already surgically challenging group of patients. Positive findings would equate to a major medical advance with a new use for anti-TNF- α agents.

5.2 INTRODUCTION

The local production of tumour necrosis factor alpha (TNF- α) is thought to have a key role in the initiation and propagation of Crohn's disease. Production of TNF- α in the intestinal mucosa, serum and stool is increased in patients with Crohn's disease (207).

There are numerous biological activities that are attributed to TNF-activation. These activities include induction of the pro inflammatory cytokines such as IL1, IL6 and enhancement of leukocyte movement or migration from the blood vessels into the tissues by increasing the permeability of the endothelial layer of blood vessels.

In animal models, antibodies to TNF- α prevent or reduce inflammation (208-212) , suggesting that therapy with such antibodies may be useful for chronic inflammatory disorders (213) .

Infliximab is a genetically constructed IgG1 murine – human chimeric monoclonal anti TNF antibody that inhibits a broad range of biological activities of TNF- α , potentially by blocking the interaction of TNF- α with its receptors, or causing lysis of cells that produce TNF- α (207).

Infliximab has been approved for treating ankylosing spondylitis, Crohn's disease, fistulising Crohn's disease, psoriatic arthritis, psoriasis, rheumatoid arthritis, and Ulcerative colitis. It has also been used in the treatment of Behçet's disease (214) and sciatica due to slipped discs (215).

Infliximab was first used for closure of fistula in Crohn's disease in 1999. In a phase II clinical trial with 94 patients who had draining abdominal or perianal fistula, Infliximab was effective in closing fistula in 56-68% of patients (207) .

A Phase III clinical trial (ACCENT 2) showed that infliximab was additionally beneficial in maintaining closure of fistula, with almost two-thirds of all patients treated with the three initial doses of infliximab having a fistula response after 14 weeks, and 36% of patients maintaining closure of fistula after a year, compared with 19% who received placebo therapy (216).

It is likely that persistent inflammation prevents adequate healing of these fistulas and that TNF- α and the subsequent stimulation of the pro-inflammatory cascade promotes their existence. This may be along with the inhibition of growth factors which studies have demonstrated a beneficial effect of many e.g. platelet-derived growth factors, fibroblast growth factors, and granulocyte-macrophage colony stimulating factor on the healing process.

Infliximab is currently not indicated for ECF caused by conditions other than IBD. A small case series of three patients has reported healing of persistent ECF not associated with IBD following a single infusion of infliximab (175) . There are no other studies to support this series in the use of infliximab for non-IBD ECF. The inflammatory activity of non-IBD ECF has not been assessed in the past and the level of TNF- α activity in these cases is not known.

There is also no study of association of TNF- α with dendritic cells (DC) in non-IBD ECF. DC are potent antigen presenting cells that bridge the adaptive and innate immune systems to induce and regulate immune responses. They present as ‘immature’ cells specialised for antigen uptake in most tissues of the body, particularly at sites of interface with the external environment such as the skin and mucosa and ECF. A property that distinguishes DC from other types of antigen

presenting cell (macrophages and B-cells) is their potency in activating naïve T cells, which interaction generally occurs in secondary lymphoid tissue.

DC are predominantly thought to originate from bone marrow derived precursor cells which are seeded into the circulation, although local renewal of DC subsets in the peripheral tissues has also been reported (217). DC precursors become tissue resident immature cells at anatomical sites where they are likely to encounter antigenic stimuli such as the skin, GI tract or respiratory tract. At these sites, DC display efficient endocytic activity, allowing them to sample environmental antigens for intracellular processing and subsequent presentation (218).

DC sample an Ag by capturing it via various receptors such as Toll like or dendrites. They internalise the antigen and then fragments are uploaded on MHC Class II. MHC Class I proteins are associated with internal antigens and disordered cells whereas MHC Class II protein molecules are associated with external antigens.

DC go via lymphatic's to the draining lymph nodes and stimulate naïve T cells via the MHC Class II as well as imprinting homing markers such as b7 (gut homing marker) or skin homing marker cutaneous leukocyte antigen (CLA).

This is described as providing a postcode, and hence directing the T cells where to go i.e. the gut or the skin. The T cells will then go to where the antigen was encountered. When the T cells arrive at the skin or gut they produce and release the cytokines such as $\text{TNF-}\alpha$

DC are the major educators of the immune system. They teach immune cells what to do. They are essentially like a safety device because if we can inhibit the initial step of $\text{TNF-}\alpha$ on DC, then this will prevent the next step of $\text{TNF-}\alpha$ from T cells occurring.

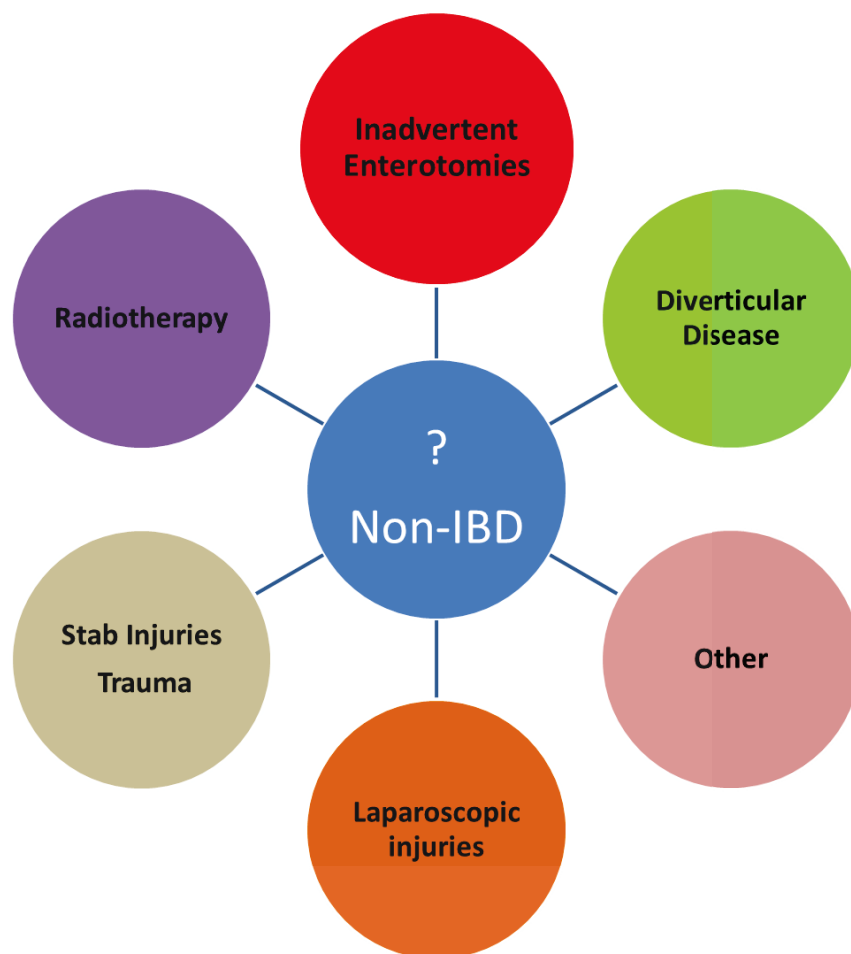
Once DC get to the lymph nodes and have stimulated the naïve T cells, the DC then die. They are cleared by macrophages. The DC on skin are from the blood, and have come from the bone marrow. The T cells go from the lymph node via blood to the e.g. skin.

The DC already on the skin simply come from the bone marrow and then blood. DC do not recirculate, although T cells do. The DC on the skin will have CLA and b7. Areas with greater inflammation will have more DC present.

DC which reside in the mucosa and cutaneous epithelium are ideally situated to perform their sentinel duties since they are both closely associated with sites of likely pathogen entry into the body and are capable of detecting antigens of both foreign and self-origin (219). However, naïve T-cells are comparatively rare in the peripheral tissues and DC are unlikely to encounter antigen specific clones suitable for expansion at these sites. DC therefore migrate to the draining lymph nodes via lymphatic vessels and interact with the re-circulating pool of naïve T-cells before exerting their effect (220). Once situated in the tissue draining lymph nodes, DC present antigen peptides in the context of MHC and stimulate antigen specific T-cell clones to proliferate and differentiate. The newly activated T-cell clones then re-enter the circulation and home back to the target organ along gradients of specific chemokine's that emanate from the destination tissues. After infiltrating the peripheral tissues, the antigen specific T-helper cells then perform a variety of different functions that support the activities of effector leukocytes in eliciting the adaptive immune response.

Upon activation DC produce pro-inflammatory cytokines such as IL12, IL6 and TNF- α . They also up-regulate and imprint homing markers such as cutaneous leukocyte antigen (CLA), also known as a skin homing marker and β 7, a gut homing marker, on T cells (221).

Figure 5.1: Causes of non-IBD ECF



5.3 HYPOTHESIS

Immunological factors and their interaction are in operation within non-IBD ECF. The level of TNF- α activity is elevated in non-IBD ECF and IBD ECF when compared with control small bowel.

5.4 AIM

The aim of this study was to assess the inflammatory activity, with a particular emphasis on TNF- α of non-IBD ECF when compared with IBD ECF and control small bowel.

5.5 METHODS

Immunological factors involved in the pathogenesis in non-IBD ECF were assessed prospectively. Immunological factors in three groups of patients were compared;

- Group (i) non-IBD ECF
- Group (ii) IBD ECF
- Group (iii) normal small bowel mucosa from terminal ileum (control group)

5.5.1 Inclusion criteria

For Group (i) non-IBD ECF:

- Consent to the study
- Male or female
- 18+ years
- Single or multiple ECF for at least three months duration as a result of non-IBD, and not responding to standard treatments

- ECF confirmed to be due to non-IBD by radiography, endoscopy or pathological examination

For Group (ii) IBD ECF:

- Consent to the study
- Male or female
- 18+ years
- Single or multiple ECF for at least three months duration as a result of IBD and not responding to standard treatments
- ECF confirmed to be due to IBD by radiography, endoscopy or pathology
- Patients with stable IBD

For Group (iii) normal bowel mucosa (control group):

- Consent to the study
- Male or female
- 18+ years
- Healthy volunteers with normal endoscopic examination of the bowel
- No prior history of IBD or ECF

5.5.2 Exclusion criteria

For Group (i) and Group (iii):

- Current sepsis or abscess
- Previous treatment with infliximab, investigational agents or any medication which reduces the concentration of TNF- α
- History of Crohn's disease or Ulcerative Colitis
- Pregnancy

For Group (ii):

- Current sepsis or abscess
- Previous treatment with infliximab, investigational agents or any medication which reduces the concentration of TNF- α
- Patients taking steroids for IBD within one month prior to start of the study
- Patients with acute flare up of IBD
- Pregnancy

5.5.3 Design

All eligible patients were identified at referral, seen in the outpatient clinic or ward and offered participation in this study. The study was explained, discussed and patients encouraged to ask questions for discussion. A patient information sheet was provided. After completing Good Clinical Practice (GCP) training, informed consent was obtained for the study. Patients, who declined to enter the study, were treated with the normal standard of clinical care provided.

For patients who entered the study, the number and location of the ECF were noted. Drawings as well as photographs were used to document the sites of disease.

5.5.4 Sample Collection

5.5.4.1 Tissue

Most patients with ECF were recruited prior to undergoing surgery on the ward or in outpatient's clinic. A few patients with ECF did not undergo surgery but were recruited on the intestinal failure ward. Tissue was obtained in these patients using tissue biopsy with local anaesthetic. Healthy control patients were recruited prior to undergoing screening colonoscopy.

5.5.4.1.1 Surgery - Laparotomy with enterocutaneous fistula resection

At the time of operation, biopsies were taken from the tissue within the ECF tracts of non-IBD and IBD patients: Groups (i) and (ii).

Patients undergoing laparotomy with enterocutaneous fistula resection were placed in the supine position. Following a general anaesthetic and antibacterial skin preparation, surgery was commenced. Once the ECF specimen had been resected, this was placed in a sterile surgical bowl. Forceps and scalpel were used to retrieve the ECF tract biopsies for immunology and placed in tissue medium. Up to ten two mm samples were taken.

Biopsies of the ECF tract were taken to assess for baseline pro and anti-inflammatory cytokine markers including TNF- α level.

5.5.4.1.2 Local anaesthetic ECF tissue biopsy

A few patients with non - IBD ECF not undergoing surgery were recruited from the intestinal failure ward. After cleaning the abdominal wall with antibacterial skin preparation, local anaesthetic was applied injected into the tissue around the ECF tract. Using a sterile technique, biopsy forceps were used to take tissue samples under direct vision from within the fistula tract. Up to ten two mm samples were taken.

5.5.4.1.2.3 Colonoscopy

Healthy patients undergoing screening colonoscopy with normal mucosa endoscopically and histologically were used as controls. A maximum of ten biopsies were taken from the terminal ileum in addition to those required for clinical evaluation: Group (iii). The terminal ileum was selected as the choice of biopsy, as in our experience the majority of ECF occurred with small bowel ileum tissue.

5.5.4.2 Peripheral blood sample collection

Peripheral blood (10-20 ml) was obtained from all three groups of patients.

5.5.5 Primary outcome measure

Cytokine profiles in the mucosa from all three groups was assessed using multiplex bead analysis. Simultaneously analysis of the following cytokines, chemokine's and growth factors was performed: IL2, IL4, IL6, IL10, TNF- α , IFN γ and IL17a.

5.5.6 Secondary outcome measures

Flow cytometer technique was used to identify whether the homing markers CLA and $\beta 7$ were expressed on blood T cells and DC in the three groups. In addition, the on-

going production of the pro-inflammatory cytokines TNF- α , IL12, IL17A and IFN γ in T cells and DC from blood and tissue in these groups was determined.

5.5.7 Sample size justification

The study was powered to determine a 60% increase in TNF- α expression between non-IBD ECF and the control group. With a 5% significance level and 90% power, 14 patients are required in each group. Statistical analyses was carried out using Sigma Stat software (SPSS Inc., Chicago, IL, USA). The Student T-test for non-paired data was used. Error bars used represent standard error of the mean (SEM). Values of $p < 0.05$ were considered significant.

5.5.8 Safety and Monitoring

There was potential for increased pain and discomfort from the blood tests and biopsies of the fistula tract. In both cases the patient was be informed during the initial explanation, consent stage, and prior to the procedure, that they may feel a very 'small pinch or prick' sensation as the blood test or biopsy was being performed. There was minimal light bleeding following either test, and this was controlled as is in normal standard clinical practice with the application of gentle pressure until any bleeding subsided. No untoward or adverse event took place.

There was a small risk of an infection occurring at the site of blood taking and this was minimised by the use of standard hospital guidelines in relation to use of alcohol hand gel and sterile technique as is part of normal standard clinical care. No untoward or adverse event took place.

5.6 Ethics approval

The research has received ethics approval from the National Research Ethics Service, North London REC 1. REC reference: 08/H0717/24. Protocol number cro1043.

Date of approval: 26th July 2010.

5.7 Funding

Funding was secured from the Bowel Disease Research Foundation (BDRF). BDRF is the only UK charity which funds research into all bowel diseases. An independent charity, BDRF functions as the research arm of the Association of Coloproctology of Great Britain and Ireland (ACPGBI).

5.8 ACPGBI / BDRF website

http://www.acpgbi.org.uk/BDRF/projects_funded/2011/rahbour

5.9 IMMUNOLOGY METHODS

Patients

Patients with non-IBD ECF and IBD ECF were recruited as described above and underwent a planned operation, laparotomy with resection of ECF, or had tissue obtained using biopsy forceps under local anaesthetic. Control patients in the experiments had no history or evidence of malignancy or inflammatory bowel disease on colonoscopy and had been referred with symptoms of per rectal bleeding or a change in bowel habit, or a screening follow up.

5.9.1 FACS sample processing

5.9.1.1 Tissue collection and transport

Biopsies were obtained from the surface of the enterocutaneous fistula tract at operation from non-IBD ECF patients, IBD ECF patients and from the terminal ileum in normal screening colonoscopy control patients. Tissues were placed in complete medium (appendix), placed on ice and transported immediately for processing.

5.9.1.2 ‘Walk out’

Samples were removed from the transport medium using sterile forceps and processed within a Class-I safety cabinet. The samples were washed in complete medium to remove blood. One or two of these pieces were then placed in a test tube with 0.5ml of complete medium and incubated overnight at 37°C with 5% CO₂ for subsequent freezing and multiplex analysis.

The other samples were weighed and then separated out into two tubes with 1ml of complete medium in each and also incubated overnight at 37°C with 5% CO₂. These samples were used for intra cellular staining (ICS) and then FACS analysis.

5.9.1.3 Intra cellular staining (ICS)

After 24 hours incubation, the two test tube samples were removed. The first tube was labelled ‘Control’ and to this an additional 100µl of complete medium was added. To the second test tube 100µl of Monensin was added. Both test tubes were then returned to the incubator for an additional 4 hours.

The Monensin solution had been prepared the day before. Vials containing 10µl of Monensin were stored at -80 °C. One vial was taken out of the freezer and 990µl of RPMI added. This was then stored in a 4°C refrigerator, ready to be used the following day.

Monensin blocks the surface of the Golgi apparatus. During the 4 hour incubation, any cytokines including TNF alpha which were produced from the Golgi apparatus could not escape and hence could not enter into the cytoplasm of the cell. This technique is useful for assessing the intracellular on-going production of cytokines.

Following 4 hours, both tubes were removed from the incubator. The supernatant was removed from each tube and placed into a 50ml test tube labelled 'Control' or 'Monensin' respectively. A cell strainer was used to drain all excess supernatant from the tissue. The tissue was then discarded.

The 50 ml tubes were then centrifuged at 250g for 5 minutes and the supernatant was discarded. The tubes were then washed with 2ml of FACS buffer and the cells were re-suspended by flicking the tube to obtain as many cells as possible. The two test tubes were then centrifuged at 200g for 5 minutes and the fluid was discarded. The cells were again re-suspended. FACS buffer (200 µl) was then added to each of the 50 ml tubes. From the 'Control' tube 100µl was placed each into FACS tubes labelled 1-Control and 3-Control. Similarly 100µl was removed from the 'Monensin' 50ml test tube and placed into FACS tubes labelled 2-Monensin and 4-Monensin.

Surface marker antibodies (highlighted red in Table 5.1) were added as shown in the Table 5.1, and left for 30 minutes. These surface marker antibodies were: Lineage

cocktail (CD3, CD14, CD16, CD19 and CD34), CD3 and HLA-DR. DC were identified as Lin negative and HLA-DR positive as explained below.

Table 5.1: ICS labelling grid with antibodies and isotype controls for surface markers and cytokines

Tube		FIT C	PE	PC5	APC
1 - Control	2 - Monensin	TNF- α 10 μ l	IL12 5 μ l	Lineage Cocktail 10 μ l	HLA-DR 5 μ l
3 - Control	4 - Monensin	TNF- α 10 μ l	IL17A 5 μ l	CD3 5 μ l	Interferon- γ 5 μ l

Lineage cocktail (Lin) = CD3, CD14, CD16, CD19 and CD34

After 30 minutes the cells were centrifuged in 2ml of FACS buffer (200g, 5 minutes).

The fluid was discarded and the cells re-suspended.

Cells were fixed in 1% paraformaldehyde for 15 minutes using 100 μ l of Leucoperm Reagent A (Serotec, UK). The cells were then centrifuged in 2ml of FACS buffer (200g, 5 minutes). The supernatant was then discarded and the cells re-suspended.

The cells were made permeable using 50 μ l Leucoperm Reagent B (Serotec, UK). This was added along with cytokines antibodies as in Table 5.1 (shown in black). These cytokines antibodies were: TNF- α , IL12, IL17A and Interferon- γ .

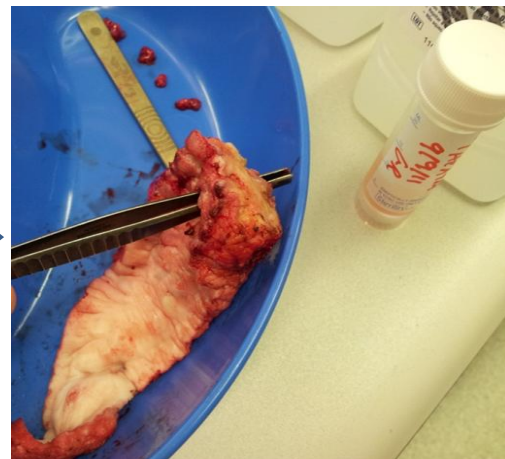
This procedure allows access of the antibodies for labelling cytokines intracellularly. After 15 minutes the cells were centrifuged in 2ml of FACS buffer (200g, 5 minutes). The fluid was discarded and the cells resuspended in 300 μ l of 1% paraformaldehyde. The samples were stored at 4°C in the refrigerator until acquisition on the flow cytometer within 48 hours.

As described above, Monensin has the ability to trap the cytokine within the Golgi apparatus. We add a fixative (Reagent A) which makes the cells rigid and hence not 'collapse.' Following this, Reagent B is added which makes the cells more permeable. Reagent B acts as a detergent soap. If only Reagent B was added the cell would simply 'collapse.' An analogy is to think of a cell as a balloon. If we inflate a balloon and then make holes in it, it would just collapse. Therefore we need to make the surface 'rigid' by 'freezing' it and then make the holes. We then wash the cells after four hours. This washes away the non-specific binding of the Ab's to TNF- α . We can then subtract the non-specific binding of the Ab to the TNF- α from the specific binding.

Fig 5.2: Intra cellular staining of cells from tissue



Patient being prepared for Laparotomy with ECF resection



Resected ECF specimen with forceps passing through a tract. Biopsies placed in complete tissue medium



24 hours incubation

MULTIPLEX ANALYSIS

Samples separated into two tubes

***Monensin* added to one, and complete medium to the other**

4 hours further incubation



INTRA CELLULAR STAINING (ICS)

5.9.1.4 Blood collection and transport

Five to ten millilitres of human whole blood was obtained by venepuncture from non-IBD ECF patients, IBD ECF patients and from normal healthy control volunteers. Blood was placed into sodium-heparin Vacutainers (Beckton-Dickinson), placed on ice and transported immediately for processing in the laboratory.

5.9.1.5 Blood processing

The whole blood was then diluted with an equal volume (1:1 ratio) of enrichment agent RPMI-1640 Dutch modification. The solution was gently mixed and slowly added to an equal volume of Ficoll-paque density gradient. This was centrifuged at 250g for 30 minutes at room temperature. Brakes were positioned to off during this centrifugation to prevent the sudden stop after 30 minutes, and rather to allow a gradual slowing down.

Peripheral blood mononuclear cells (PBMC's) were collected from the interface that resulted. PMBC's include lymphocytes (T cells, B cells, natural killer cells), monocytes, macrophages and dendritic cells. An equal volume (1:1 ratio) of complete medium was added to the PBMC's (Figure 5.3).

The tube was then centrifuged at 250g for 5 minutes and the supernatant was discarded. The cells were re-suspended and complete medium was added to the cells to make a 5 ml solution.

Trypan blue (50µl of 0.4%) was added to a single well of a 96 well plate with 150µl of complete medium and 50µl of the cells solution was added to this well and mixed. A sample from this well was placed on a slide and microscopy used to count the

number of dye excluding cells present, followed by calculation of the number of cells present in the 5ml solution of cells.

The cells were then divided for use in cell surface marking, intra cellular staining and multiplex analysis as explained below.

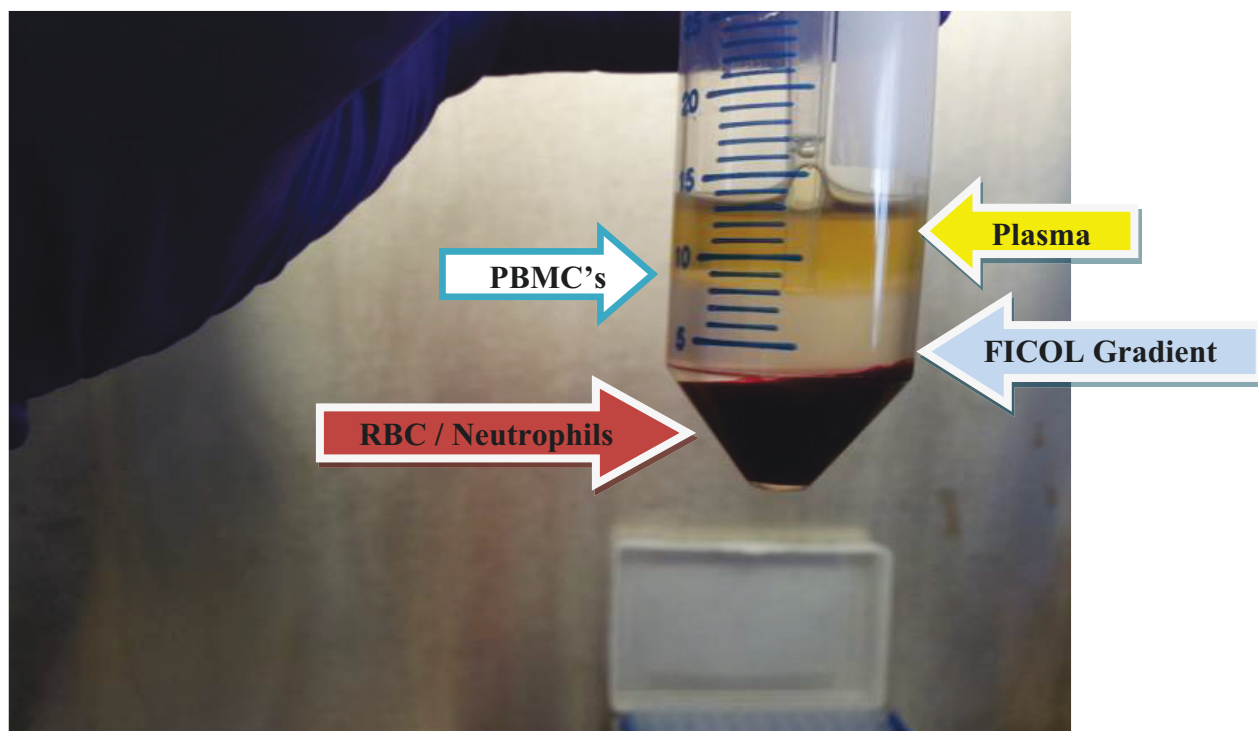


Fig 5.3: Peripheral blood mononuclear cells (PBMC's) being collected

5.9.1.6 Blood cell surface markers

FACS buffer (8mls) was added to PBMC's. They were then centrifuged at 200g for 5 minutes and the supernatant was discarded and the cells were re-suspended

1600µl of FACS buffer was then added. 400µl was then placed into each of four FACS tubes. Surface marker antibodies and isotype controls were added as shown in Table 5.2, and left for 20 minutes on ice.

After 20 minutes the cells were centrifuged in 2ml of FACS buffer (200g, 5 minutes). The fluid was discarded and the cells were re-suspended in 300µl of 1% paraformaldehyde. The samples were stored at 4°C in the refrigerator until acquisition on the flow cytometer within 48 hours.

Table 5.2: Cell surface marking labelling grid and isotype controls

Tube	FIT C	PE	PC5	APC
1	CLA 10µl	β7 5 µl	Lineage cocktail 10 µl	HLA-DR 5 µl
2	r1γm 2 µl	ry2A 2 µl	Lineage cocktail 10 µl	HLA-DR 5 µl
3	CLA 10 µl	β7 5 µl	CD3 5 µl	-
4	r1γm 2 µl	ry2A 2 µl	γ1 5 µl	-

See Appendix V for definitions of antibodies

5.9.1.7 Blood Intra cellular staining

Cells (500µl) were placed into each of four FACS tubes. 500µl of complete medium was then added into FACS tubes labelled 1-Control and 3-Control. Monensin (100µl) was added into FACS tubes labelled 2-Monensin and 4-Monensin. Into both of these FACS tubes, 400µl of complete medium was added.

All four FACS tubes were then placed in the incubator for 4 hours.

Following 4 hours, all four FACS tubes were removed from the incubator and 2ml of FACS buffer added to each.

The tubes were then centrifuged at 200g for 5 minutes and the fluid was discarded. The cells were re-suspended by flicking the tube to obtain as many cells as possible. FACS buffer (100 µl) was then added to each of the four FACS tubes.

Surface marker antibodies (highlighted red in Table 5.3) were added as shown in the Table 5.3, and left for 15 minutes. These surface marker antibodies were: Lineage cocktail (CD3, CD14, CD16, CD19 and CD34), CD3 and HLA-DR.

Table 5.3: ICS labelling grid with antibodies and isotype controls for surface markers and cytokines

Tube		FIT C	PE	PC5	APC
1 - Control	2 - Monensin	TNF- α 10 µl	IL12 5 µl	Lineage Cocktail 10 µl	HLA-DR 5 µl
3 - Control	4 - Monensin	TNF- α 10 µl	IL17A 5 µl	CD3 5 µl	Interferon- γ 5 µl

Lineage cocktail (Lin) = CD3, CD14, CD16, CD19 and CD34

After 15 minutes the cells were again centrifuged in 2ml of FACS buffer (200g, 5 minutes). The fluid was discarded and the cells re-suspended by flicking the tube to obtain as many cells as possible.

Cells were fixed in 1% paraformaldehyde for 15 minutes using 100µl Leucoperm Reagent A (Serotec, UK). The cells were then centrifuged in 2ml of FACS buffer (200g, 5 minutes). The supernatant was then discarded and the cells re-suspended by flicking the tube to obtain as many cells as possible.

The cells were made permeable using 50µl Leucoperm Reagent B (Serotec, UK). This was added along with cytokines antibodies as in the above table (shown in black). These cytokines antibodies were: TNF- α , IL12, IL17A and Interferon- γ .

After 15 minutes the cells were centrifuged in 2ml of FACS buffer (200g, 5 minutes). The fluid was discarded and the cells agitated by flicking the bottom of the test tubes to obtain as many cells as possible. The cells were suspended in 300µl of 1% paraformaldehyde. The samples were stored at 4°C in the refrigerator until acquisition on the flow cytometer within 48 hours.

5.9.1.8 Beads for compensation in flow cytometry

Two drops each of positive and negative antibody compensation beads were added to each of four FACS tubes. Each bead has a set number of arbitrary binding sites which allows counting of how much antibody binds, and how much was positive or negative. Fluorochrome (10µl) antibodies FITC, PE, PC5 and APC were added respectively to FACS tubes labelled 1, 2, 3 and 4. Each FACS tube was then vortexed for a few seconds to mix the solution in each tube, and then left for 20-30 minutes.

Following this, 500µl of PBS was added to each of the four FACS tubes. 20µl of counting beads were added to each of the experiment tubes.

5.9.1.9 Calibration / performance check

PBS (350µl) was added to a single FACS tube. 2 drops of cytometer setup and tracking beads (CST beads) were added. Using the CST beads a performance check was then performed on the FACS machine. CST beads are used for setting up the machine to the same settings every day.

The purpose of calibration is because we want to have the same settings always in every experiment. We want to have the same settings mechanically and for the lasers, and have standardised results. Hence any difference found will be due to biological findings or reagent differences.

5.9.1.10 Flow cytometry

Multi-colour flow cytometry allows characterisation of individual cells in suspension by measuring size and granularity as well as identifying the wavelength of light given off by a fluorophore (FitC, PE, PC5 or APC) attached to cells as they pass through one or more focused laser beams.

Twenty microliters of flow-count fluorospheres (Beckham Coulter, High Wycombe, UK) at a known concentration was added to each tube to enable absolute cell number assessment. Samples were acquired for approximately 5 minutes at high flow rate until all cells had been analysed. Compensation samples were acquired until between 20000 and 50000 events had occurred. All data were then saved as list-mode data files, stored and transferred to a computer for subsequent analysis.

5.9.1.11 FACS analysis

Data were acquired using FACS Canto II (Becton Dickinson, England). Further compensation and analysis of list-mode data were carried out subsequently using Win List software (Verity Software House, Maine). To enable compensation, a region was made around lymphocytes, identified by their size and granularity on the side scatter (SSC) versus forward scatter (FSC) plot and, in each channel, the CD3 for PC5 marker was evaluated and compensation applied with the compensation tool on Win List. Live cells (again, based on size and granularity) were analysed on a plot of HLA-DR +ve versus lineage cocktail -ve staining which identifies DC (Figure 5.4 and 5.5). Lineage cocktail comprised specific monoclonal antibody labels for T cells (CD3), monocytes (CD14), macrophages (CD16), B cells (CD19), and stem cells (CD34) (Table 5.1). T cells, macrophages, B cells and others were also identified by gating on viable cells and then determining the proportion of cells within this region positive for the flourophore corresponding to the cell marker in that tube. Both Region Gating and Enhanced Normalized Subtraction (ENS) were used to analyse the flow cytometry data (Figure 5.6). Data was then plotted on multi graphs and saved.

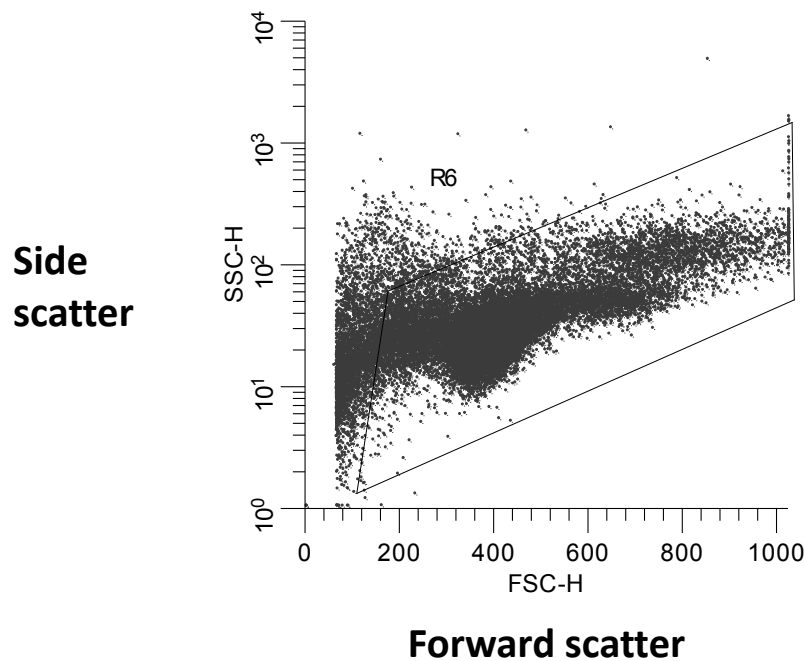


Fig 5.4: Identification of cells by their size and granularity – Side scatter (SSC) versus forward scatter (FSC): Win List software (Verity Software House, Maine) was used to identify cells by their size and granularity on the side scatter (SSC) versus forward scatter (FSC) plot.

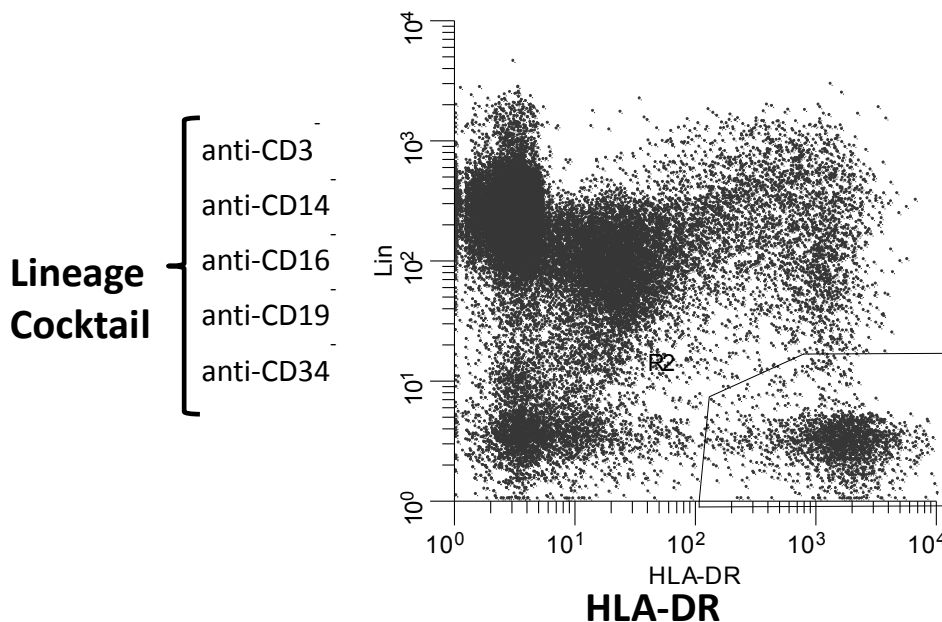


Fig 5.5: Region Gating: Identification of DC. Cells staining HLA-DR +ve and lineage cocktail -ve identify DC: Live cells were analysed on a plot of HLA-DR +ve versus lineage cocktail -ve staining which identifies DC. Lineage cocktail comprised specific monoclonal antibody labels for T cells (CD3), monocytes (CD14), macrophages (CD16), B cells (CD19), and stem cells (CD34).

Region gating is performed by drawing a region that excludes isotype matched control antibody non-specific staining. This region can then be applied to the labelling histogram. All events that fall within this region exceed the isotype control staining intensity and can be regarded as positively labelled cells. The proportion of positive cells as a percentage of the total population is then calculated. Region gating reduces the impact of differences in fluochrome, antibody binding and variation between batches of antibody.

In immunohistochemistry by looking at the cells we want to see if an Ab has specific binding to the markers on the cells. For example we may have ten cells staining for a particular marker. We then add an isotype control (which is an immunoglobulin) and it may for example bind to three random cells. Therefore we can say that this has non-specific binding. We can subtract: $10 - 3 = 7$ cells. Therefore 70% of the cells have specific binding. The same principle is used for the FACS.

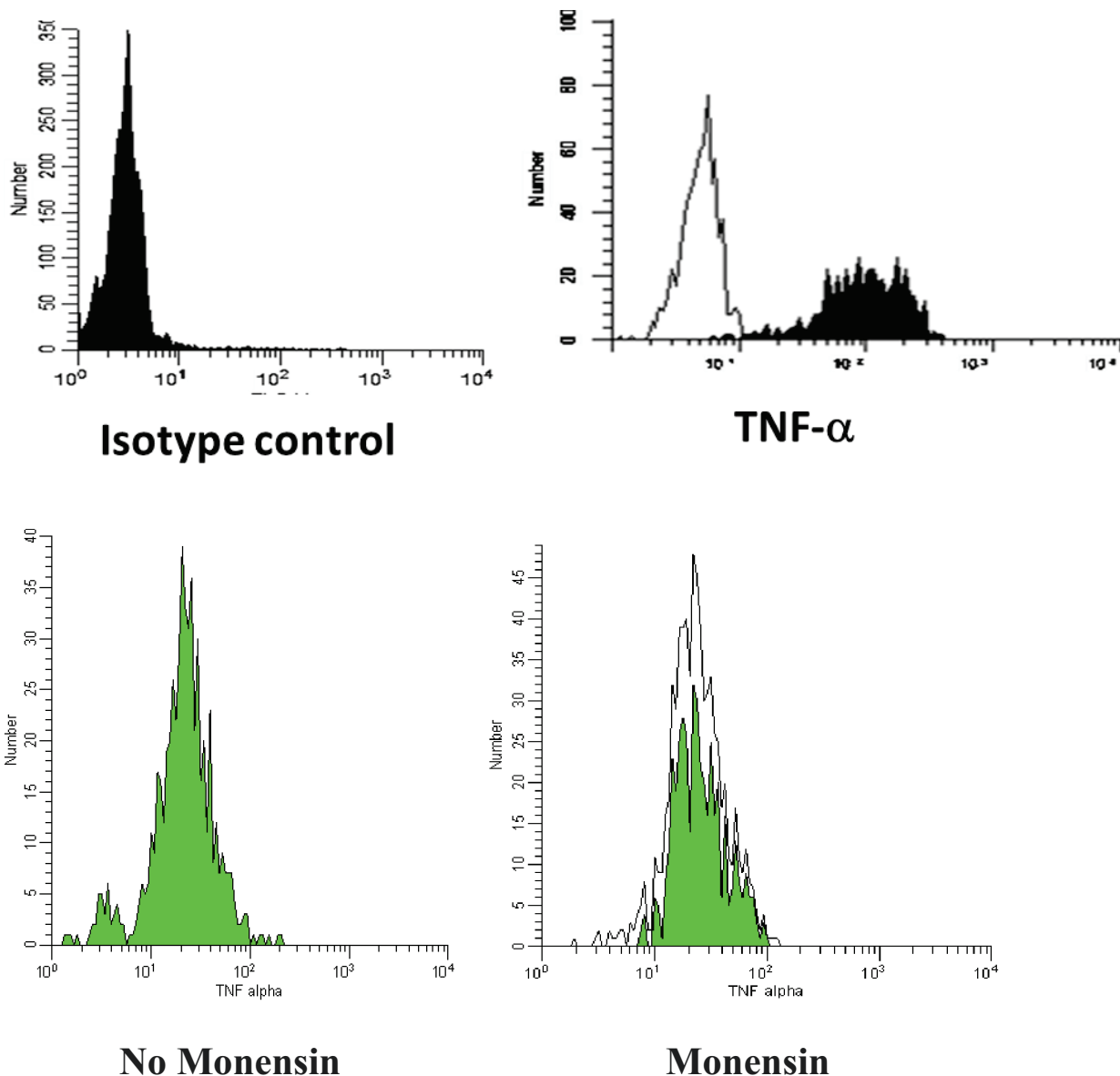


Fig 5.6: Enhanced Normalized Subtraction (ENS): An isotype control represents the level of non-specific staining achieved using an isotype matched control antibody. Staining for a surface marker represents the specific labelling for the target antigen combined with an element of non-specific binding. The software allows the subtraction of the non-specific binding component from the labelling histogram. The median channel of fluorescence and distribution of each histogram is compared and the proportion of positive events calculated. The staining intensity of the positive events is expressed as a ratio relative to the staining intensity of the control events. This allows the identification of positive events, even low positive events that fall within the control histogram, and hence may give a more accurate measure of staining intensity than the mean of a gated population. ENS was also used for ICS analysis by subtracting cells to which no monensin was added from cell to which monensin was added. Subtraction results thus provided data of the % +ve of cells which had the on-going production of a particular cytokine.

Compensation is done after the experiment and when we load the FACS tubes into the FACS machine. We have different fluorochromes to the Ab's; FitC, PE, PC5 and APC. The compensation is to make sure that all the Ab's with the fluorochromes are falling within the parameters of the histograms. Hence we do this to all of the fluorochrome Ab's. This does not change any of the results, but simple makes the graphical results more visible. We apply the same to all cells of all experiments in exactly the same way.

5.9.2 Multiplex sample processing

5.9.2.1 'Walk out'

The one to two tissue biopsy samples set aside for cytokine analysis were placed in a FACS tube with 0.5ml of complete medium and incubated overnight at 37°C with 5% CO₂.

After 24 hours the cells were centrifuged at 200g for 5 minutes. The supernatant was removed into a 1.2ml cryovial, labelled and placed into a freezer at -80°C until multiplex analysis was undertaken on all samples examining for the presence of IL2, IL4, IL6, IL10, TNF- α , IL17A and Interferon- γ .

5.9.2.1.1 Blood Multiplex

PBMC's (500 μ l) were placed in a FACS tube and incubated overnight at 37°C with 5% CO₂.

The following day the cells were centrifuged, labelled and stored, ready for multiplex analysis in exactly the same manner as described above for 'Walk out'

5.9.2.1.2 Multiplex methods

All cryovials containing supernatant were thawed at room temperature and then vortexed. Supernatant (50µl) was placed into labelled sample tubes containing 50µl of vortexed human cytokine capture beads (BD Biosciences, UK). Human Detection Reagent (BD Biosciences, UK) (50µl) was added to each tube and incubation at room temperature occurred for three hours, with the tubes protected from light using silver foil sealed bags. 1ml of buffer wash (BD Biosciences, UK) was then added to each tube before five minutes of centrifugation at 200g. The supernatant was discarded and 300µl of wash buffer was added to re-suspend the precipitate.

All samples were then acquired using FACS Canto II flow cytometer (Becton Dickinson, England) as described above until all events had been captured. The resultant data was analysed using FCAP Array software (BD Biosciences, UK).

Multiplex experiments are all done on the same day to avoid variation. Each sample contains cytokines which have moved out of the tissue and into the supernatant, and which has then been stored at -80°C. Multiplex involves beads which are cells. The beads already have a fixed coated amount of a particular Ab such as TNF- α . We add these beads separately coated with TNF- α Ab, IL12 Ab and as required, to a sample supernatant. Each bead has 0.5 picco grams of Ab on its surface. Therefore when this Ab beads are added to the sample supernatant, we can find out how much of a particular cytokine is present in the solution.

5.10 RESULTS

5.10.1 Tissue Biopsy

Results are available on tissue biopsy of ten patients with non-IBD ECF and ten control patients. The percentage positive (% +ve) of cells which were labelled following ICS were recorded in Figures 5.7 and 5.9

5.10.1.1 Comparison of TNF- α and IL12 production by non-IBD ECF and control cells

Results of intracellular staining on biopsies taken from ten non-IBD ECF patients are shown in Table 5.4 and 5.5. Production of TNF- α by DC from non-IBD ECF tissue was significantly higher than that by DC from control terminal ileum tissue (Figure 5.7). All viable cells from non-IBD ECF tissue also produced significantly higher level of TNF- α compared with all viable cells from control terminal ileum tissue (Figure 5.7).

Production of IL12 by DC from non-IBD ECF tissue was significantly higher than that by DC from control terminal ileum tissue (Figure 5.7). All viable cells from non-IBD ECF tissue also produced significantly higher level of IL12 compared with all viable cells from control terminal ileum tissue (Figure 5.7).

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in DC % +ve	84.35	49.19	0	22.89	67.04	77.52	61.11	74.62	54.14	75.99
IL12 In DC % +ve	74.66	45.24	0	31.12	62.83	73.97	76.2	0	56.26	0
TNF-α in ALL cells % +ve	56.12	72.84	73.46	29.15	36.57	55.92	71.96	80.24	55.64	55.83
IL12 in ALL cells % +ve	41.4	82.08	4.32	67.16	45.53	73.83	72.36	3.85	46.55	64.9

Table 5.4: Non - IBD ECF Patients DC and all cells % +ve ICS: Results of ICS on biopsies taken from ten non-IBD ECF patients. TNF- α and IL12 antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in DC % +ve	56.69	26.75	8.45
IL12 in DC % +ve	42.03	32.17	10.17
TNF-α in ALL cells % +ve	58.77	16.51	5.22
IL12 in ALL cells % +ve	50.20	27.76	8.78

Table 5.5: Mean, SD & SEM of Table 5.4 data: Summary of results for data in Table 5.4 with mean, SD and SEM applied.

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in DC % +ve	0	97.09	0	78.18	0	0	0	0	0	0
IL12 in DC % +ve	78.06	90.53	0	3.88	0	0	0	0	0	0
TNF-α in ALL cells % +ve	0	3.64	4.05	58.69	94.58	60.65	0	0	74.94	52.68
IL12 in ALL cells % +ve	0	74.55	0	73.76	62.34	76.76	0	0	63.22	14.69

Table 5.6: Control Patients (Endoscopy Terminal Ileum Biopsy) DC and all cells % +ve ICS: Results of ICS on biopsies taken from ten control patients. TNF- α and IL12 antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in DC % +ve	17.53	37.22	11.77
IL12 in DC % +ve	17.25	35.48	11.22
TNF-α in ALL cells % +ve	34.92	36.96	11.69
IL12 in ALL cells % +ve	36.53	35.97	11.37

Table 5.7: Mean, SD & SEM of Table 5.6 data: Summary of results for data in Table 5.5 with mean, SD and SEM applied.

	Non - IBD ECF Patients (Mean of all patients)	Control Patients (Means of all patients)
TNF-α in DC % +ve	56.685	17.527
IL12 in DC % +ve	42.028	17.247
TNF-α in ALL cells % +ve	58.773	34.923
IL12 in ALL cells % +ve	50.198	36.532

Table 5.8: Comparison of Non - IBD ECF Patients and Control DC and all cells % +ve ICS Means of all patients: Side by side results of intracellular staining with TNF- α and IL12 antibodies.

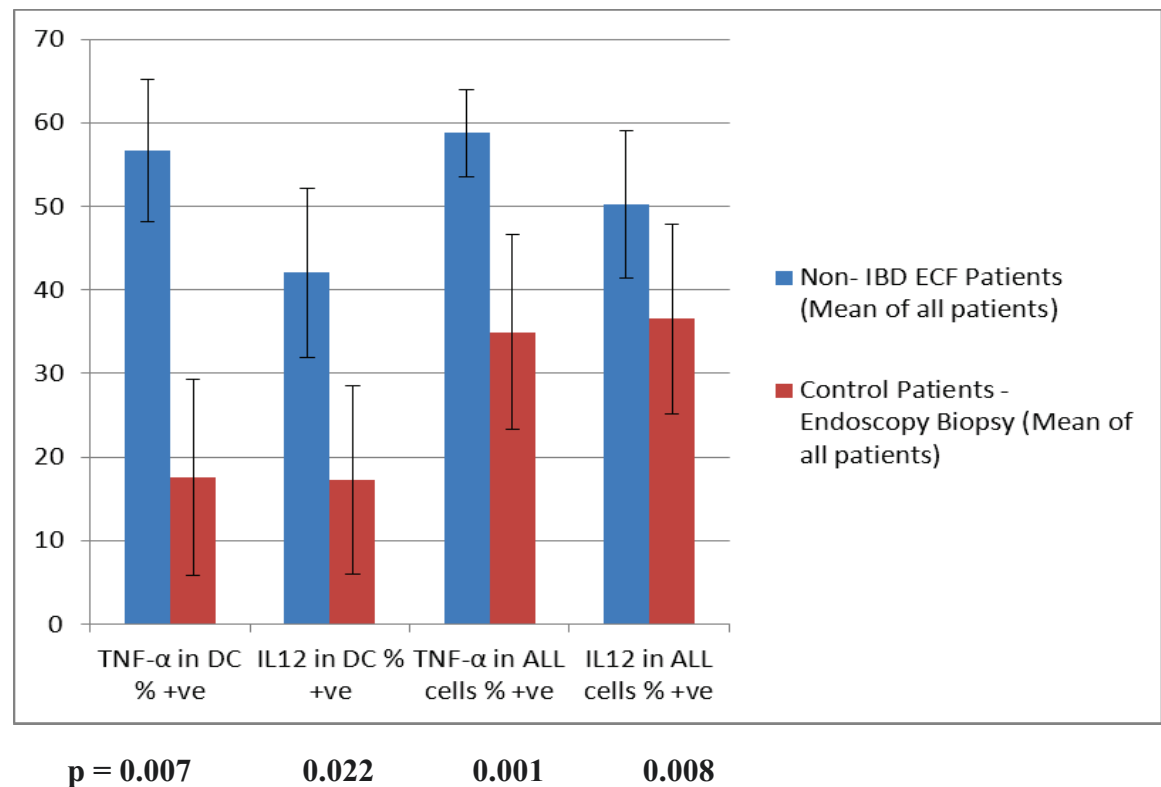


Fig 5.7: The means of on-going intracellular production of cytokines from DC and all cells % +ve: Bar chart with SEM comparing the mean % +ve of DC and all cells with TNF- α and IL12 in non-IBD ECF versus control patients.

5.10.1.2 Comparison of Intensity Ratio (IR) of TNF- α and IL12 intracellular production by non-IBD ECF and control patients DC and all cells

Results of ten non-IBD patient's intensity ratios are provided in Table 5.9 with summary results in Table 5.10. Results of ten control patient's intensity ratios are provided in Table 5.11 with summary results in Table 5.12. Table 5.13 provides side by side comparison of the mean intensity ratios between the two groups.

There was significant difference in the intensity ratios of IL12 in all viable cells from non-IBD ECF tissue compared with control terminal ileum tissue (Figure 5.8). TNF- α was increased in DC and all viable cells of non-IBD ECF tissue, though not significantly.

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in DC IR	1.6	1.45	0	1.36	1.52	1.65	1.18	1.1	1.4	1.48
IL12 in DC IR	2.04	2.64	0	0	1.59	2.35	1.21	0	1.36	0
TNF-α in ALL cells IR	1.02	2.78	1.06	1.94	2.18	1.85	1.23	1.11	1.46	1.22
IL12 in ALL cells IR	1.1	3.89	2.66	1.82	2.32	2	1.3	2.82	1.44	1.3

Table 5.9: Non - IBD ECF Patients DC and all cells Intensity Ratio (IR) ICS: Results of ICS on biopsies taken from ten non-IBD ECF patients. TNF- α and IL12 antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in DC IR	1.27	0.48	0.15
IL12 in DC IR	1.29	0	0
TNF-α in ALL cells IR	1.59	0.58	0.18
IL12 in ALL cells IR	2.07	0.87	0.28

Table 5.10: Mean, SD & SEM of Table 5.9 data: Summary of results for data in Table 5.9 with mean, SD and SEM applied.

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in DC IR	0	0	0	0	0	0	0	0	0	0
IL12 in DC IR	1.64	1.92	0	0	0	0	0	0	0	0
TNF-α in ALL cells IR	0	2.72	2.91	1.6	1.14	1.09	0	0	3.42	1.28
IL12 in ALL cells IR	0	1.38	0	1.32	1.22	1.12	0	0	4.54	0

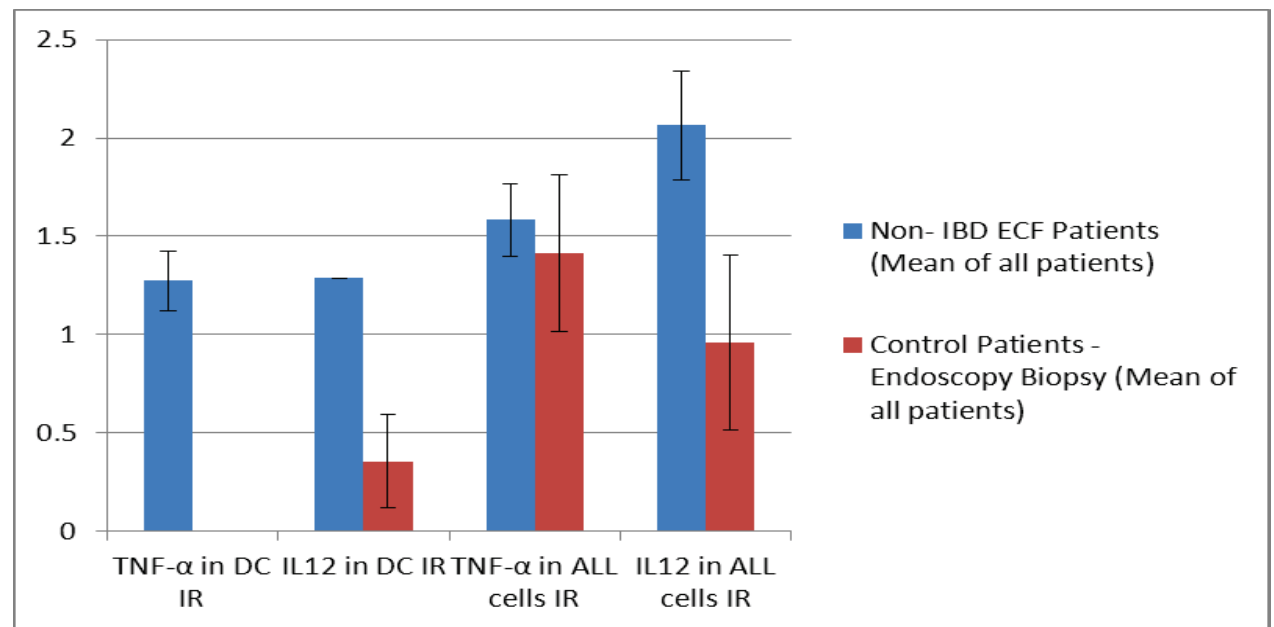
Table 5.11: Control Patients (Endoscopy Terminal Ileum Biopsy) DC and all cells ICS Intensity Ratio (IR): Results of ICS on biopsies taken from ten control patients. TNF- α and IL12 antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in DC IR	0	0	0
IL12 in DC IR	0.35	0.75	0.23
TNF-α in ALL cells IR	1.42	1.26	0.39
IL12 in ALL cells IR	0.95	1.41	0.44

Table 5.12: Mean, SD & SEM of Table 5.11 data: Summary of results for data in Table 5.11 with mean, SD and SEM applied.

	Non- IBD ECF Patients (Mean of all patients)	Control Patients (Mean of all patients)
TNF-α in DC IR	1.27	0
IL12 in DC IR	1.29	0.35
TNF-α in ALL cells IR	1.59	1.42
IL12 in ALL cells IR	2.07	0.96

Table 5.13: Comparison of Non - IBD ECF Patients and Control DC and all cells IR ICS Means of all patients: Side by side results of ICS with TNF- α and IL12 antibodies.



p = 0.278 0.352 **0.024**

Fig 5.8: The means for Intra cellular staining in DC and all cells IR: Bar chart with SEM comparing the mean intensity ratio of DC and all cells with TNF- α and IL12 in non-IBD ECF versus control patients.

5.10.1.3 TNF- α , IL17a, IFN γ production by non-IBD ECF T cells and all cells

Results of ten non-IBD patient's mean % +ve following intracellular staining are provided in Table 5.14 with summary results in Table 5.15. Results of ten control patients are provided in Table 5.16 with summary results in Table 5.17. Table 5.18 provides side by side comparison of the mean % +ve's between the two groups.

There were significant differences in the production of TNF alpha by T cells and all viable cells in non-IBD ECF compared with control terminal ileum tissue (Figure 5.9).

There was no significant differences in the production of IL17a by T cells in non-IBD ECF compared with control terminal ileum tissue. However the on-going production of IL17a in all viable cells from non-IBD ECF was significantly higher compared with all viable cells from control terminal ileum tissue (Figure 5.9).

Also on-going production of IFN γ was significantly different in non-IBD ECF T cells compared with control terminal ileum tissue. However there was no significant difference in IFN γ in all viable cells from non-IBD ECF compared with all viable cells from control terminal ileum tissue (Figure 5.9).

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in T cells % +ve	49.09	9.48	0	73.08	35.62	24.6	29.37	54.38	52.25	55.37
IL17a in T cells % +ve	74.56	54.38	0	70.96	74.69	69.47	47.23	63.98	52.77	46.31
IFNγ in T cells % +ve	70.38	58.47	48.24	68.5	2.01	0	46.34	63.3	64.34	0
TNF-α in All cells % +ve	56.12	65.46	6.77	31.99	49.26	30.73	16.32	16.63	58.05	51.01
IL17a in All cells % +ve	10.57	73.16	29.17	63.4	79.86	58.78	38.67	50.21	68.87	49.27
IFNγ in All cells % +ve	51.27	59.63	47.11	54.24	0.1	0	41.16	27.74	57.26	0

Table 5.14: Non - IBD ECF Patients T cells and all cells % + ve ICS: Results of ICS on biopsies taken from ten non-IBD ECF patients. TNF- α , IL17a and IFN γ antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in T cells % +ve	38.32	22.71	7.18
IL17a in T cells % +ve	55.44	22.31	7.05
IFNγ in T cells % +ve	42.16	29.66	9.37
TNF-α in All cells % +ve	36.57	20.47	6.47
IL17a in All cells % +ve	47.18	21.37	6.76
IFNγ in All cells % +ve	31.08	25.01	7.91

Table 5.15: Mean, SD & SEM of Table 5.14 data: Summary of results for data in Table 5.14 with mean, SD and SEM applied.

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in T cells % +ve	0	0	24.51	2	0	20.7	71.22	0	37.74	36.08
IL17a in T cells % +ve	0	0	38.69	74.13	0	6.61	96.99	0	71.29	68.03
IFNγ in T cells % +ve	0	52.7	34.4	21.91	0	0.16	0	45.5	0.05	0.43
TNF-α in All cells % +ve	0	0	4.05	71.32	0	45.9	0	0	49.89	18.8
IL17a in All cells % +ve	0	0	0	59.94	0	39.36	0	0	35.76	55.08
IFNγ in All cells % +ve	0	7.24	54.09	33.52	0	0.16	53.16	0	19.7	28.96

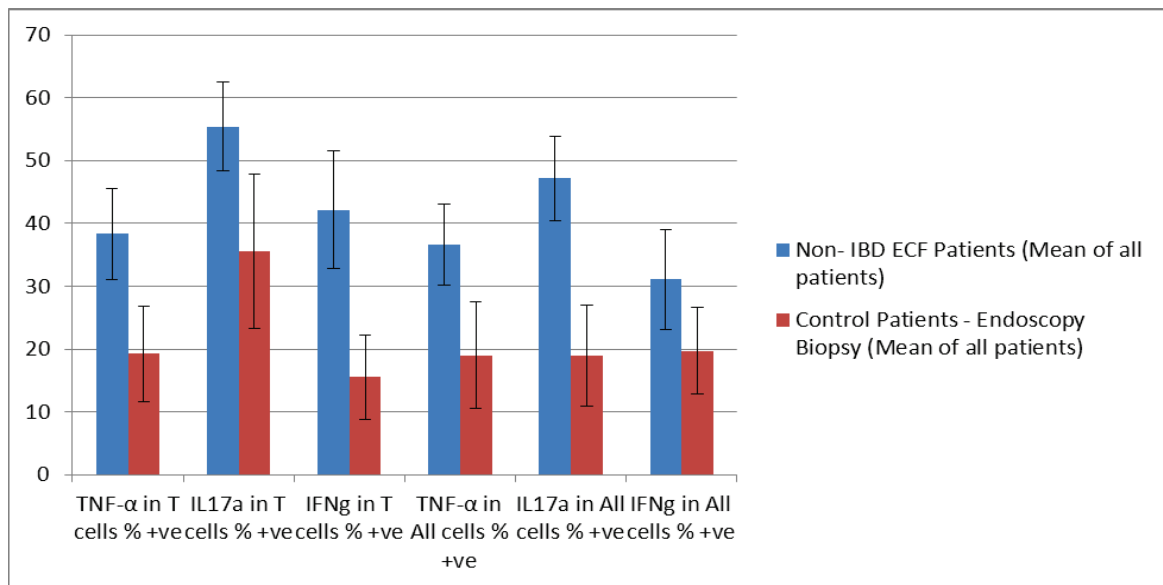
Table 5.16: Control Patients (Endoscopy Terminal Ileum Biopsy) T cells and all cells % +ve ICS: Results of ICS on biopsies taken from ten control patients. TNF- α , IL17a and IFN γ antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in T cells % +ve	19.22	23.89	7.55
IL17a in T cells % +ve	35.57	38.73	12.25
IFNγ in T cells % +ve	15.52	21.35	6.75
TNF-α in All cells % +ve	18.99	26.76	8.46
IL17a in All cells % +ve	19.01	25.47	8.05
IFNγ in All cells % +ve	19.68	21.81	6.89

Table 5.17: Mean, SD & SEM of Table 5.16 data: Summary of results for data in Table 5.16 with mean, SD and SEM applied.

	Non- IBD ECF Patients (Mean of all patients)	Control Patients (Mean of all patients)
TNF-α in T cells % +ve	38.32	19.23
IL17a in T cells % +ve	55.44	35.57
IFNγ in T cells % +ve	42.16	15.52
TNF-α in All cells % +ve	36.57	18.99
IL17a in All cells % +ve	47.18	19.01
IFNγ in All cells % +ve	31.08	19.68

Table 5.18: Comparison of Non - IBD ECF Patients and Control T cells and all cells % +ve ICS Means of all patients: Side by side results of ICS with TNF- α , IL17a and IFN γ antibodies.



p = 0.041 0.088 0.016 0.043 0.002 0.096

Fig 5.9: The means of on-going intracellular production from T cells and all cells % +ve: Bar chart with SEM comparing the mean % +ve of T cells and all cells with TNF- α , IL17a and IFN γ in non-IBD ECF versus control patients.

5.10.1.4 Comparison of Intensity Ratio (IR) of TNF- α , IL17a and IFN γ intracellular production by non-IBD ECF and control patients T cells and all cells

Results of ten non-IBD patient's intensity ratios are provided in Table 5.19 with summary results in Table 5.20. Results of ten control patient's intensity ratios are provided in Table 5.21 with summary results in Table 5.22. Table 5.23 provides side by side comparison of the mean intensity ratios between the two groups.

The mean intensity ratio for cytokine TNF- α , was greater though not significantly non-IBD ECF T cells and all viable cells in comparison to control tissue (Figure 5.10).

IL17a was significantly increased in T cells of non-IBD ECF though not all viable cells (Figure 5.10).

In contrast IFN γ was significantly increased in non-IBD ECF all viable cells but not in T cells alone (Figure 5.10).

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in T cells IR	1.55	1.43	0	2.1	1.35	2.02	6.13	2.45	1.22	1.24
IL17a in T cells IR	1.2	1.46	0	1.91	1.86	1.48	4.41	2.48	1.2	1.37
IFNγ in T cells IR	1.79	1.84	1.66	4.1	0.88	0	3.23	4.33	1.64	0
TNF-α in ALL cells IR	2.19	2.79	1.35	2.04	1.42	2.16	4.91	1.12	1.32	1.24
IL17a in All cells IR	1.09	3.7	1.58	2.38	2.04	1.66	3.37	1.24	1.32	1.35
IFNγ in All cells IR	3.16	4.67	1.74	1.96	0	0	2.29	1.43	2.08	0

Table 5.19: Non - IBD ECF Patients T cells and all cells Intensity Ratio (IR) ICS: Results of ICS on biopsies taken from ten non-IBD ECF patients. TNF- α , IL17a and IFN γ antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in T cells IR	1.70	1.61	0.50
IL17a in T cells IR	1.48	1.13	0.35
IFNγ in T cells IR	1.51	1.52	0.48
TNF-α in ALL cells IR	1.94	1.14	0.36
IL17a in All cells IR	1.84	0.91	0.28
IFNγ in All cells IR	1.73	1.49	0.47

Table 5.20: Mean, SD & SEM of Table 5.19 data: Summary of results for data in Table 5.19 with mean, SD and SEM applied.

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α in T cells IR	0	0	2.77	0	0	3.68	1.08	0	1.08	1.08
IL17a in T cells IR	0	0	1.3	1.23	0	0	1.08	0	1.08	1.08
IFNγ in T cells IR	0	1.03	1.62	1.17	0	0	0	1.62	0	4.22
TNF-α in ALL cells IR	0	0	2.91	1.49	0	1.18	0	0	1.93	1.43
IL17a in All cells IR	0	0	0	1.44	0	1.16	0	0	4.43	3.41
IFNγ in All cells IR	0	1.25	1.45	1.43	0	0	1.26	0	0.99	1.25

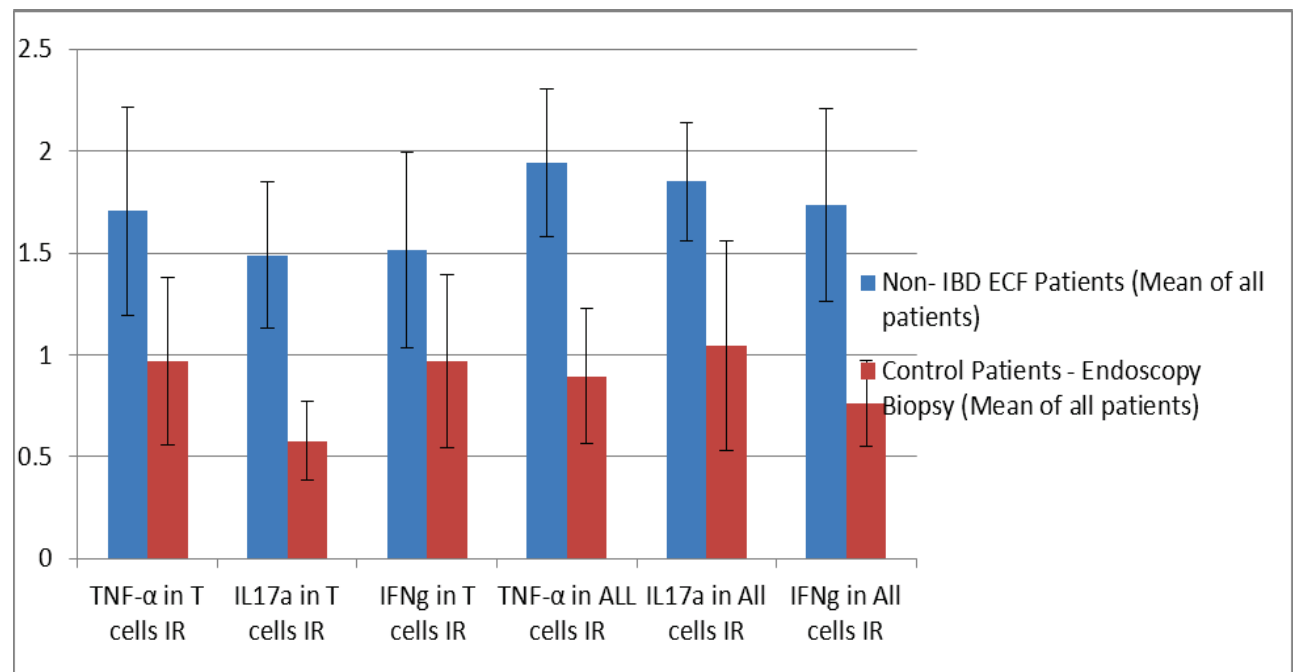
Table 5.21: Control Patients (Endoscopy Terminal Ileum Biopsy) T cells and all cells ICS Intensity Ratio (IR): Results of ICS on biopsies taken from ten control patients. TNF- α , IL17a and IFN γ antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α in T cells IR	0.97	1.30	0.41
IL17a in T cells IR	0.57	0.61	0.19
IFNγ in T cells IR	0.97	1.34	0.42
TNF-α in ALL cells IR	0.89	1.04	0.33
IL17a in All cells IR	1.04	1.63	0.51
IFNγ in All cells IR	0.76	0.67	0.21

Table 5.22: Mean, SD & SEM of Table 5.21 data: Summary of results for data in Table 5.21 with mean, SD and SEM applied.

	Non- IBD ECF Patients (Mean of all patients)	Control Patients (Mean of all patients)
TNF-α in T cells IR	1.70	0.97
IL17a in T cells IR	1.48	0.57
IFNγ in T cells IR	1.51	0.97
TNF-α in ALL cells IR	1.94	0.89
IL17a in All cells IR	1.84	1.04
IFNγ in All cells IR	1.73	0.76

Table 5.23: Comparison of Non - IBD ECF Patients and Control T cells and all cells IR ICS Means of all patients: Side by side results of ICS with TNF- α , IL17a and IFN γ antibodies.



p = 0.076 **0.005** 0.072 **0.014** 0.066 **0.039**

Fig 5.10: The means of on-going intracellular production of cytokines from T cells and all cells IR: Bar chart with SEM comparing the mean intensity ratio of T cells and all cells with TNF- α , IL17a and IFN γ in non-IBD ECF versus control patients.

5.10.2 Blood

5.10.2.1 CLA and $\beta 7$ homing markers on DC and all cells from blood of non-IBD ECF versus control patients

Results of CLA and $\beta 7$ homing markers on DC and all cells from blood of ten non-IBD ECF patients are provided in Table 5.24 with summary results in Table 5.25.

Results of ten control patients are provided in Table 5.26 with summary results in Table 5.27. Table 5.28 provides side by side comparison of the mean intensity ratios between the two groups.

There was a greater percentage of DC, though non-significant ($p = 0.196$), which had the presence of CLA surface marker in non - IBD ECF patient's blood, then from control patient's blood. There was no significance difference for surface marker between the two groups ($p = 0.425$) (Figure 5.11).

CLA surface marker was significantly present on all viable cells from non - IBD ECF patient's blood ($p = 0.036$). We were unable to detect CLA surface marker on any of the ten control patient's blood (Figure 5.11).

The experiments have shown there to be a significance difference ($p = 0.002$) with greater percentage of cells having the $\beta 7$ surface marker on all cells from the control group in comparison to the non-IBD ECF group. This would suggest that control blood had a greater percentage of gut homing markers in comparison to non-IBD ECF blood, or we could say that there is a lack of gut homing cells in non-IBD ECF tissue (Figure 5.11).

Patient	1	2	3	4	5	6	7	8	9	10
CLA on DC % +ve	56.00	37.54	1.47	31.67	17.20	0	45.51	0	0	93.12
CLA on DC IR	9.17	2.71	8.69	6.85	2.01	0	2.87	0	0	6.09
b7 on DC % +ve	88.32	0	88.45	58.92	33.62	31.78	16.06	46.68	18.77	84.34
b7 on DC IR	12.47	0	5.55	6.00	2.18	3.41	6.80	3.52	9.71	15.67
CLA on ALL cells % +ve	1.15	0	0.59	0.58	23.27	0	0	0	9.13	12.91
CLA on ALL cells IR	1.26	0	12.76	4.67	1.49	0	0	0	4.20	3.29
b7 on ALL cells % +ve	11.72	0	44.52	18.74	36.93	52.06	10.12	64.22	31.63	25.66
b7 on ALL cells IR	1.09	0	3.72	1.60	1.87	4.36	9.75	2.08	5.38	8.43

Table 5.24: Non - IBD ECF Patients DC and all cells % +ve Surface Markers:
Results assessing the presence of CLA and β 7 homing markers from blood of ten non-IBD ECF patients.

	Mean	SD	SEM
CLA on DC % +ve	28.25	30.92	9.77
CLA on DC IR	3.83	3.58	1.13
b7 on DC % +ve	46.69	32.20	10.18
b7 on DC IR	6.53	4.83	1.52
CLA on ALL cells % +ve	4.76	7.93	2.50
CLA on ALL cells IR	2.76	3.94	1.24
b7 on ALL cells % +ve	29.56	20.25	6.40
b7 on ALL cells IR	3.82	3.21	1.01

Table 5.25: Mean, SD & SEM of Table 5.24 data: Summary of results for data in Table 5.24 with mean, SD and SEM applied.

Patient	1	2	3	4	5	6	7	8	9	10
CLA on DC % +ve	68.36	18.09	0	0	0	0	76.32	0	0	0
CLA on DC IR	2.33	4.87	0	0	0	0	1.45	0	0	0
b7 on DC % +ve	96.97	59.35	39.84	47.36	61.22	51.29	66.59	70.94	0	0
b7 on DC IR	31.81	22.87	6.43	9.76	24.87	10.36	19.81	34.91	0	0
CLA on ALL cells % +ve	0	0	0	0	0	0	0	0	0	0
CLA on ALL cells IR	0	0	0	0	0	0	0	0	0	0
b7 on ALL cells % +ve	52	2.87	55.92	69.33	60.82	61	63.07	52.77	21.82	59.91
b7 on ALL cells IR	6.91	10.10	1.83	4.81	6.81	4.1	7.72	3.89	11.55	7.3

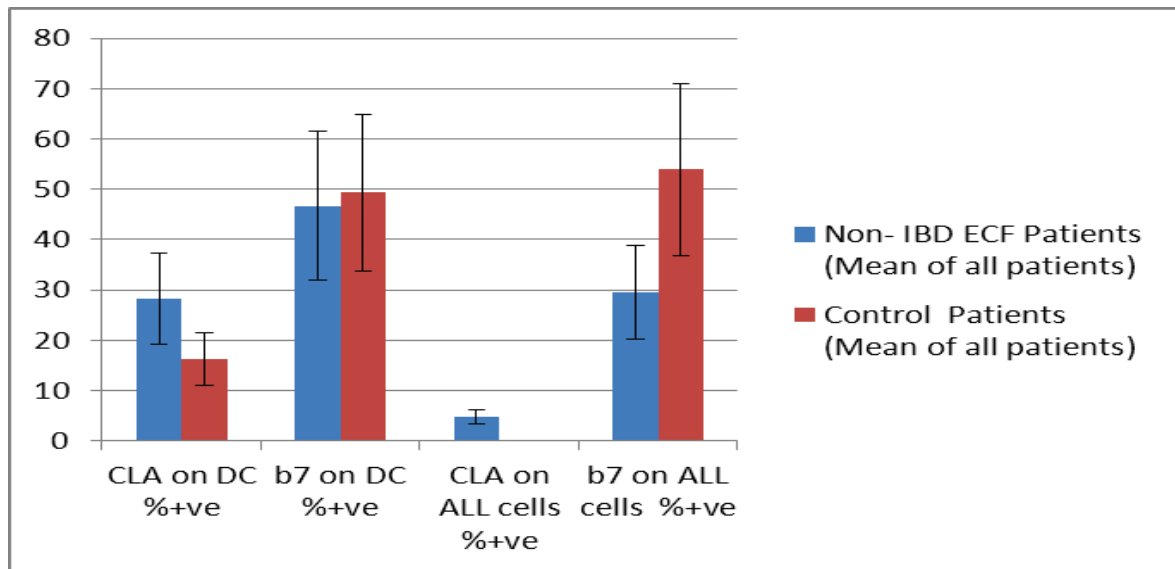
Table 5.26: Control Patients DC and all cells % +ve Surface Markers: Assessing the presence of CLA and $\beta 7$ homing markers from blood of ten control patients.

	Mean	SD	SEM
CLA on DC % +ve	16.27	30.13	9.53
CLA on DC IR	0.86	1.62	0.51
b7 on DC % +ve	49.35	30.25	9.56
b7 on DC IR	16.08	12.57	3.97
CLA on ALL cells % +ve	0	0	0
CLA on ALL cells IR	0	0	0
b7 on ALL cells % +ve	53.95	13.40	4.23
b7 on ALL cells IR	6.50	2.94	0.93

Table 5.27: Mean, SD & SEM of Table 5.26 data: Summary of results for data in Table 5.26 with mean, SD and SEM applied.

	<i>Non- IBD ECF Patients (Mean of all patients)</i>	<i>Control Patients (Mean of all patients)</i>
CLA on DC % +ve	28.25	16.27
b7 on DC % +ve	46.69	49.35
CLA on ALL cells % +ve	4.76	0
b7 on ALL cells % +ve	29.56	53.95

Table 5.28: Comparison of Non - IBD ECF Patients and Control DC and all cells % +ve Surface Markers Means of all patients: Side by side results of CLA and β 7 homing markers from blood of non-IBD ECF versus control patients.



p = 0.196 0.425 **0.036** **0.002**

Fig 5.11: The means of % +ve Surface Markers on DC and all cells: Bar chart with SEM comparing the mean % +ve's of DC and all cells with CLA and β 7 homing markers in non-IBD ECF versus control patients.

5.10.2.2 CLA and $\beta 7$ homing markers on T cells and all cells from blood of non-IBD ECF versus control patients

Results of CLA and $\beta 7$ homing markers on DC and all cells from blood of ten non-IBD ECF patients are provided in Table 5.29 with summary results in Table 5.30. Results of ten control patients are provided in Table 5.31 with summary results in Table 5.32. Table 5.33 provides side by side comparison of the mean intensity ratios between the two groups.

There was a greater percentage of T cells and all cells, though non-significant ($p = 0.085$) and ($p = 0.089$) respectively, which had the presence of CLA surface marker in non - IBD ECF patient's blood, then from control patient's blood (Figure 5.12).

The experiments have shown there to be a greater percentage of cells having the $\beta 7$ surface marker on T cells ($p = 0.012$) and all cells ($p = 0.310$) from the control group blood in comparison to the non-IBD ECF group blood. This would suggest that non-IBD ECF blood has less gut homing markers then normal control patient's blood or we could say that there is a lack of gut homing cells in non-IBD ECF blood (Figure 5.12).

Patient	1	2	3	4	5	6	7	8	9	10
CD3 % +ve	78.88	30.49	50.02	53.13	56.29	60.84	38.03	50.89	54.98	0
CD3 IR	54.48	5.65	3.73	21.28	3.74	4.98	48.58	39.47	56.92	0
CLA on T cells % +ve	26.79	2.38	0	9.72	0	0	0	0	0	0
CLA on T cells IR	1.66	1.33	0	0.99	0	0	0	0	0	0
b7 on T cells % +ve	47.13	0	49.38	44.17	36.13	51.54	38.85	15.79	44.1	43.02
b7 on T cells IR	10.65	0	3.86	4.83	3.65	4.92	8.16	9.4	6.29	7.95
CLA on ALL cells % +ve	15.68	0	0	5.22	1.22	0	0	0	0	0
CLA on ALL cells IR	1.18	0	0	0.39	1.73	0	0	0	0	0
b7 on ALL cells % +ve	14.83	0	38.43	45.87	30.85	43.07	0	53.85	45.66	34.09
b7 on ALL cells IR	1.17	0	3.41	1.52	2.78	4.32	0	1.43	4.1	5.17

Table 5.29: Non - IBD ECF Patients T cells and all cells % +ve Surface

Markers: Results assessing the presence of CLA and $\beta 7$ homing markers on T cells and all cells from blood of ten non-IBD ECF patients.

	Mean	SD	SEM
CD3 % +ve	47.35	21.03	6.65
CD3 IR	23.88	23.47	7.42
CLA on T cells % +ve	3.88	8.60	2.72
CLA on T cells IR	0.39	0.66	0.20
b7 on T cells % +ve	37.01	16.42	5.19
b7 on T cells IR	5.97	3.16	1.00
CLA on ALL cells % +ve	2.21	5.00	1.58
CLA on ALL cells IR	0.33	0.61	0.19
b7 on ALL cells % +ve	30.66	19.29	6.10
b7 on ALL cells IR	2.39	1.83	0.57

Table 5.30: Mean, SD & SEM of Table 5.29 data: Summary of results for data in Table 5.29 with mean, SD and SEM applied.

Patients	1	2	3	4	5	6	7	8	9	10
CD3 % +ve	69.42	65.02	45.6	63.99	78.57	84.66	79.08	70.33	69.68	76.46
CD3 IR	27.41	23.41	19.54	23.12	58.37	33.12	30.02	122.97	166.73	247.09
CLA on T cells % +ve	0	0	0	0	0	0	0	0	0	0
CLA on T cells IR	0	0	0	0	0	0	0	0	0	0
b7 on T cells % +ve	39.64	42.94	38.59	75.43	50.87	42.65	67.05	63.83	49.04	62.00
b7 on T cells IR	8.53	12.75	6.36	8.81	11.45	9.90	12.22	11.02	8.18	11.74
CLA on ALL cells % +ve	0	0	0	0	0	0	0	0	0	0
CLA on ALL cells IR	0	0	0	0	0	0	0	0	0	0
b7 on ALL cells % +ve	42.66	41.16	14.78	50.1	36.92	0	56.71	35.25	31.45	38.06
b7 on ALL cells IR	7.06	10.03	8.66	7.44	10.8	0	11.56	10.66	8.39	11.37

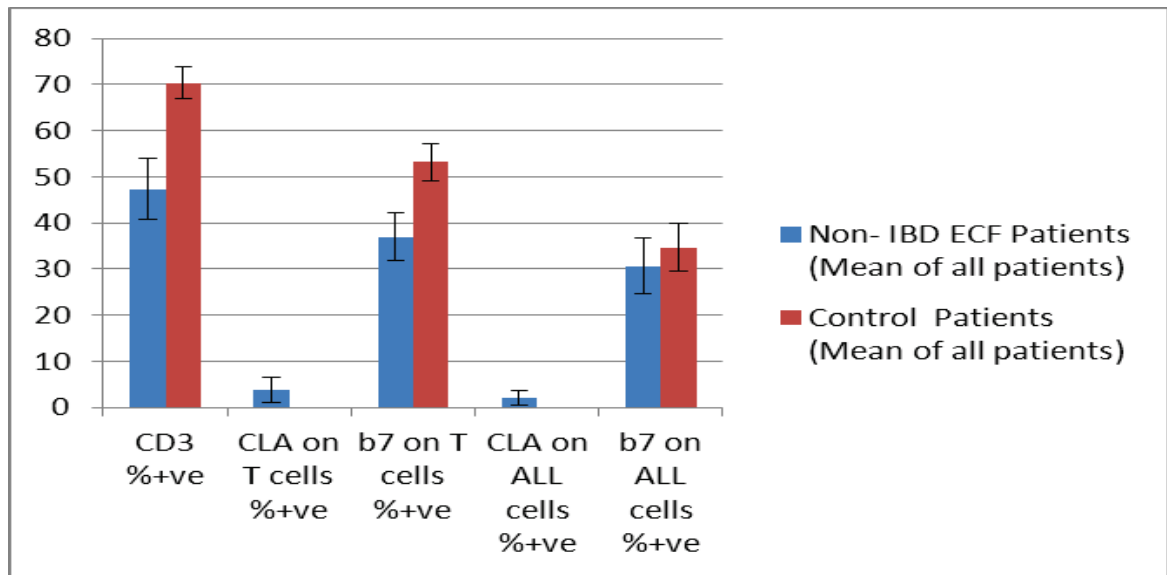
Table 5.31: Control Patients T cells and all cells % +ve Surface Markers:
Results assessing the presence of CLA and $\beta 7$ homing markers on T cells and all cells from blood of ten control patients.

	Mean	SD	SEM
CD3 % +ve	70.28	10.89	3.44
CD3 IR	75.17	78.22	24.73
CLA on T cells % +ve	0	0	0
CLA on T cells IR	0	0	0
b7 on T cells % +ve	53.20	12.96	4.10
b7 on T cells IR	10.09	2.07	0.65
CLA on ALL cells % +ve	0	0	0
CLA on ALL cells IR	0	0	0
b7 on ALL cells % +ve	34.70	16.51	5.22
b7 on ALL cells IR	8.59	3.42	1.08

Table 5.32: Mean, SD & SEM of Table 5.31 data: Summary of results for data in Table 5.31 with mean, SD and SEM applied.

	Non- IBD ECF Patients (Mean of all patients)	Control Patients (Mean of all patients)
CD3 % +ve	47.35	70.28
CLA on T cells % +ve	3.88	0
b7 on T cells % +ve	37.01	53.20
CLA on ALL cells % +ve	2.21	0
b7 on ALL cells % +ve	30.66	34.70

Table 5.33: Comparison of Non - IBD ECF Patients and Control T cells and all cells % +ve Surface Markers Means of all patients: Side by side results of CLA and β 7 homing markers from blood of non-IBD ECF versus control patients.



p = 0.003 0.085 0.012 0.089 0.310

Fig 5.12: The means of % +ve Surface Markers on T cells and all patients: Bar chart with SEM comparing the mean % +ve's of T cells and all cells with CLA and β 7 homing markers from blood of non-IBD ECF versus control patients.

5.10.2.3 TNF- α and IL12 production by non-IBD ECF blood cells

Results of ten non-IBD patient's blood mean % +ve following intracellular staining are provided in Table 5.34 with summary results in Table 5.35. Results of ten control patients are provided in Table 5.36 with summary results in Table 5.37. Table 5.38 provides side by side comparison of the mean % +ve's between the two groups.

Production of TNF- α by DC from non-IBD ECF blood cells was significantly higher ($p = 0.0008$) than that by DC from control blood patients. All viable cells from non-IBD ECF blood patients also produced significantly higher level of TNF- α compared with all viable cells from control blood patients ($p = 0.001$) (Figure 5.13).

Production of IL12 by DC from non-IBD ECF blood patients was significantly ($p = 0.025$) higher than that by DC from control blood patients. All viable cells from non-IBD ECF blood also produced higher level, though non-significant, of IL12 ($p = 0.088$) compared with all viable cells from control blood patients (Figure 5.13).

Patient	1	2	3	4	5	6	7	8	9	10
TNF-α on DC % +ve	16.28	5.75	68.33	30.12	64.87	66.24	73.65	0	51.57	76.3
TNF-α on IR	1.19	4.92	1.12	2.41	1.5	3	1.09	0	2.90	1.98
IL12 on DC % +ve	0	13.84	73.46	29.10	67.86	74.62	0	0	48.23	46.64
IL12 on DC IR	0	2.85	1.18	1.34	1.28	1.99	0	0	1.53	1.71
TNF-α on ALL cells % +ve	0	52.94	0	17.64	51.45	60.59	6.31	0.69	62.62	58.37
TNF-α on ALL cells IR	0	1.39	0	0.46	1.28	1.38	3.09	1.61	1.43	1.81
IL12 on ALL cells % +ve	0.78	11.57	23.23	11.86	0	32.25	10.91	0	68.51	49.71
IL12 on ALL cells IR	1.68	1.63	1.13	1.48	0	1.21	0.84	0	1.11	1.36

Table 5.34: Non - IBD ECF Patients DC and all cells % +ve ICS: Results of ICS on blood taken from ten non-IBD ECF patients. TNF- α and IL12 antibodies were used for labelling cytokines intracellularly.

Experiment Number	Mean	SD	SEM
BLOOD			
TNF-α on DC % +ve	45.31	29.52	9.33
TNF-α on IR	2.01	1.37	0.43
IL12 on DC % +ve	35.37	30.90	9.77
IL12 on DC IR	1.18	0.94	0.29
TNF-α on ALL cells % +ve	31.06	28.19	8.91
TNF-α on ALL cells IR	1.24	0.92	0.29
IL12 on ALL cells % +ve	20.88	23.04	7.28
IL12 on ALL cells IR	1.04	0.60	0.19

Table 5.35: Mean, SD & SEM of Table 5.34 data: Summary of results for data in Table 5.34 with mean, SD and SEM applied

Patients	1	2	3	4	5	6	7	8	9	10
TNF-α on DC % +ve	0	0	0	0	0	54.40	0	0	0	0
TNF-α on IR	0	0	0	0	0	1.99	0	0	0	0
IL12 on DC % +ve	0	0	2.57	82.72	0	0	0	0	0	0
IL12 on DC IR	0	0	3.83	1.03	0	0	0	0	0	0
TNF-α on ALL cells % +ve	0	0	0	0	0	0	0	0	0	0
TNF-α on ALL cells IR	0	0	0	0	0	0	0	0	0	0
IL12 on ALL cells % +ve	47.48	0.85	0.6	29.08	0.7	0	0	4.1	0	0
IL12 on ALL cells IR	1.05	0.84	5.96	1.05	5.78	0	0	5.75	0	0

Table 5.36: Control Patients DC and all cells % +ve ICS: Results of ICS on blood taken from ten control patients. TNF- α and IL12 antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
TNF-α on DC % +ve	5.44	17.20	5.44
TNF-α on IR	0.19	0.62	0.19
IL12 on DC % +ve	8.52	26.08	8.24
IL12 on DC IR	0.48	1.21	0.38
TNF-α on ALL cells % +ve	0	0	0
TNF-α on ALL cells IR	0	0	0
IL12 on ALL cells % +ve	8.28	16.44	5.19
IL12 on ALL cells IR	2.04	2.64	0.83

Table 5.37: Mean, SD & SEM of Table 5.36 data: Summary of results for data in Table 5.36 with mean, SD and SEM applied.

	<i>Non- IBD ECF Patients (Mean of all patients)</i>	<i>Control Patients (Mean of all patients)</i>
TNF-α on DC % +ve	45.31	5.44
IL12 on DC % +ve	35.37	8.52
TNF-α on ALL cells % +ve	31.06	0
IL12 on ALL cells % +ve	20.88	8.28

Table 5.38: Comparison of Non - IBD ECF Patients and Control DC's and all cells % +ve ICS Means of all patients: Side by side results of intracellular staining with TNF- α and IL12 antibodies.

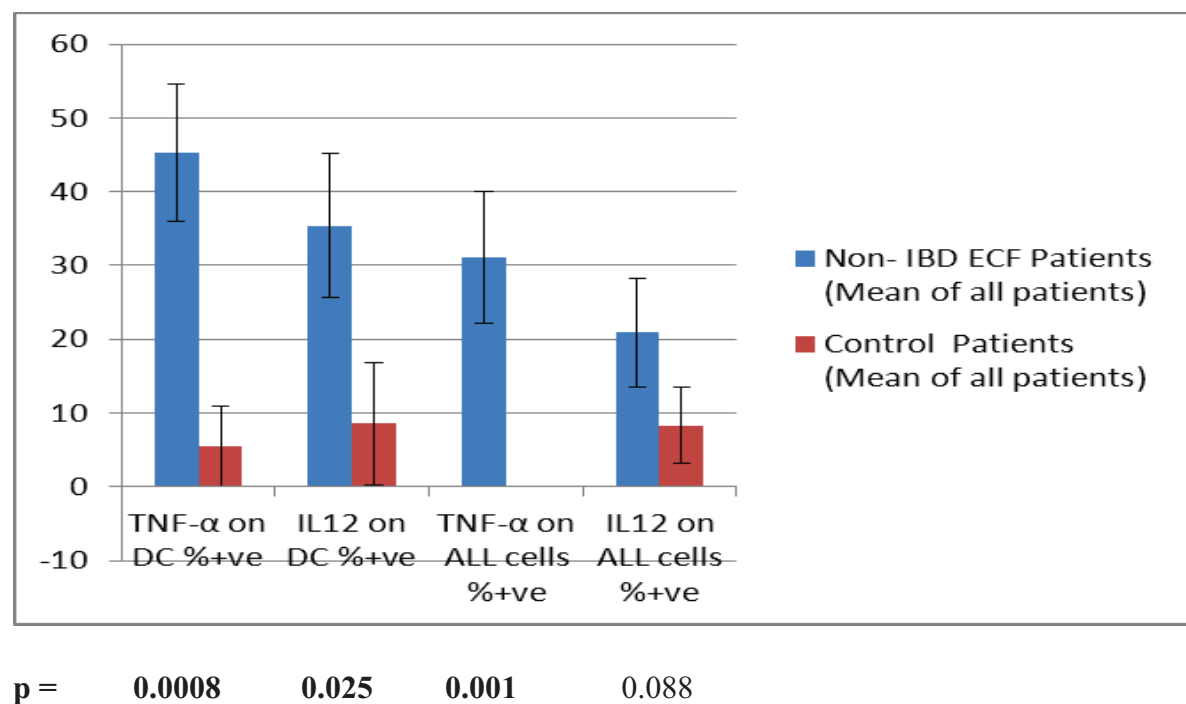


Fig 5.13: The means of TNF- α and IL12 on DC and all cells: Bar chart with SEM comparing the mean % +ve of DC and all cells in blood with TNF- α and IL12 in non-IBD ECF versus control patients.

5.10.2.4 Comparison of production of TNF- α , IL17a, IFN γ from Non - IBD ECF and Control patient's blood T cells and all cells

Results of ten non-IBD patient's blood mean % +ve following intracellular staining are provided in Table 5.39 with summary results in Table 5.40. Results of ten control patient's blood are provided in Table 5.41 with summary results in Table 5.42. Table 5.43 provides side by side comparison of the mean % +ve's between the two groups.

There were significant differences in the production of TNF- α by T cells ($p = 0.0005$) and all viable cells ($p = 0.0003$) in non-IBD ECF blood compared with control blood patients (Figure 5.14).

There were also significant differences in the production of IL17a by T cells ($p = 0.009$) and all viable cells ($p = 0.0001$) in non-IBD ECF blood compared with control blood patients (Figure 5.14).

Also the on-going production of IFN γ was significantly different by T cells ($p = 0.015$) and all viable cells ($p = 0.0008$) in non-IBD ECF blood compared with control blood patients (Figure 5.14).

Patient	1	2	3	4	5	6	7	8	9	10
CD3 % +ve	0	44.16	32.66	0	0	0	45.71	0.43	0	31.73
CD3 IR	0	3.64	3.66	0	0	0	2.03	12.12	0	1.55
TNF-α on T cells % +ve	54.35	90.89	0	48.41	65.25	42.71	40.14	0	61.51	61.77
TNF-α on T cells IR	1.37	5.29	0	2.22	1.12	1.25	1.51	0	1.42	1.49
IL17a on T cells % +ve	19.30	91.99	35.68	48.99	44.04	27.96	31.13	0	46.47	14.77
IL17a on T cells IR	1.15	5.53	1.33	2.67	1.14	1.14	1.26	0	1.11	12.84
IFNγ on T cells % +ve	1.23	48.7	22.15	24.02	1.65	66.4	36.89	40.36	0	41.73
IFNγ on T cells IR	65.09	2.63	1.47	23.06	0.83	4.43	1.31	3.21	0	2.02
TNF-α on All cells % +ve	0	81.93	0	27.31	49.26	50.61	51.82	0	63.73	60.16
TNF-α on ALL cells IR	0	3.99	0	1.33	1.2	1.26	1.4	0	1.36	1.67
IL17a on All cells % +ve	0	83.28	37.49	40.25	58.92	48.51	39.74	0	69.36	51.06
IL17a on All cells IR	0	4.18	1.18	1.78	1.23	1.11	1.2	0.3	1.1	1.27
IFNγ on All cells % +ve	38.64	48.7	40.43	42.59	4.32	60.91	0	0	25.87	67.96
IFNγ on All cells IR	1.25	2.63	1.28	1.72	1.12	2.89	0	0	1.05	1.14

Table 5.39: Non - IBD ECF Patients T cells and all cells % +ve ICS: Results of ICS on blood taken from ten non-IBD ECF patients. TNF- α , IL17a and IFN γ antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
CD3 % +ve	30.93	18.21	5.76
CD3 IR	4.6	4.30	1.36
TNF-α on T cells % +ve	46.50	28.33	8.95
TNF-α on T cells IR	1.56	1.47	0.46
IL17a on T cells % +ve	36.03	24.92	7.88
IL17a on T cells IR	2.81	3.82	1.20
IFNγ on T cells % +ve	28.31	22.52	7.12
IFNγ on T cells IR	10.40	20.36	6.43
TNF-α on All cells % +ve	38.48	29.82	9.43
TNF-α on ALL cells IR	1.22	1.17	0.37
IL17a on All cells % +ve	42.86	26.69	8.44
IL17a on All cells IR	1.33	1.12	0.35
IFNγ on All cells % +ve	32.94	24.67	7.80
IFNγ on All cells IR	1.30	0.94	0.29

Table 5.40: Mean, SD & SEM of Table 5.39 data: Summary of results for data in Table 5.39 with mean, SD and SEM applied.

Patients	1	2	3	4	5	6	7	8	9	10
CD3 % +ve	71.13	58.79	0	4.42	52.7	48.23	44.01	0	76.92	0
CD3 IR	1.31	1.48	0	3.15	1.68	1.7	1.01	0	1.81	0
TNF-α on T cells % +ve	0	0	0	0	0	39.75	0	30.78	0	0
TNF-α on T cells IR	0	0	0	0	0	1.10	0	1.06	0	0
IL17a on T cells % +ve	0	0	23.76	0	0	0	0	0	26.30	56.27
IL17a on T cells IR	0	0	3.50	0	0	0	0	0	1.14	1.10
IFNγ on T cells % +ve	35.61	0	0	0	0	0	0	33.98	2.16	13.03
IFNγ on T cells IR	1.01	0.33	0	0	0	0	0	1	0.94	1.07
TNF-α on All cells % +ve	0	0	0	0	0	3.07	0	0	0	0
TNF-α on ALL cells IR	0	0	0	0	0	4.17	0	0	0	0
IL17a on All cells % +ve	6.74	0.63	5.58	0	0	0	0	0	0	0
IL17a on All cells IR	1.64	43.38	3.45	0	0	0	0	0	0	0
IFNγ on All cells % +ve	4.27	6.26	0	0	0	4.9	9.32	4.13	2.16	8.27
IFNγ on All cells IR	0.94	1.01	0	0	0	1.04	1.2	0.84	0.94	2.12

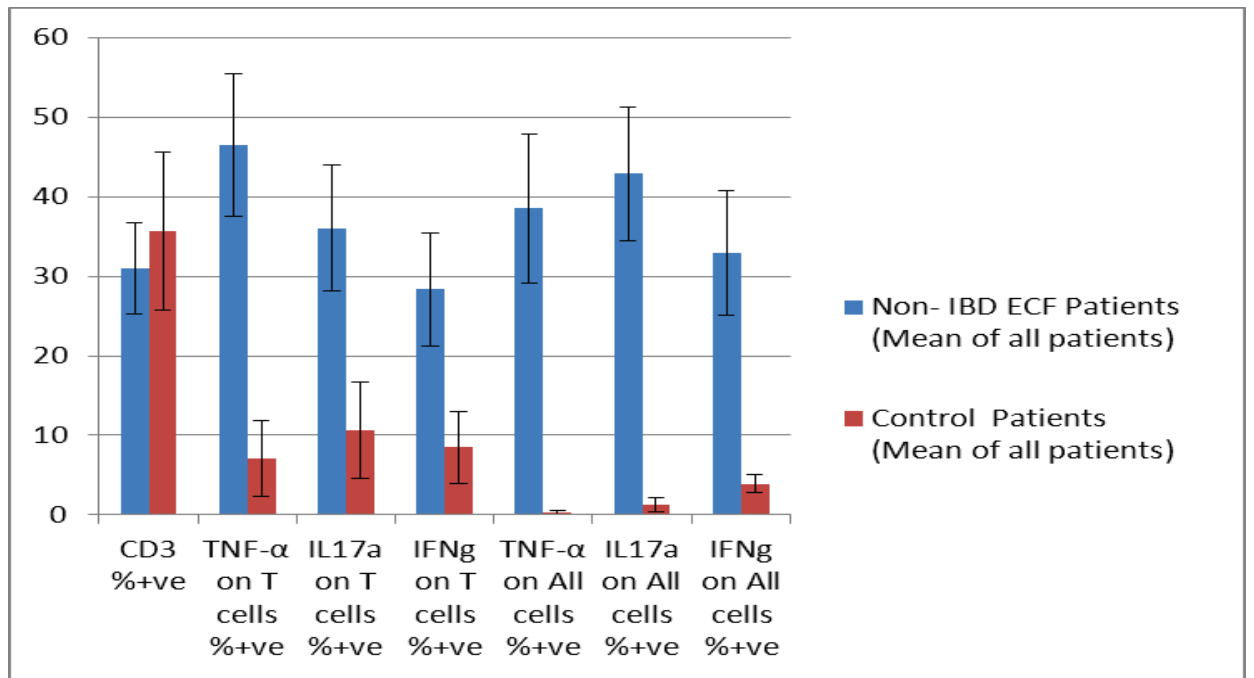
Table 5.41: Control Patients T cells and all cells % +ve ICS: Results of ICS on blood taken from ten control patients. TNF- α , IL17a and IFN γ antibodies were used for labelling cytokines intracellularly.

	Mean	SD	SEM
CD3 % +ve	35.62	31.27	9.89
CD3 IR	1.21	1.00	0.31
TNF-α on T cells % +ve	7.05	15.01	4.74
TNF-α on T cells IR	0.21	0.45	0.14
IL17a on T cells % +ve	10.63	19.12	6.04
IL17a on T cells IR	0.57	1.12	0.35
IFNγ on T cells % +ve	8.47	14.44	4.56
IFNγ on T cells IR	0.43	0.50	0.15
TNF-α on All cells % +ve	0.30	0.97	0.30
TNF-α on ALL cells IR	0.41	1.31	0.41
IL17a on All cells % +ve	1.29	2.58	0.81
IL17a on All cells IR	4.84	13.58	4.29
IFNγ on All cells % +ve	3.93	3.39	1.07
IFNγ on All cells IR	0.80	0.66	0.20

Table 5.42: Mean, SD & SEM of Table 5.41 data: Summary of results for data in Table 5.41 with mean, SD and SEM applied.

	Non- IBD ECF Patients (Mean of all patients)	Control Patients (Mean of all patients)
CD3 % +ve	30.93	35.62
TNF-α on T cells % +ve	46.50	7.05
IL17a on T cells % +ve	36.03	10.63
IFNγ on T cells % +ve	28.31	8.47
TNF-α on All cells % +ve	38.48	0.30
IL17a on All cells % +ve	42.86	1.29
IFNγ on All cells % +ve	32.94	3.93

Table 5.43: Comparison of Non - IBD ECF Patients and Control T cells and all cells % +ve ICS Means of all patients: Side by side results of ICS with TNF- α , IL17a and IFN γ antibodies.



p = 0.382 0.0005 0.009 0.015 0.0003 0.0001 0.0008

Fig 5.14: The means of TNF- α , IL17a, IFN γ on T cells and all cells: Bar chart with SEM comparing the mean % +ve of T cells and all cells with TNF- α , IL17a and IFN γ in non-IBD ECF versus control patient's blood.

5.10.3 Results of ICS on tissue biopsy and blood from IBD ECF patient

Only one patient was recruited with IBD ECF from whom tissue from the ECF tract and blood was taken. The results are shown in Tables 5.44 and 5.45.

TNF-α in DC % +ve	0
TNF-α in DC IR	0
IL12 in DC % +ve	0
IL12 in DC IR	0
TNF-α in ALL cells % +ve	0
TNF-α in ALL cells IR	0
IL12 in ALL cells % +ve	0
IL12 in ALL cells IR	0
CD3 % +ve	
CD3 IR	
TNF-α in T cells % +ve	81.08
TNF-α in T cells IR	1.1
IL17a in T cells % +ve	0
IL17a in T cells IR	0
IFNγ in T cells % +ve	75.27
IFNγ in T cells IR	1.24
TNF-α in All cells % +ve	73.62
TNF-α in ALL cells IR	1.29
IL17a in All cells % +ve	0
IL17a in All cells IR	0
IFNγ in All cells % +ve	68.42
IFNγ in All cells IR	1.52

Table 5.44: Tissue Biopsy: IBD ECF DC, T and all cells % +ve ICS: 1 patient:
Results of intracellular staining on biopsy taken from one IBD ECF patients. TNF- α and IL12 antibodies were used for labelling cytokines intracellularly on DC and all cells. TNF- α , IL17a and IFN γ antibodies were used for labelling cytokines intracellularly on T cells and all cells.

CLA on DC % +ve	0
CLA on DC IR	0
b7 on DC % +ve	67.65
b7 on DC IR	7.74
CLA on ALL cells % +ve	0
CLA on ALL cells IR	0
b7 on ALL cells % +ve	53.36
b7 on ALL cells IR	6.81
CD3 % +ve	65.8
CD3 IR	113.47
CLA on T cells % +ve	0
CLA on T cells IR	0
b7 on T cells % +ve	64.67
b7 on T cells IR	7.66
CLA on ALL cells % +ve	0
CLA on ALL cells IR	0
b7 on ALL cells % +ve	52.98
b7 on ALL cells IR	6.9
TNF- α in DC % +ve	0
TNF- α in DC IR	0
IL12 in DC % +ve	0
IL12 in DC IR	0
TNF- α in ALL cells % +ve	0
TNF- α in ALL cells IR	0
IL12 in ALL cells % +ve	0
IL12 in ALL cells IR	25.67
TNF- α in T cells % +ve	0
TNF- α in T cells IR	0
IL17a in T cells % +ve	0
IL17a in T cells IR	1339.67
IFN γ in T cells % +ve	0
IFN γ in T cells IR	1207.77
TNF- α in All cells % +ve	0
TNF- α in ALL cells IR	0
IL17a in All cells % +ve	0.06
IL17a in All cells IR	15.49
IFN γ in All cells % +ve	0.08
IFN γ in All cells IR	64.67

Table 5.45: Blood: IBD ECF DC, T and all cells % +ve Surface Markers and ICS: 1 patient: CLA and on blood DC, T cells and all cells. TNF- α , IL12, IL17a and IFN γ antibodies were used for labelling cytokines intracellularly.

5.10.4 Multiplex

5.10.4.1 Multiplex Tissue Results

Multiplex analysis was performed on tissue obtained from each of six patients with non-IBD ECF (Table 5.47) and control patients (Table 5.48), as well as the one IBD ECF patient (Table 5.49).

IL2, IL4, IL10, TNF- α , IFN γ and IL17a were all present in greater quantities, though not significantly, in non-IBD ECF tissue compared with normal control terminal ileum tissue (Figure 5.15).

IL6 was significantly ($p = 0.00009$) present in greater quantity in in non-IBD ECF tissue patients compared with normal control terminal ileum tissue patients (Figure 5.16).

PROT (pg/ml)							Mean	SD	SEM
IL2	6.53	6.53	6.57	7.34	20.70	6.53	9.04	5.72	2.33
IL4	11.97	10.65	11.81	12.99	27.97	9.41	14.13	6.88	2.81
IL6	3353.25	2378.69	3725.24	3684.33	2173.01	1036.49	2725.17	1057.46	431.70
IL10	8.42	8.75	79.74	11.92	10.40	7.37	21.10	28.77	11.74
TNF-α	6.42	6.24	6.64	9.76	9.73	6.25	7.50	1.74	0.71
IFNγ	9.97	9.97	9.78	13.58	16.26	10.05	11.60	2.70	1.10
IL17a	2.27	2.27	13.62	3.75	2.46	2.18	4.43	4.54	1.85

Table 5.46: Multiplex analysis showing tissue production of cytokines from Non-IBD ECF Tissue: Multiplex results in pg/ml of six patient's non-IBD ECF tissue. Cytokines assessed (IL2, IL4, IL10, TNF- α , IFN γ and IL17a).

PROT (pg/ml)							Mean	SD	SEM
IL2	6.59	6.65	6.53	6.56	8.21	6.55	6.85	0.66	0.27
IL4	9.76	10.35	9.89	10.09	13.30	9.69	10.51	1.38	0.56
IL6	121.22	66.46	192.71	154.96	565.82	203.45	217.44	177.83	72.60
IL10	10.88	8.71	8.43	9.76	16.35	10.79	10.82	2.89	1.18
TNF-α	6.24	6.37	6.31	6.41	7.00	6.45	6.46	0.27	0.11
IFNγ	11.64	10.47	9.98	10.79	12.56	10.07	10.92	1.00	0.40
IL17a	2.48	2.20	2.18	2.25	2.19	2.19	2.25	0.11	0.04

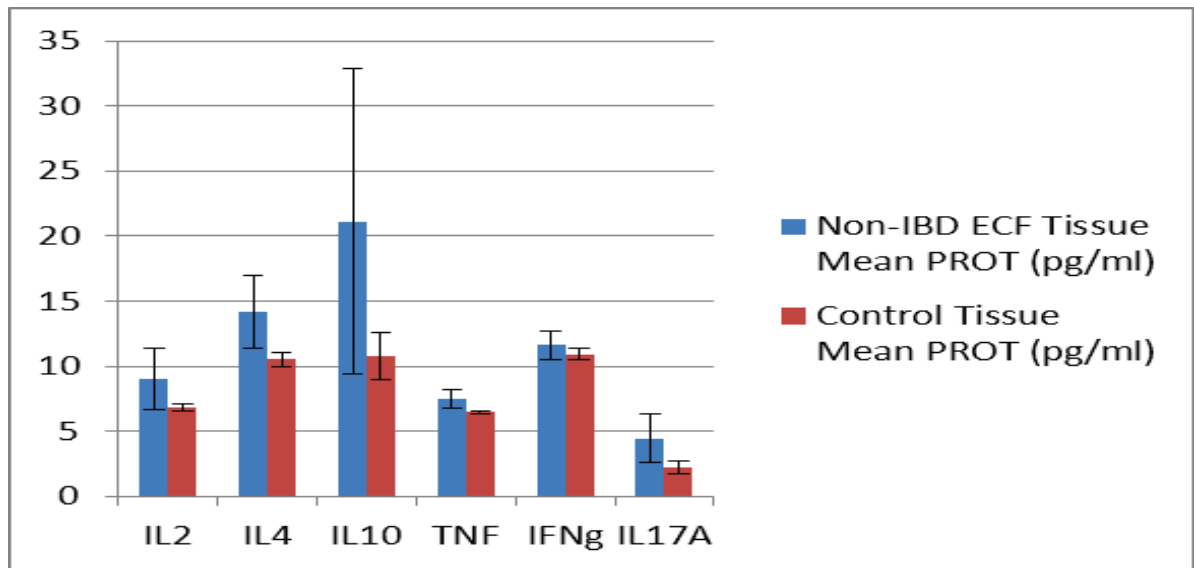
Table 5.47: Multiplex analysis showing tissue production of cytokines from Control Tissue: Multiplex results in pg/ml of six patients control tissue. Cytokines assessed (IL2, IL4, IL10, TNF- α , IFN γ and IL17a).

PROT (pg/ml)	
IL2	6.53
IL4	9.41
IL6	17.49
IL10	9.83
TNF-α	6.44
IFNγ	10.17
IL17a	2.26

Table 5.48: Multiplex analysis showing tissue production of cytokines from IBD ECF Tissue: Multiplex results in pg/ml of one patient IBD tissue. Cytokines assessed (IL2, IL4, IL10, TNF- α , IFN γ and IL17a).

	Non-IBD ECF Tissue Mean PROT (pg/ml)	Control Tissue Mean PROT (pg/ml)
IL2	9.04	6.85
IL4	14.13	10.51
IL10	21.10	10.82
TNF-α	7.50	6.46
IFNγ	11.60	10.92
IL17a	4.43	2.25

Table 5.49: Comparison of Non-IBD ECF Tissue and Control Tissue Means: Side by side comparison of multiplex cytokine results for non-IBD versus control patient tissue.

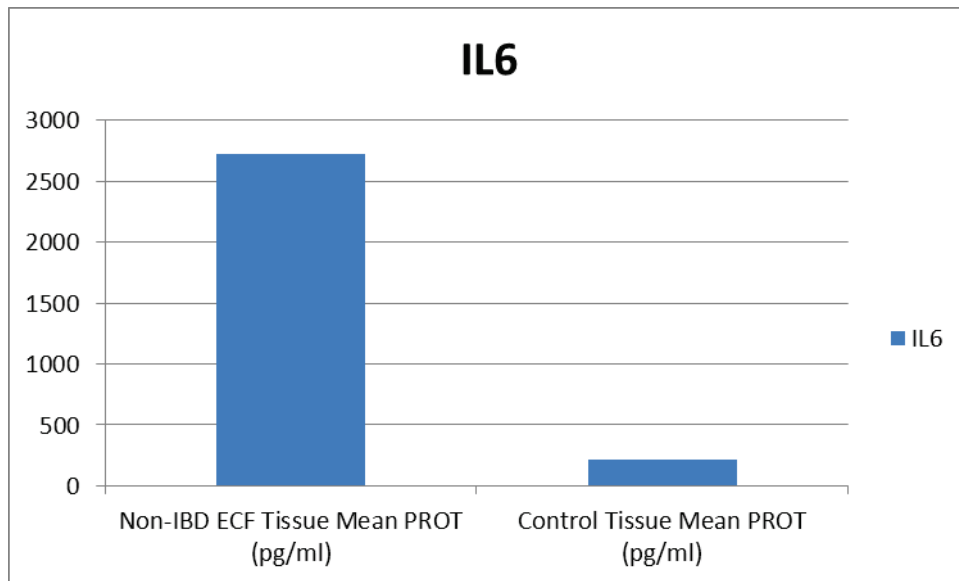


p = 0.187 0.117 0.202 0.089 0.287 0.134

Fig 5.15: Multiplex analysis showing mean tissue production of cytokines: Bar chart with SEM showing multiplex cytokine (IL2, IL4, IL10, TNF- α , IFN γ and IL17a) mean pg/ml results for non-IBD versus control patient tissue.

	Non-IBD ECF Tissue Mean PROT (pg/ml)	Control Tissue Mean PROT (pg/ml)
IL6	2725.17	217.44

Table 5.50: IL6 tissues multiplex analysis: Side by side comparison of multiplex cytokine IL6 results for non-IBD versus control patient tissue. (Shown in separate table to other cytokine results as values for both groups is proportionally much greater than for any of the other cytokines present in Table 5.49).



p = 0.00009

Fig 5.16: The mean of cytokine IL6: Bar chart with SEM showing multiplex cytokine IL6 mean pg/ml results for non-IBD versus control patient tissue.

5.10.4.2 Multiplex Blood Results

Multiplex analysis was performed on blood obtained from seven patients with non-IBD ECF (Table 5.51) and four control patients (Table 5.52), as well as the one IBD ECF patient (Table 5.53).

IL2, IL10, TNF- α , IL17a and IL6 were all present in greater quantities, though not significantly, in non-IBD ECF blood compared with normal control blood patients (Figure 5.17 and 5.18).

IL4 and IFN γ were not significantly but very mildly present, and in greater quantity in control blood than non-IBD ECF patients' blood (Figure 5.17).

PROT (pg/ml)								Mean	SD	SEM
IL2	7.600	6.538	6.538	7.811	7.031	6.538	6.538	6.942	0.555	0.209
IL4	9.589	9.419	11.051	9.419	9.419	10.706	10.288	9.985	0.691	0.261
IL6	44.716	48.379	3721.322	10.577	34.363	2492.788	3252.561	1372.101	1706.282	644.91
IL10	10.024	10.439	151.373	9.599	14.378	15.753	66.965	39.790	53.341	20.161
TNF-α	6.380	6.453	23.361	6.245	6.307	6.287	6.318	8.765	6.436	2.432
IFNγ	11.489	9.870	10.124	14.580	10.242	9.616	10.174	10.871	1.739	0.657
IL17A	2.296	2.176	2.745	3.711	2.254	2.171	2.188	2.506	0.568	0.214

Table 5.51: Multiplex analysis showing blood production of cytokines from Non-IBD Blood: Multiplex results in pg/ml of non-IBD ECF patient's blood. Cytokines assessed (IL2, IL4, IL10, TNF α , IFN γ and IL17a).

PROT (pg/ml)					Mean	SD	SEM
IL2	6.550	6.538	6.538	6.548	6.543	0.006	0.003
IL4	10.125	10.476	9.419	10.763	10.196	0.579	0.289
IL6	1681.084	2397.398	11.169	43.771	1033.356	1197.818	598.909
IL10	16.287	46.814	8.693	10.734	20.632	17.747	8.873
TNF-α	6.610	6.297	6.245	9.642	7.198	1.636	0.818
IFNγ	10.124	9.785	9.836	16.434	11.545	3.262	1.631
IL17A	2.217	2.214	2.166	6.860	3.364	2.330	1.165

Table 5.52: Multiplex analysis showing blood production of cytokines from Control Blood: Multiplex results in pg/ml of control patient's blood. Cytokines assessed (IL2, IL4, IL10, TNF- α , IFN γ and IL17a).

PROT (pg/ml)	
IL2	6.538
IL4	9.508
IL6	790.017
IL10	8.425
TNF-α	6.703
IFNγ	20.278
IL17a	3.241

Table 5.53: Multiplex analysis showing blood production of cytokines from IBD Blood: Multiplex results in pg/ml of one patient IBD blood. Cytokines assessed (IL2, IL4, IL10, TNF- α , IFN γ and IL17a).

	Non-IBD Blood Mean PROT (pg/ml)	Control Blood Mean PROT (pg/ml)
IL2	6.942	6.543
IL4	9.985	10.196
IL10	39.790	20.632
TNF-α	8.765	7.198
IFNγ	10.871	11.545
IL17a	2.506	3.364

Table 5.54: Comparison of Non-IBD Blood and Control Blood Mean PROT (pg/ml): Side by side comparison of multiplex cytokine results for non-IBD versus control patient blood.

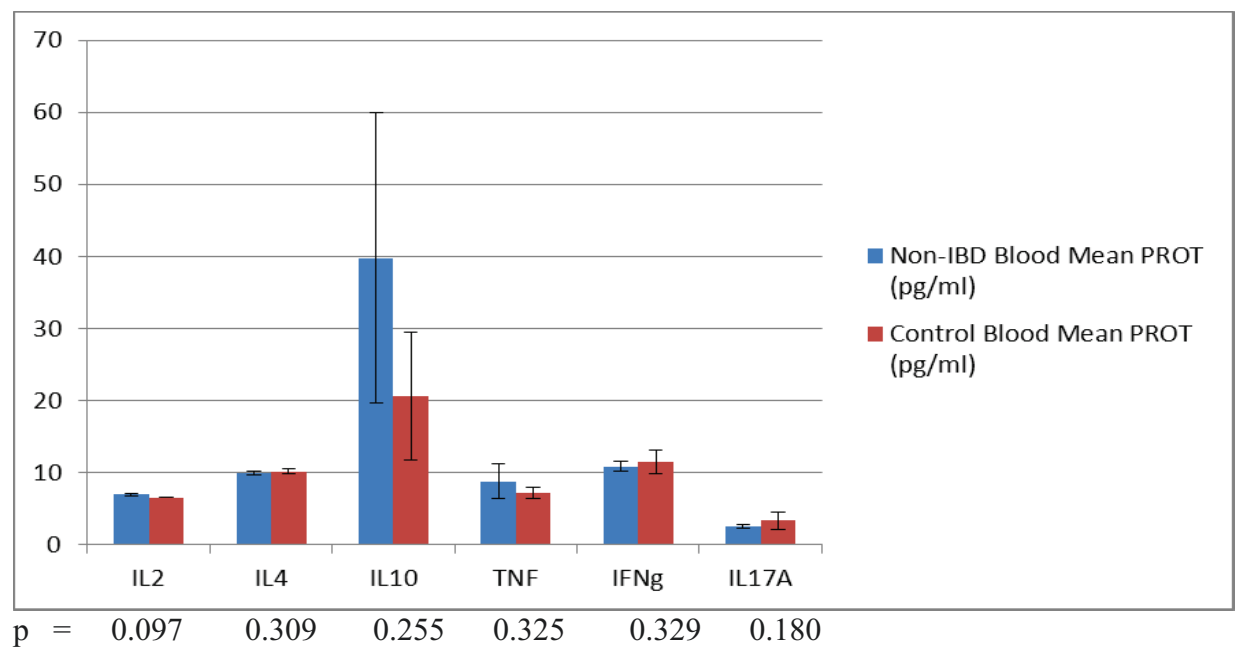
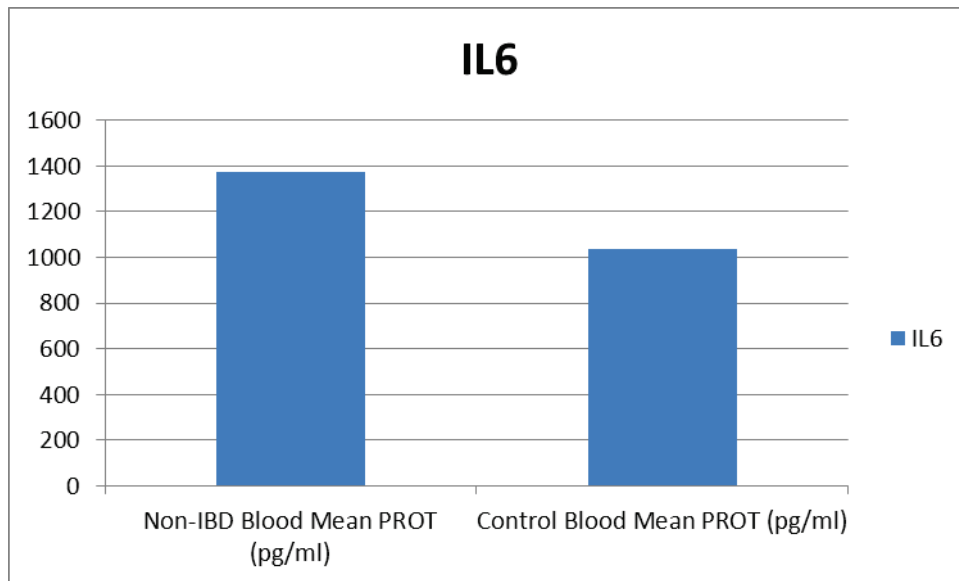


Fig 5.17: Multiplex analysis showing the means of blood production of cytokines: Bar chart with SEM showing multiplex cytokine (IL2, IL4, IL10, TNF- α , IFN γ and IL17a) mean pg/ml results for non-IBD versus control patient blood.

	Non-IBD Blood Mean PROT (pg/ml)	Control Blood Mean PROT (pg/ml)
IL6	1372.101	1033.356

Table 5.55: IL6 blood multiplex analysis: Side by side comparison of multiplex cytokine IL6 results for non-IBD versus control patient tissue. (Shown in separate table to other cytokine results as values for both groups is proportionally much greater than for any of the other cytokines present in Table 5.54).



$p = 0.368$

Fig 5.18: The mean of cytokine IL6: Bar chart with SEM showing multiplex cytokine IL6 mean pg/ml results for non-IBD versus control patient blood.

SUMMARY OF RESULTS

TNF- α

Tissue: On-going production of TNF- α by DC ($p = 0.0007$), all viable cells ($p = 0.001$), and T cells ($p = 0.04$) was significantly higher in non-IBD ECF than that from control tissue.

Blood: On-going production of TNF- α by DC ($p = 0.0008$), all viable cells ($p = 0.001$) and T cells ($p = 0.0005$) was significantly higher from non-IBD ECF blood compared with control.

IL12

Tissue: On-going production of IL12 by DC ($p = 0.022$) and all viable cells ($p = 0.008$) was significantly higher in non-IBD ECF than from control tissue.

Blood: On-going production of IL12 by DC ($p = 0.025$) from non-IBD ECF blood was significantly higher than from control.

IFN γ

Tissue: On-going production of IFN γ by T cells ($p = 0.016$) from non-IBD ECF was significantly higher than control tissue.

Blood: On-going production of IFN γ by T cells ($p = 0.015$) and all viable cells ($p = 0.0008$) was significantly higher in non-IBD ECF blood compared with control.

IL17a

Tissue: On-going production of IL17a in all viable cells ($p = 0.002$) was significantly higher from non-IBD ECF compared with control tissue.

Blood: On-going production of IL17a by T cells ($p = 0.009$) and all viable cells ($p = 0.0001$) was significant higher in non-IBD ECF blood compared with control blood.

CLA surface marker

Blood: There was a greater percentage of DC, T cells, and significantly all viable cells ($p = 0.036$), which had the presence of CLA surface marker in non - IBD ECF patient's blood, then from controls.

Multiplex

Tissue: IL2, IL4, IL10, TNF- α , IFN γ , IL17A and IL6 were all present in greater quantities, in non-IBD ECF tissue compared with normal control terminal ileum tissue.

Blood: IL2, IL10, TNF- α , IL17A and IL6 were all present in greater quantities, in non-IBD ECF blood compared with normal control blood patients.

5.11 DISCUSSION

To the best of our knowledge, there are no studies in the literature regarding the assessment of immunological response in ECF of IBD or non-IBD ECF.

This study has shown the on-going production of TNF- α from dendritic cells (DC) ($p = 0.0007$), putative monocyte and B cell populations ($p = 0.04$) and CD3+ T cells ($p = 0.04$) was significantly higher in non-IBD ECF than that from control tissue. Larger sample numbers will be required to confirm statistical validity.

The production of TNF- α by DC and all viable cells from non-IBD ECF patients was significantly higher ($p = 0.0008$ and $p = 0.001$ respectively) than that by from control blood patients.

From blood ICS experiments, there were significant increased difference in the production of TNF- α by T cells ($p = 0.0005$) and all viable cells ($p = 0.0003$) in non-IBD ECF blood compared with control blood patients.

DC undergo maturation and migrate to draining lymph nodes in response to signals that indicate local “danger” or tissue damage. This would be the case in non-IBD ECF. These signals include cytokines such as TNF- α . During maturation, DC up regulate their expression of MHC class II and costimulatory molecules and acquire the ability to stimulate naïve T cells. Communication between DC and T cells is a two way process with important interactions occurring particularly between members of the TNF and TNF receptor protein families. These interactions serve to further activate the DC and regulate their survival (222).

The population of gut DC is a dynamic one, with cells constantly trafficking into and out of the gut compartment. Migration of gut DC can be further increased by inflammatory stimuli, including TNF- α (222).

IL12 production was also significantly higher from DC and all viable cells in non-IBD ECF tissue. On-going production of IL17a, not in T cells, but in all viable cells from non-IBD ECF was significantly higher compared with all viable cells from control terminal ileum tissue.

Production of IL12 by DC from non-IBD ECF blood patients was significantly ($p = 0.025$) higher than that by DC from control blood patients. All viable cells from non-IBD ECF blood also produced non-significantly higher level of IL12 ($p = 0.088$) compared with all viable cells from control blood patients.

Hart et al showed that in IBD, DC are activated, and more DC produce pathologically relevant cytokines (223). The study also showed that in Crohn's disease, more colonic DCs produced IL12 and IL6. As our study has revealed a higher level of IL12 produced from DC in non-IBD ECF compared to control tissue, it would be useful to compare the level directly with IBD ECF in a future study. Our study did not investigate the production of IL6 from DC tissue.

IFN γ was significantly higher in T cells, though not in all viable cells, from non-IBD ECF compared with control terminal ileum tissue. Also the on-going production of IFN γ was significantly higher by T cells ($p = 0.015$) and all viable cells ($p = 0.0008$) in non-IBD ECF blood compared with control blood patients. Murine Peyer's patch DC up regulate IL10 and generate T cells that produce high levels of IL4 and IL10 and less IFN γ . In contrast, DC from the spleen generate T cells producing

predominately IFN γ (222). Patients with IBD even in an inactive state of disease exert an increased capacity for IFN γ induction in T cells mediated by intestinal epithelial cells (224).

From blood experiments, there was a greater percentage of DC, T cells, and significantly on all viable cells ($p = 0.036$), which had the presence of CLA surface marker in non - IBD ECF patient's blood, then from control patient's. There was no significant difference in the gut homing marker. Mann et al has shown that blood DC mostly express both gut and skin homing markers, indicating potential to migrate to both major immune surface organs, and induced multi-homing T cells (225).

There were also significant differences with increased production of IL17a by T cells ($p = 0.009$) and all viable cells ($p = 0.0001$) in non-IBD ECF blood compared with control blood patients.

The main function of IL17a appears to be the regulation of local tissue inflammation via the coordinated expression of pro-inflammatory and neutrophil-mobilizing cytokines and chemokine's. High concentrations of IL17a are associated with inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.

Therapeutic monoclonal antibodies against human IL17a have shown promise for the treatment of psoriasis and rheumatoid arthritis. This is particularly useful as one-third of rheumatoid arthritis patients do not respond to current TNF inhibition treatments and to the patients that lose the initially good response to TNF inhibition treatment.

Wedebye et al found that colitis development was associated with an increase in IL17a producing TH17 cells in spleen, mesenteric lymph nodes, and lamina propria.

The study suggests that IL17a blockade as a potential strategy in inflammatory bowel disease therapy (226).

There were limitations to the number of samples on which multiplex analysis could be performed. Samples from the non-IBD and control groups were selected consecutively to avoid any bias.

IL2, IL4, IL10, TNF- α , IFN γ , IL17A and IL6 were all present in greater quantities, in non-IBD ECF tissue compared with normal control terminal ileum tissue. IL2, IL10, TNF- α , IL17A and IL6 were all present in greater quantities, in non-IBD ECF blood compared with normal control blood patients. Thus results of our multiplex analysis for both tissue and blood experiments have shown greater quantities of pro-inflammatory cytokines to be present in the non-IBD ECF group as one would expect in response to inflammation.

Although the production of TNF- α in the intestinal mucosa is increased in patients with Crohn's disease, this study is the first to show the presence of TNF- α in a non-IBD ECF. Hence these data may explain the clinical results of Infliximab used in the small case series of three patients which reported healing of persistent ECF not associated with IBD to have healed following a single infusion of infliximab (227).

The aim of this study was to assess and compare the immunological factors in three groups of patients. Unfortunately as the study progressed it became evident that this would be difficult to do, and this was one of the limitations of the study. Patients with inflammatory bowel disease ECF had at some stage used medications including steroids, immunosuppressive drugs such as Azathioprine, Mesalazine or 5-aminosalicylic acid (5-ASA), or used anti-TNF- α agents such as Infliximab or

Humera (Adalimumab). We decided to continue with the study to compare patients with non-IBD ECF with normal control patients. Although an IBD-ECF group was not used, it is well known that TNF- α levels are higher in Crohn's disease tissue compared to healthy controls and anti-TNF- α therapy has shown efficacy in the closure of fistula associated with IBD (228).

We acknowledge that this study compared tissue from the ECF in the non-IBD group and tissue from small bowel and from normal control group. Ideally tissue should be compared from similar sites, but in essence as control patients are 'normal' then no ECF is available in these cases. For future work one could compare ECF tissue and small bowel tissue from the same patient.

The study was limited to ten patients in each of non-IBD ECF and the control group. This was due in difficulty in co-ordinating times for when patients were undergoing a laparotomy and surgical resection of the ECF or colonoscopy and the availability of laboratory facilities. For each patient, after specimens were taken laboratory facilities were required for three to four days continuously.

Experiments were also only possible when all antibodies and FACS reagents were available, and this had to be identified prior to taking specimens from patients.

5.12 CONCLUSION

A trend has been found with a higher on-going production of cytokines in non-IBD ECF compared with control terminal ileum tissue. The addition of a greater number of samples may show significant differences between the two groups of patients and provide validity to this trend.

Data from this study may provide evidence for the potential use of anti-TNF- α agents such as Infliximab in the treatment of non-IBD ECF patients. Recommendations are for a pilot study to assess whether this can be an alternative option to benefit an already surgically challenging group of patients. A research protocol will have to be developed for the safe administration of an anti-TNF- α agent. This will have to be submitted and approved by the MHRA (Medicines and Healthcare Products Regulatory Agency) as the product though licensed for use, would have to be used out of its indication in patients with non-IBD ECF. Any positive findings would equate to a major medical advance with a new use for anti-TNF- α agents.

In conclusion, this data may provide evidence for the potential use of anti-TNF- α agents in the treatment of non-IBD ECF.

CHAPTER 6

DISCUSSION

This thesis reviewed and investigated several aspects of the management of enterocutaneous fistula.

This thesis has shown that the best management of ECF, particularly complex fistulas, is in specialist units where there is a multidisciplinary structured approach. Surgical management of ECF at St. Mark's National IF Unit is safe and improving.

The highest incidence of ECF development occurs following abdominal surgery particularly in the presence of Crohn's disease. The audit confirmed this in our unit, with the most common aetiology for ECF formation being in surgery performed in patients with a background of inflammatory bowel disease.

Management of persistent ECF has been divided into five main categories; specialised nutritional support, control of sepsis, judicious use of pharmacological agents, wound / stoma care and surgery.

Definitive surgery with ECF resection should be deferred until there is re-epithelisation of the abdominal wall, eradication of sepsis, maturation of adhesions, full bowel mapping has been performed and attainment of an adequate nutritional and general medical status. Adequate nutritional status is when the patient is anabolic, evidenced by gaining weight, increasing muscle mass and muscle strength. General medical status relates to stability of the underlying conditions as well as correction of any electrolyte and metabolic derangements. This process can take over six months.

A period of home leave prior to a planned date for surgery can be both mentally and physically beneficial.

Vacuum assisted pumps are avoided in our unit due to experience of increased ECF formation. The use of non-absorbable meshes is also avoided because ECF surgery is often performed in contaminated or grossly infected wounds. When required a Vicryl (Ethicon, Somerville, NJ, USA) mesh or Strattice™ (Lifecell Corporation, Branchburg, NJ, USA) are used. Either a stapled and over sewn or hand sewn interrupted anastomosis with 4-0 Ethibond (Ethicon, USA) are performed.

The retrospective audit of our unit revealed the majority of patients underwent definitive surgery. There were improved overall healing rates following both surgery and conservative management in the current series compared with the previous series, with also improvement in the 30 day post fistula resection mortality

Multivariate analysis revealed co-morbidity, source of referral and aetiology had significant associations with ECF healing.

Patients from external sources were less likely to heal. This may be due to a greater number of operations being performed at external institutions prior to the decision to refer to specialist care.

This thesis has included the first study and design of a scoring system for predicting ECF healing. The scoring systems devised were both validated. They can now be applied on all patients at their first presentation with an ECF. This is without knowing if the patient will require medical treatment alone or progress to requiring surgery.

It is noted that the course of Crohn's fistula and fistula associated with UC are different. The total number of patients with UC related fistula was low in our series and it was difficult to independently assess this. Assessing their individual impact on clinical outcome would be useful for any future study as well as the potential impact on the scoring system.

The scoring systems can provide further information to aid clinical management and decision making for the multidisciplinary team and predict the possible long term outcome for patients at external institutions. They have the potential to predict ECF healing and can be a useful decision making tool. It would be useful to assess the scoring systems at other centres in this regard to see whether they have a role in future clinical practice.

Future work will involve contacting units both nationally and internationally to apply the scoring system. This will allow assessment of its effectiveness and modifications to take place.

Meta-analyses of nine randomised control trials have revealed somatostatin and octreotide increase the likelihood of fistula closure. Both were shown to be beneficial in reducing the time to fistula closure. Neither has an effect on mortality. The risk ratio for somatostatin was higher than the risk ratio for analogues. This may suggest that somatostatin could be better than analogues in relation to the number of fistulas closed and time to closure. The best results were following the use of somatostatin or long acting somatostatin analogue, lanreotide.

Our meta-analysis is limited due to several studies having small numbers of patient's recruited, wide variability in study designs and lack of clear definitions relating to

recording of study outcomes: fistula closure, time of fistula closure, fistula output reduction, complication rate and mortality. The findings may be compounded by limitations such as different treatment protocols, patient selection, population characteristics and methodology used to determine the actual time of closure of fistula for each trial. Further studies are warranted directly comparing both these agents to establish whether there is a significant difference between them and to corroborate these apparent findings.

Recommendation is for a large randomised control trial with adequate patient numbers or a multi-centre trial with well-defined criteria and agreed parameters between the participating centres. If a clear option is identified this would benefit an already surgically challenging group of patients associated with a high morbidity and mortality where it is deemed standard medical treatment has failed.

The immunological studies reveal encouraging results and a trend has been found in the on-going production of cytokine, in particular TNF- α , in non-IBD ECF compared with control terminal ileum tissue. The data is encouraging and may provide evidence for the potential use of anti-TNF- α agents in the treatment of non-IBD ECF.

In section 6.1 a future pilot study has been designed with the aim to identify a potential novel therapy for patients with persistent enterocutaneous fistula not associated with inflammatory bowel disease.

FUTURE DESIGN PROTOCOL FOR A PILOT STUDY

6.1 INTRODUCTION

Our pilot study aims to identify a potential novel therapy for patients with persistent enterocutaneous fistula not associated with inflammatory bowel disease. This would be an alternative option for the treatment of a surgically challenging group of patients who have a high morbidity and mortality with operative treatment.

It has been suggested in a single article that it is possible to treat non-IBD ECF patients with Infliximab (175).

6.2 Hypothesis

Anti-TNF- α antibody (Infliximab) is effective in the treatment of patients with persistent enterocutaneous fistula due to non-inflammatory bowel disease who have failed to heal following supportive therapy.

6.3 Aim

- To heal persistent enterocutaneous fistula in non-inflammatory bowel disease patients using infliximab.
- To assess the cytokine milieu in non-inflammatory bowel diseases patients with enterocutaneous fistula and their response to infliximab.

6.3.1 Primary end points

- The primary end point is defined as a reduction of 50 per cent or more in the number of draining fistula observed at ten weeks as compared to baseline.

6.3.2 Secondary end points

- Closure of all fistulas.
- Reduction in fistula volume output.
- Quality of life scores to assess morbidity pre and post treatment with infliximab. SF-36 (short form health survey with 36 questions), and visual analogue scores will be assessed.
- Serum levels of pro and anti-inflammatory cytokine markers including TNF- α , pre and post treatment with infliximab.
- Levels of pro and anti-inflammatory cytokine markers including TNF- α , in enterocutaneous fistula mucosa, fistula output and stool, pre and post treatment with infliximab.
- Magnetic Resonance Imaging (MRI) to assess radiological changes in response to treatment with infliximab.

6.4 METHODS

6.4.1 Inclusion criteria

- Consent
- 18+ years
- Single or multiple enterocutaneous fistula for at least three months duration as a result of non-inflammatory bowel disease, not responding to standard treatments.
- Enterocutaneous fistula confirmed to be due to non-inflammatory bowel disease by radiography, endoscopy or pathological examination.

6.4.2 Exclusion criteria

- Current sepsis or abscess
- Previous treatment with infliximab.
- History of allergy to murine proteins.
- Treatment with investigational agents or the use of any medication which reduces the concentration of TNF- α within three months before enrolment.
- History of Crohn's disease or Ulcerative Colitis.
- Patients will also be excluded if there is evidence of latent or active tuberculosis (they will require a clear chest X-ray and evidence of previous BCG vaccine)
- Females who are pregnant or breast feeding.
- Moderate to severe heart failure.
- Claustrophobia or other contraindication to MRI.
- Malignancy.
- Evidence of distal bowel obstruction from the enterocutaneous fistula.

6.4.4 Study design

This will be a prospective pilot study of patients with enterocutaneous fistula secondary to non-inflammatory bowel disease, who undergo this novel treatment with infliximab. We aim to pilot this study in 20 patients.

Following consultation with a statistician we conclude that 13 patients are required for a statistically significant study. The primary outcome is change in the number of draining fistula. The sample size was based on showing a change in the number of fistula from pre to post treatment. Patients would have a mean of two fistulas before treatment, and it is expected that there will be a 50% reduction to a mean of one fistula after treatment. It is expected that the standard deviation for the change in the number of fistula will be 1.25. With a 5% significance level and 80% power, it is calculated that 13 patients are required for the study.

The change in the number of fistula from pre to post treatment will be examined using the paired T-test or the Wilcoxon matched pairs test, depending on the distribution of the change in number of fistula over time.

6.4.4 Study Procedure

An eligible patient will be identified in the outpatient or ward setting and offered participation in this study.

The study will be explained and Infliximab and the alternative options will be discussed and patients encouraged to ask questions for discussion. A trial information sheet will be provided.

Patients who decide to enter the study will be required to sign a consent form. The patients, who decline to enter the study, will be treated with the normal standard of clinical care.

Participants will be given the opportunity for freely informed consent, in accordance with the declaration of Helsinki.

Patients entering the study will require to undergo a full medical history and clinical examination. Base characteristics as follows will be noted; age (years), weight (kg), BMI, ethnicity, male/female, duration of non-inflammatory bowel disease, area of involvement (gastric, duodenum, jejunum, ileum, colon), details of previous operative procedures and segmental resections. A prior history of vaccination with BCG, history of Tuberculosis (TB) or exposure to TB will be confirmed.

A routine physical examination will be performed and the number and location of the enterocutaneous fistulae will be noted. All fistulas will be distinctly identifiable; drawings as well as photographs will be used to document the sites of disease.

A chest X-ray (CXR) and routine bloods including full blood count (FBC), urea and electrolytes (U&E's), liver function tests (LFT's), C-reactive protein, Erythrocyte sedimentation rate (ESR) will be performed, as part of standard clinical care.

Patients will be contacted and at an appropriate time and requested to attend the Infliximab infusion unit at St. Mark's Hospital.

0 weeks: (Start of study)

Patients will be asked to complete a quality of life questionnaire prior to treatment.

We will use the validated and reliable SF-36. This is a short-form health survey with 36 questions. It uses an eight scale profile of functional health and wellbeing scores as well as psychometrically based physical and mental health summary measures and a preference based health utility index. It is a generic measure, as opposed to one that targets a specific age, disease, or treatment group.

Visual analogue scores will be used to evaluate quality of life with respect to bowel function (e.g. loose stools and abdominal pain), systemic symptoms (fatigue and altered sleep pattern), social function (work attendance and the need to cancel social events), emotional status (angry, depressed, or irritable), and their perspective on body image.

A blood test will be taken as part of standard clinical care, for baseline FBC, U&E's, LFT's including albumin, CRP, ESR. We will also test for pro and anti-inflammatory cytokine markers including TNF - α levels.

Magnetic Resonance Imaging (MRI) will be performed as part of standard clinical care to image and assess the anatomy of the enterocutaneous fistula.

A biopsy of the enterocutaneous fistula tract will be taken to assess for baseline pro and anti-inflammatory cytokine markers including TNF - α levels (Biopsy 1). Biopsy forceps will be used to take 1mm tissue samples under direct vision from the fistula tract. This procedure requires no anaesthetic and is performed routinely and frequently as part of standard clinical care during procedures such as oesophago gastric duodenoscopy (OGD), flexible sigmoidoscopy and colonoscopy.

Following this, a standard 5mg of Infliximab per kilogram bodyweight will be administered intravenously as defined by the manufacturer, and according to departmental guidelines (Dose 1). This will be administered in the Infliximab infusion unit by a dedicated Inflammatory Bowel Disease Clinical Nurse Specialist. Routine monitoring and surveillance will be performed as is part of normal standard clinical care before, during and after administration of infliximab.

A daily record of fistula output volume will be kept. We will then arrange for the patient to return to the Infliximab infusion unit at two weeks from the start date.

At 2 weeks

A blood test will be taken as part of standard clinical care, for FBC, U&E's, LFT's including albumin, CRP, ESR. We will also test for pro and anti-inflammatory cytokine markers including TNF - α levels.

The second dose of 5mg of Infliximab per kilogram bodyweight will be administered intravenously as defined by the manufacturer, and according to departmental guidelines (Dose 2). This will be administered again in the Infliximab infusion unit by a dedicated nurse with experience in Infliximab infusion. Routine monitoring and surveillance will be performed as is part of normal standard clinical care before, during and after administration of Infliximab.

A daily record of fistula output volume will be kept. We will then arrange for the patient to return to the Infliximab infusion unit at six weeks from the start date.

At 6 weeks

A blood test will be taken as part of standard clinical care, for FBC, U&E's, LFT's including albumin, CRP, ESR. We will also test for pro and anti-inflammatory cytokine markers including TNF- α levels.

The third and final dose of 5mg of Infliximab per kilogram bodyweight will be administered intravenously as defined by the manufacturer, and according to departmental guidelines (Dose 3). This will be administered in the Infliximab infusion unit by a dedicated nurse. Routine monitoring and surveillance will be performed as is part of normal standard clinical care before, during and after administration of infliximab.

A daily record of fistula output volume will be kept. We will then arrange for the patient to return to the Infliximab infusion unit at eight weeks from the start date

At 8 weeks

A blood test will be taken for FBC, U&E's, LFT's including albumin, CRP, ESR. We will also test for pro and anti-inflammatory cytokine markers including TNF- α levels.

A biopsy of the enterocutaneous fistula tract will be taken (if still present) to assess for baseline pro and anti-inflammatory cytokine markers including TNF- α levels (Biopsy 2). Biopsy forceps will be used to take 1mm tissue samples under direct vision from the fistula tract. This procedure again requires no anaesthetic and is performed routinely and frequently as part of standard clinical care during procedures such as oesophago gastric duodenoscopy (OGD), flexible sigmoidoscopy and colonoscopy.

We will then arrange for the patient to return at ten weeks from the start date.

At 10 weeks

Patients will be asked to complete both the post treatment SF-36 survey and Visual analogue score questionnaire, just as they had done prior to commencement of treatment.

A blood test will be taken for FBC, U&E's, LFT's including albumin, CRP, ESR. We will also test for pro and anti-inflammatory cytokine markers including TNF- α levels.

A post treatment MRI will be performed as part of standard clinical care to assess the anatomy and degree of healing of the enterocutaneous fistula which has taken place. We will be able to ascertain information relating to tract length, diameter and volume.

At 14, 18, and 26 weeks

Patients will be reviewed in outpatient clinic for routine follow up and assessment at 14, 18 and 26 weeks from the start date.

If at any point the treatment does not seem to be working or the patient does not want to continue, they can leave the trial and be treated exactly as they normally would.

Table 6.1: Summary of proposed study protocol*Weeks*

	Screening	0	2	6	8	10	14	18	26
Clinical test / Management									
Assess eligibility	✓								
Consent for study	✓								
SF-36 Survey		✓				✓			
Visual analogue scores		✓				✓			
CXR	✓								
Bloods: FBC, U&E's, LFT's including albumin, CRP, ESR	✓	✓	✓	✓	✓	✓			
Pro and anti -inflammatory cytokine markers including TNF- α levels		✓	✓	✓	✓	✓			
MRI		✓				✓			
Fistulae tract mucosa biopsy		✓			✓				
Infusion of infliximab at unit		✓	✓	✓					
Outpatient Clinic Follow up					✓	✓	✓	✓	✓

✓ : Clinical test / Management to take place

6.5 Safety and Monitoring

There is potential for increased pain and discomfort from the additional blood tests and biopsies of the fistula tract. In both cases the patient will be informed during the initial explanation, consent stage, and prior to the procedure, that they may feel a very 'small pinch or prick' sensation as the blood test or biopsy is being performed. There is minimal risk of very light bleeding following either test, and this would be controlled as is in normal standard clinical practice with the application of gentle pressure until any bleeding subsides.

There is a small risk of an infection occurring at the site of blood taking and this will be minimised by the use of standard hospital guidelines in relation to use of alcohol hand gel and sterile technique as is part of normal standard clinical care.

There is potential for inconvenience by the attendance for an extra MRI scan (post treatment scan) and attendance for extra outpatient clinic appointments.

There is risk of ionising radiation from the one CXR performed. If a recent CXR has been performed there will be no need for a further CXR.

The CXR estimated dose constraint is ~ 0.02 mSv (Effective Dose) per individual. Risk assessment has been completed by Medical Physicist & Head of Physics Department Consultant. The average annual natural background radiation dose in the UK is 2.2 mSv. In this context, therefore, the study "Dose Constraint" of ~ 0.02 mSv is equivalent to about three days of exposure to the UK natural background radiation. Any associated risk would be considered to be trivial.

Safety will be assessed in terms of the incidence of adverse events and changes in vital signs and routine laboratory measures. Patients will be monitored for adverse events during each infusion and at each study visit.

Patients are pre medicated with Hydrocortisone 200mg, approximately 30 minutes prior to commencement of the Infliximab Infusion. Infliximab is prepared as per manufactures guidelines and given in 250mls of Normal Saline. Infliximab will be given over two hours using a giving set and 1.2-micron filter via an infusion pump as per departmental protocol.

All patients who are admitted for Infliximab will have a set of observation done before commencement of the infusion, as per departmental guidelines. Observations will include: BP, pulse, temperature, respiratory rate, and oxygen saturation.

Observations will be performed every 15 minutes for the 1st hour and every 30 minutes for the 2nd hour. Hourly observations for two hour post infusion. Intravenous access will remain in-situ for each patient for the appropriate post infusion period. Resuscitation facilities will be available and easily accessible as in normal standard of care.

6.6 Infliximab experience and risks

Besides infusion reactions and delayed hypersensitivity reactions, other infrequent but serious toxicities related to TNF- α neutralizing therapies have emerged. The most worrying are infectious complications, such as tuberculosis (TB) and respiratory tract infections, and the theoretical risk of cancer and lymphoma (229-231). In particular, an increased risk for TB has been disclosed in post-marketing experience (229, 232), but training, screening and monitoring procedures, together with prophylaxis can markedly reduce the incidence (229, 233). We will closely monitor for infections including TB before, during and after treatment with infliximab.

A large-scale post-marketing surveillance study was carried out to determine the safety profile of Infliximab in 5000 Japanese patients with rheumatoid arthritis. Serious infusion reactions were observed in 0.5% (234).

Five hundred patients treated with Infliximab at the Mayo Clinic were reviewed. They concluded that short and long term Infliximab therapy was generally well tolerated.

However, clinicians must be vigilant for the occurrence of infrequent but serious events, including opportunistic infection and sepsis, and autoimmune disorders (230).

The underlying risk of developing malignancies among patients with severe or chronically active Crohn's disease in need of Infliximab treatment is not known but the finding of a 1.5% annual incidence of lymphoma emphasises the need for vigilant surveillance with respect to this malignant complication (235). Discussion regarding the risk of malignancy versus the length of the course of treatment will take place.

The TREAT registry has prospectively evaluated patients for pre specified safety related outcomes in Crohn's disease. 6290 patients were enrolled. Mortality rates were similar between Infliximab - and non-Infliximab treated patients. The increased risk for serious infection observed with Infliximab likely was owing to disease severity and prednisone use (236).

Details of Infliximab and recommendations are available from the Summary of product characteristics (SmPC) (231).

Infliximab has been associated with acute infusion-related reactions, including anaphylactic shock, and delayed hypersensitivity reactions. Acute infusion reactions including anaphylactic reactions may develop during (within seconds) or within a few hours following infusion. If acute infusion reactions occur, the infusion will be immediately stopped. Emergency equipment, such as adrenaline, antihistamines, corticosteroids and an artificial airway are available in the Infliximab infusion unit.

Patients may be pre-treated with an antihistamine, hydrocortisone and/or paracetamol to prevent mild and transient effects. In our unit we pre-treat using hydrocortisone as standard.

Infusion-related reactions: An infusion-related reaction was defined in clinical studies as any adverse event occurring during an infusion or within one to two hours after an infusion. In clinical studies, approximately 20% of Infliximab-treated patients compared with approximately 10% of placebo treated patients experienced an infusion-related effect. Approximately 3% of patients discontinued treatment due to infusions reactions and all patients recovered with or without medical therapy (231).

Patients will be monitored closely for infections including tuberculosis before, during and after treatment with Infliximab. Because the elimination of infliximab may take up to six months, monitoring will be continued throughout this period. Patients would be reviewed at outpatient clinic as is the normal clinical practice.

Suppression of TNF- α may mask symptoms of infection such as fever. Therefore early recognition of atypical clinical presentations of serious infections is critical in order to minimize delays in diagnosis and treatment.

Patients taking TNF-blockers are more susceptible to serious infections. Tuberculosis, bacterial infections, including sepsis and pneumonia, invasive fungal infections, and other opportunistic infections have been observed in patients treated with infliximab. Some of these infections have been fatal; the most frequently reported opportunistic infections with a mortality rate of > 5% include pneumocystosis, candidiasis, listeriosis and aspergillosis (230, 231, 235).

Patients who develop a new infection while undergoing treatment with Infliximab will be monitored closely and managed accordingly. Administration of Infliximab will be discontinued if a patient develops a new serious infection or sepsis, and appropriate antimicrobial or antifungal therapy will be initiated until the infection is controlled.

In clinical studies 36% of infliximab-treated patients were treated for infections compared with 25% of placebo-treated patients (236).

Before starting treatment with Infliximab, all patients will be evaluated for both active and inactive ('latent') tuberculosis. This evaluation will include a detailed medical history with personal history of tuberculosis or possible previous contact with tuberculosis and previous and/or current immunosuppressive therapy. A CXR will be performed in patients as is the current departmental guideline.

If active or latent tuberculosis is diagnosed, Infliximab therapy will not be initiated. The patient will not be eligible for the trial and a physician with expertise in the treatment of tuberculosis will be consulted for further management.

All patients will be informed to seek medical advice if signs/symptoms suggestive of tuberculosis (e.g. persistent cough, wasting/weight loss, low-grade fever) appear during or after Infliximab treatment.

Reactivation of hepatitis B has occurred in patients receiving a TNF-antagonist including infliximab, who are chronic carriers of this virus. Patients at risk for HBV infection will be evaluated for prior evidence of HBV infection before initiating Infliximab therapy.

Very rare cases of jaundice and non-infectious hepatitis, some with features of autoimmune hepatitis, have been observed in the post-marketing experience of Infliximab. Isolated cases of liver failure resulting in liver transplantation or death have occurred (231). Patients with symptoms or signs of liver dysfunction will be evaluated for evidence of liver injury. If jaundice and/or ALT elevations ≥ 5 times

the upper limit of normal develop(s), Infliximab will be discontinued, and a thorough investigation of the abnormality will be undertaken.

The relative deficiency of TNF- α caused by anti-TNF- α therapy may result in the initiation of an autoimmune process. If a patient develops symptoms suggestive of a lupus-like syndrome following treatment with Infliximab and is positive for antibodies against double-stranded DNA, further treatment with Infliximab will not be given.

Infliximab and other agents that inhibit TNF- α have been associated in rare cases with optic neuritis, seizure and new onset or exacerbation of clinical symptoms and/or radiographic evidence of central nervous system demyelinating disorders, including multiple sclerosis, and peripheral demyelinating disorders, including Guillain-Barré syndrome. In patients with pre-existing or recent onset of demyelinating disorders, the benefits and risks of Infliximab treatment should be carefully considered before initiation of Infliximab therapy.

During clinical studies of Infliximab across all approved indications the incidence of lymphoma in Infliximab-treated patients was higher than expected in the general population, but the occurrence of lymphoma was rare. Furthermore, there is an increased background lymphoma risk in rheumatoid arthritis patients with longstanding, highly active, inflammatory disease, which complicates the risk estimation.

With the current knowledge, a risk for the development of lymphomas or other malignancies in patients treated with a TNF-blocking agent cannot be excluded. Caution should be exercised when considering TNF-blocking therapy for patients

with a history of malignancy or when considering continuing treatment in patients who develop a malignancy.

Rare post-marketing cases of hepatosplenic T-cell lymphoma have been reported in patients treated with TNF-blocking agents including infliximab. This rare type of T-cell lymphoma has a very aggressive disease course and is usually fatal. All cases have occurred in patients with Crohn's disease or Ulcerative Colitis and the majority were reported in adolescent or young adult males. All of these patients had received treatment with AZA or 6-MP concomitantly with or immediately prior to Infliximab. Our patients are non-Crohn's patients and would not have received either of these treatments.

Malignancies and lymph proliferative disorders: In clinical studies with Infliximab in which 5,780 patients were treated, representing 5,494 patient years, five cases of lymphomas and 26 non-lymphoma malignancies were detected as compared with no lymphomas and one non-lymphoma malignancy in 1,600 placebo-treated patients representing 941 patient years (234).

In long-term safety follow-up of clinical studies with infliximab of up to five years, representing 6,234 patients-years (3,210 patients), five cases of lymphoma and 38 cases of non-lymphoma malignancies were reported.

From August 1998 to August 2005, 1,909 cases of suspected malignancies have been reported from post-marketing, clinical studies and registries (321 in Crohn's disease patients, 1,302 in rheumatoid arthritis patients and 286 in patients with other or unknown indications). Among those there were 347 lymphoma cases. During this period, the estimated exposure is 1,909,941 patient years since first exposure (231).

6.7 CONCLUSION

In this future research pilot study we are replicating work which has been performed and published in several prominent peer reviewed journals (including the New England Journal of Medicine), but using a different cohort of patients (non-inflammatory bowel disease patients instead of patient with inflammatory bowel disease such as Crohn's disease).

Our patient's safety and monitoring will be as is the normal standard of care when Infliximab is administered to patients with inflammatory bowel disease at our Infliximab infusion unit.

We hope to counter the potential increase in pain, distress or inconvenience caused by this trial by preventing the extensive risks, morbidity and mortality involved in the current options of considering further surgery or living with this debilitating condition for the remainder of their life.

We hope that a major positive lifestyle change will result during the Infliximab treatment stage.

This research protocol and study plan has been presented to the pharmaceutical research companies: Merck (MSD) and Schering Plough. Currently we are in this process of collaboration to conduct a pilot study with the use of Infliximab on a small number of patients with non-inflammatory bowel disease ECF.

For patients with inflammatory bowel disease, five mg/kg is given as an intravenous infusion followed by additional 5 mg/kg doses at two and six weeks after the first dose, and then every eight weeks thereafter.

A 100-mg vial of Infliximab costs £419.62p (excluding VAT). The drug cost differs between individuals because the dose is adjusted to each person's body weight. For example, if it is assumed that vials are not shared between patients, for a person weighing 73 kg the cost per infusion would be £1678, corresponding to four 100-mg vials needed for a dose of 365 mg.

As the above has shown, a key component of conducting the pilot study will be in obtaining the drug Infliximab or funding for this.

Any positive or significant findings from the pilot study would equate to a major medical advance and a new market for the use of Infliximab and other anti-TNF- α agents. It would provide an alternative option and benefit an already surgically challenging group of patient.

Future Work

In addition to the pilot study discussed in 6.1 and future study comparing somatostatin and octreotide in a well-designed randomised control trial, there is potential for further studies in the management of patients with ECF.

One avenue is to further the research completed looking into the effect of injected Botox on the function of high output stomas and ECF. The idea is based on studies that show blockage of smooth muscle neuromuscular activity and is a method already being employed in the treatment of anal fissures. Current means to manage high output stomas or output from ECF can be divided into measures acting to reduce the output itself and measures employed to increase functionality of the remaining bowel. The aim of future work would be to use Botox to produce an increase in bowel function by reducing smooth muscle activity and transit time. This would be a Phase III trial comparing Botox against a placebo.

Poggioli et al has recommended that local injection of infliximab for the treatment of perianal Crohn's disease in selected cases is safe and feasible. This treatment could represent an effective option for those patients with perianal fistulas who cannot undergo intravenous infliximab treatment especially if there is associated stenosing ileal Crohn's disease. However, further randomised studies are needed to evaluate the effectiveness and the correct indications for this technique (237). In a similar approach, there may be the possibility of injecting anti-TNF- α agents down the ECF tract to enhance the local effect, and thus avoiding the degree of systemic side effects and complications which can arise from intravenous administration.

In summary, this thesis has reviewed several aspects of enterocutaneous fistula healing. Through this work, there is now potential for new studies, including application of the ECF scoring systems, RCT of somatostatin and its analogues, and immunological / clinical therapeutic studies. Hence, there is a great deal to look forward to.

APPENDIX

APPENDIX I

Enterocutaneous Fistula Proforma



Hospital No.
Surname, Forename
Sex

1) Age (years):

2) Degree of co-morbidity / (ASA):

None (ASA 1) ☐

Minor (ASA 2) ☐

Major (ASA ≥ 3) ☐

	Minor Co-morbidity	Major Co-morbidity
Cardiovascular	Controlled Hypertension or AF	IHD, CCF, PVD, Stroke
Respiratory	Treated tuberculosis	COPD, Pulmonary Fibrosis
Gastrointestinal	Alcohol dependence without cirrhosis or hepatitis	Short Bowel Syndrome Cirrhosis with portal HTN
Metabolic		Diabetes mellitus, Addison's
Renal		Chronic Renal Failure
Neurological		Dementia, Encephalopathy
Psychiatric	Depression	

3) Source of Referral: Internal (St. Mark's) ☐ External ☐

4) Time elapsed between initial fistulation and referral to the IF team (months):

5) Aetiology of fistulas (six groups):

- (a) Post-operative in the setting of inflammatory bowel disease (IBD) ☐
- (b) Post-operative (other causes) e.g. ischaemic bowel, trauma ☐
- (c) Secondary to malignancy ☐
- (d) Secondary to radiotherapy ☐
- (e) Spontaneous fistula in patients with IBD ☐
- (f) Other (spontaneous) ☐

6) Origin of fistula/anatomical site:

- (a) Gastric ☐
- (b) Duodenal / jejunal ☐
- (c) Ileal ☐
- (d) Colonic ☐

(e) Unknown ☐

7) Complexity of fistula:

(a) Simple ☐

(b) Complex (multiple /multiple bowel loops and/or
internal abscess cavity ☐

(c) Unknown ☐



8) Fistula Output (High if output before treatment was > 500ml in a 24 hr.):

(a) Low ☐ (b) High ☐ (c) Unknown ☐

9) Presence of a laparostomy at the time of first consultation:

(a) Yes ☐ (b) No ☐ (b) Unknown ☐

10) Albumin level at the time of first consultation (g/dl):

11) BMI (body mass index) at the time of first consultation:

12) Radiology for anatomy:

Small bowel contrast follow through ☐

Fistulography ☐

Retrograde study of distal bowel ☐

CT/US drainage investigation ☐

Radiological drainage ☐

OGD ☐

Colonoscopy ☐

Other (e.g. pouchogram, vaginogram etc.) ☐

13) Nutrition:

	Before	After
Parenteral	☐	☐
IVF	☐	☐
Stoma	☐	☐

14) No. of operations at previous hospital prior to referral:

15) Management at St. Mark's:

Operative management
(Definitive surgical fistula closure e.g. resection/anastomosis) ☐
Conservative Management ☐

16) Time of definitive surgery after previous major op. (days):

17) If management was 'Conservative:'

- Not suitable for surgical intervention ☐
- Did not want surgery ☐
- Laparotomy for control of sepsis without repair / resection ☐
- Open drainage ☐
- Stoma brought out ☐
- Minor surgery to control / evaluate sepsis ☐



18) Bowel defunctioned at fistula surgery (stoma proximal to the anastomosis)?

Yes ☐ No ☐

19) After definitive surgery any abdominal wall hernia surgery?

Yes ☐ No ☐

20) If hernia surgery performed: Mesh used (document type)?

Yes ☐ No ☐

21) Patient Outcome: Died?

Yes ☐ No ☐

22) If died: Days since major surgery (30 day mortality):

23) Fistula related mortality e.g. sepsis?

Yes ☐ No ☐

24) Fistula related complications?

Yes ☐ No ☐

25) Follow up duration: (months):

26) Fistula Healing:

- | | | | | |
|---------------------------|-----|--------------------------|----|--------------------------|
| Fistula healed | Yes | ☐ | No | ☐ |
| Re-fistula | Yes | ☐ | No | ☐ |
| Re-fistula treated | | | | |
| Conservatively | | ☐ | | |
| Infliximab | | ☐ | | |
| With definitive surgery | | ☐ | | |
| Surgery to control sepsis | | ☐ | | |

Following Re-fistula surgery - fistula healed?

Yes ☐ No ☐

APPENDIX II

Patient Information Sheet for Enterocutaneous Fistula Study

Immunological and microbiological factors in inflammatory bowel disease

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The purpose of this study is to understand better how the immune system and bacteria play a part in causing gut diseases, in particular inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis. To do this we are collecting samples of different tissues of the body so that we can analyse cells called dendritic cells that are present within them. We will look at slices of the tissue under a microscope and extract cells from the tissues and determine the type of immune reactions that they stimulate in test tube experiments. A small part of the tissue will be stored frozen so that we can use new tests on it as they become available.

Why have I been chosen?

You have been invited to take part because you have a fistula track (communication between bowel and skin). We would like to take some tissue from the track for our research.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

The tissue we need for our research is removed under local anaesthetic in the outpatients department or on the ward where you are being seen. After the local anaesthetic is injected, which can be a little uncomfortable, a biopsy will be taken using a scalpel or simple punch biopsy. We will take only five 2mm samples. Any bleeding will be stopped and then a dressing will be applied. If the biopsy is taken in the operating theatre local anaesthetic will not be required.

What do I have to do?

Taking part in the study will not require any additional hospital visits or commitments on your part.

What are the possible disadvantages and risks of taking part?

Removal of the tissue may cause some discomfort and the biopsy site may bleed a little for a few minutes. Because the tissue we take is right on the surface, any bleeding will be easy to control. The biopsies will not make your current or future care any harder and will not change the timing of your surgery or any medications which you are taking.

What are the possible benefits of taking part?

There is no benefit to you. The information we get from this study may help us to treat future patients with inflammatory bowel disease and enterocutaneous fistulae better.

What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

The results should be published within approximately 1-2 years of the study and you can obtain a copy of the results by contacting Dr Ailsa Hart at Imperial College London. You will not be identified in any report or publication.

Who is organising and funding the research?

The research is organised by Imperial College London. It is funded by St Mark's Hospital.

Who has reviewed the study?

This study has been reviewed in the standard manner by the Research & Ethics Committee at North West London Hospitals NHS Trust

Contact for Further Information

If you have any problems or questions relating to the study please contact Mr Goher Rahbour 02082354020 or Dr Ailsa Hart 02088695805

Thank you for taking the time to read this information. If you are happy to take part please sign the enclosed consent form. You will be given a copy of the information sheet and signed consent form to keep.

APPENDIX III



RESEARCH PROJECT CONSENT FORM FOR PARTICIPANTS

Title of Project: Immunological and microbiological factors in inflammatory bowel disease

Ethics Committee (EC) No.: 08/H0717/24
Principal Investigator: Dr Ailsa Hart
Clinical Research Fellow: Mr Goher Rahbour

PART A: TO BE COMPLETED BY THE INVESTIGATOR

I confirm that I have explained this research project to the respondent in terms which, in my judgement, are suited to his/her understanding. An Information Sheet was also given to, and read by, the respondent.

_____	_____	_____
<i>Name of Researcher</i>	<i>Signature</i>	<i>Date</i>
_____	_____	_____
<i>Name of Person taking consent (If different from researcher)</i>	<i>Signature</i>	<i>Date</i>

PART B: TO BE COMPLETED BY PATIENT AND/OR PARENT OR GUARDIAN:

Please initial box

1. I confirm that I have fully read and understand the Information Sheet for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care being affected. ☐
3. I understand that my identity will not be disclosed in any published or written data resulting from this study. ☐
4. I understand the above information and agree to take part in the above research project. ☐
5. I am happy for my GP to be informed that I am taking part in the above research project. ☐

_____	_____	_____
<i>Name of Patient</i>	<i>Signature</i>	<i>Date</i>

APPENDIX IV

FACS materials and protocols

FACS materials

Product	Company / Components
Complete medium	RPMI-1640 Dutch Modification (Sigma Aldrich, UK) containing 10% Fetal Calf Serum, 100µg/ml streptomycin, 100 units / ml penicillin, Gentamycin
FACS Buffer	PBS with 2% FCS, 0.02% sodium azide and 1mM EDTA
Phosphate Buffed Saline (PBS)	Sigma, UK
Fetal calf serum (FCS)	Tissue culture systems
Ficoll-Paque	Amersham Pharmacia Biotech AB, Uppsala, Sweden
Flow-count TM Fluorospheres	Beckman Coulter, Bucks
Paraformaldehyde (PFA) 1%	BDH chemicals, Poole

APPENDIX V

Antibodies used for labelling surface markers and cytokines

Antibody	Definition
CLA	Cutaneous Lymphocyte Antigen
$\beta 7$	Purified anti-human/mouse Integrin $\beta 7$ antibody
Lineage cocktail (CD3, CD14, CD16, CD19 and CD34)	Individual explanations are below
CD3	Anti-CD3 monoclonal antibody. for T cells
CD14	Anti-CD14 monoclonal antibody for monocytes
CD16	Anti-CD16 monoclonal antibody for macrophages
CD19	Anti-CD19 monoclonal antibody for B cells
CD34	Anti-CD34 monoclonal antibody for stem cells
HLA-DR	React with the human major histocompatibility (MHC) class II antigen HLA-DR.
r lym	Purified Rat gamma M isotype control
r γ 2A	Purified Rat IgG2a isotype control
γ 1	Gamma 1 antibody
TNF- α	Tumour necrosis factor alpha
IL12	Interleukin 12
IL17A	Interleukin 17A
Interferon- γ	Interferon gamma

PUBLICATIONS AND PRESENTATIONS EMERGED FROM THIS WORK

PUBLICATIONS

Journals

(1) Prospective study of immunological factors in non-inflammatory bowel disease enterocutaneous fistulas.

Rahbour G, Hart AL, Al-Hassi HO, Ullah MR, Gabe SM, Knight SC, Warusavitarne J, Vaizey CJ.

BMC Surgery 2011, 11:12. DOI: 10.1186/1471-2482-11-12. PMID: 21619579.

(2) A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.

Rahbour G, Siddiqui MS, Ullah MR, Gabe SM, Warusavitarne J, Vaizey CJ.

Annals of Surgery. 2012 Dec; 256(6):946-54. PMID: 22885696.

(3) Seven year experience of enterocutaneous fistula with univariate and multivariate analysis examining factors associated with healing leading to development of a validated scoring system.

Rahbour G, Gabe SM, Ullah MR, Thomas G, Al-Hassi HO, Yassin N, P Tozer, Warusavitarne J, Vaizey CJ.

Colorectal Dis. 2013 Jul 19. Doi: 10.1111/codi.12363. PMID: 23869525

Abstracts

(1) 7-year experience of enterocutaneous fistulae with an analysis of healing and mortality at St. Mark's Hospital and Academic Institute, London.

Rahbour G, Ranchod P, Hart A, Gabe SM, Vaizey CJ.

British Journal of Surgery 2010; 97(S2): 76–202.

(2) Experience of enterocutaneous fistulae with an analysis of healing and mortality at St. Mark's Hospital and Academic Institute, London.

Rahbour G, Hart A, Gabe SM, Warusavitarne J, Vaizey CJ.

British Journal of Surgery 2010; 97(S5): 1–58.

(3) New projects: Study of immunological factors in non-inflammatory bowel disease enterocutaneous fistulae.

Rahbour et al.

The newsletter of the Bowel Disease Research Foundation. The Association of Coloproctology of Great Britain and Ireland. No. 2. November 2010.

(4) New projects: Study of immunological factors in non-inflammatory bowel disease enterocutaneous fistulae. Bowel Disease Research Foundation. The Association of Coloproctology of Great Britain and Ireland.

http://www.acpgbi.org.uk/BDRF/projects_funded/2011/rahbour

(5) 8-year experience of enterocutaneous fistulae at a national intestinal failure centre.

Rahbour G, Warusavitarne J, Hart A, Gabe SM, Vaizey CJ.

Journal of Crohn's and Colitis (2011) 5, S13-S170.

(6) 8-year experience of enterocutaneous fistulae at a national intestinal failure centre.

Rahbour G, Warusavitarne J, Gabe SM, Hart A, P Tozer, N Daulatzai, Vaizey CJ.

Gut April 2011 Vol 60 Suppl 1. Doi:10.1136/gut.2011.239301.186.

(7) Univariate and multivariate analysis examining factors associated with healing with enterocutaneous fistulae healing with development of a scoring system.

Rahbour G, Gabe SM, Warusavitarne J, P Tozer, N Daulatzai, Hart A, Vaizey CJ.

Gut April 2011 Vol 60 Suppl 1. Doi:10.1136/gut.2011.239301.185.

(8) Eight-year experience of enterocutaneous fistulae at a national intestinal failure Centre.

Rahbour G, Warusavitarne J, Gabe SM, Daulatzai N, Tozer P, Hart AL & Vaizey CJ.

St. Mark's Hospital and Academic Institute, London, UK.

Colorectal Disease 2011. The Association of Coloproctology of Great Britain and Ireland. 13 (Suppl. 4), 15–47.

(9) Univariate and multivariate analysis examining factors associated with enterocutaneous fistulae healing with division of a scoring system

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St. Mark's Hospital and Academic Institute, London, UK.

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(10) 8-year experience of enterocutaneous fistula at a UK National Intestinal Failure Centre.

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Hepato – Gastroenterology 58 Supplements II (2011).

(11) Univariate and multivariate analysis examining factors associated with enterocutaneous fistula healing with development of a scoring system at a UK National Intestinal Failure Centre.

Rahbour G, Gabe SM, Warusavitarne J, Vaizey CJ.

Hepato – Gastroenterology 58 Supplements II (2011).

(12) A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.

Rahbour G, Siddiqui MS, Ullah MR, Gabe SM, Warusavitarne J, Vaizey CJ.

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(13) A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.

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SARS Annual Meeting, Nottingham. 2012.

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(14) A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.

Rahbour G, Siddiqui MS, Ullah MR, Gabe SM, Warusavitarne J, Vaizey CJ.

Proceedings of the Nutrition Society 2011 70: E295. DOI: 10.1017/S0029665111003806. Published online: 2012.

(15) Tumour Necrosis Factor Alpha in non-inflammatory bowel disease enterocutaneous fistulas – Prospective Study.’ **Rahbour G**, Al-Hassi HO, Hart AL, Ullah MR, Gabe SM, Knight SC, Warusavitarne J, Vaizey CJ.

British Journal of Surgery. Volume 100, Issue s4, pages 2–49, May 2013.

(16) Prospective study to assess tumour necrosis factor alpha in non-inflammatory bowel disease enterocutaneous fistula.

Goher Rahbour, Hafid O. Al-Hassi, Ailsa L. Hart, Muhammad R. Ullah, Simon M. Gabe, Stella C. Knight, Janindra Warusavitarne, Carolynne J. Vaizey.

International Journal of Surgery, Volume 11, Issue 8, 2013, Pages 589-590.

PRESENTATIONS

International

(1) *'8-year experience of enterocutaneous fistulae at a national intestinal failure centre.'*

6th Congress of European Crohns and Colitis Organisation (ECCO) Meeting - Inflammatory Bowel Diseases 2011, Dublin, Ireland. 24-26 February 2011.

(2) *'A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.'*

4th World Congress of Coloproctology and pelvic diseases.

Rome 19-21 June 2011.

(3) *'8-year experience of enterocutaneous fistula at a UK National Intestinal Failure Centre.'*

IASGO 2011, World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists. Tokyo, Japan. 9-12th Nov 2011.

(4) *'Univariate and multivariate analysis examining factors associated with enterocutaneous fistula healing with development of a scoring system at a UK National Intestinal Failure Centre.'*

IASGO 2011, World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists. Tokyo, Japan. 9-12th Nov 2011.

(5) *'A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.'*

IASGO 2011, World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists. Tokyo, Japan. 9-12th Nov 2011.

(6) *'Univariate and multivariate analysis examining factors associated with enterocutaneous fistula healing with development of a scoring system at a UK National Intestinal Failure Centre.'*

European Society of Surgery. 15th Annual Meeting. Krakow, Poland. 17-19th Nov 2011.

(7) *'A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.'*

European Society of Surgery. 15th Annual Meeting. Krakow, Poland. 17-19th Nov 2011.

(8) *'8-year experience of enterocutaneous fistula at a UK National Intestinal Failure Centre.'*

European Society of Surgery. 15th Annual Meeting. Krakow, Poland. Poster Presentation. 17-19th Nov 2011.

(9) *'Prospective Study to assess Tumour Necrosis Factor Alpha in non-inflammatory bowel disease enterocutaneous fistulas.'*

ASiT International Conference, Manchester. 5th-7th April 2013.

National

(1) *'7-year experience of enterocutaneous fistulae with an analysis of healing and mortality at St. Mark's Hospital, London.'*

Association of Surgeons of Great Britain and Ireland (ASGBI). Poster Presentation. 14th April 2010.

(2) *'Enterocutaneous fistulae experience at St. Mark's Hospital.'* Association of Upper Gastro Intestinal Surgeons of Great Britain and Ireland (AUGIS). Poster Presentation. 9-10th Sept 2010.

(3) *'8-year audit of enterocutaneous fistulae with univariate and multivariate analysis examining factors associated with healing with development of a scoring system.'*

Royal College of Surgeons of Edinburgh Annual Audit Symposium. 10th March 2011.

(4) *'8-year experience of enterocutaneous fistulae at a national intestinal failure centre.'*

British Society of Gastroenterology Annual Meeting. Birmingham. 14-17th March 2011. **Poster of Distinction.**

(5) *'Univariate and multivariate analysis examining factors associated with healing with enterocutaneous fistulae healing with development of a scoring system.'*

British Society of Gastroenterology Annual Meeting. Poster. 14-17th March 2011.

(6) *'Eight-year experience of enterocutaneous fistulae at a national intestinal failure centre.'*

Association of Coloproctology of Great Britain and Ireland (ACPGBI) Meeting, Birmingham. Poster. June 2011.

(7) *'Univariate and multivariate analysis examining factors associated with healing with enterocutaneous fistulae healing with development of a scoring system.'*
Association of Coloproctology of Great Britain and Ireland (ACPGBI) Meeting. Birmingham. Poster. June 2011.

(8) *'A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.'*
Royal Society of Medicine. Norman Tanner prize meeting. 5th Oct 2011.

(9) *'A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.'*
BAPEN (British Association of Parenteral and Enteral Nutrition) Annual Conference. Harrogate International Centre. Poster Presentation. 29-30th Nov 2011.

(10) *'A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.'*
SARS (Society of Academic and Research Surgery). Annual Meeting, Nottingham. 4-5th Jan 2012.

(11) *'Tumour Necrosis Factor Alpha in non-inflammatory bowel disease enterocutaneous fistulas – Prospective Study.'*
Royal Society of Medicine / SARS (Society of Academic and Research Surgery). Annual Meeting. 9-10th Jan 2013.

Regional

(1) *'A meta-analysis of outcomes following use of somatostatin and its analogues for the management of enterocutaneous fistulas.'*

Imperial College London Graduate School of Life Sciences & Medicine Summer Research Symposium. 15th July 2011.

(2) *'Studies in enterocutaneous fistulas.'*

Grand Round St. Mark's Hospital. 30th March 2012.

CAREER PROGRESSION

The work and achievements from this MD (Res) have been instrumental in obtaining a ST3 General Surgery 'Number' in London Deanery (London - North West six year rotation) through the 2013 national recruitment selection process.

RESEARCH ETHICS COMMITTEE MEMBER

Following my experience gained through this research, I volunteered and was appointed a member of the London - Harrow REC (National Research Ethics Service Member of NRES Committee on behalf of Health Research Authority). My role has involved reviewing research applications and providing an opinion on whether the research is ethical as part of the committee.

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