Confocal Laser Endomicroscopy in the Assessment of Intestinal Permeability in Acute and Chronic Pancreatitis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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Abstract

Background: The function of the gastrointestinal tract in monitoring and sealing the host interior from toxins, antigens and microbes, is termed the gut barrier. Disruptions in the gut barrier result in an increase in intestinal permeability (IP). Evidence suggests that the intestinal permeability barrier becomes compromised in acute pancreatitis (AP), chronic liver disease (CLD) and to a limited degree in chronic pancreatitis (CP). In acute pancreatitis, changes correlate with disease severity, multi-organ failure, mortality and outcome. Bacterial translocation and dysbiosis along with immunological responses have also been associated with changes in gut permeability. Confocal laser endomicroscopy (CLE) is a novel tool that has been used to investigate intestinal permeability in other gastrointestinal disease.

Aims: To determine whether CLE can be used to assess intestinal permeability, bacterial translocation and endotoxaemia in patients with chronic pancreatitis, chronic liver disease and acute pancreatitis. To assess the responses of immunology and infection in patients with acute pancreatitis whilst also assessing changes related to increasing severity of disease in acute pancreatitis.

Methods: 182 patients were recruited: 54 AP (Determinant Based Classification); 47 CP (surgically or conservatively managed); 33 CLD and 48 non-ulcer dyspepsia, rendering 13 healthy controls. Blood was sampled and duodenal fluid aspirated for culture. CLE was performed in consenting patients and fluorescein leakage scored. Endotoxin antibodies, cytokine concentrations and lactulose mannitol ratios were measured; Diamine oxidase (DAO) concentrations were assayed in AP patients undergoing CLE.

Results: Fluorescein leakage and lactulose mannitol ratios were significantly higher in patients with AP ($p=0.0346$ and $p=0.046$ respectively) and surgically managed CP ($p=0.032$, $p=0.030$) compared to healthy controls. Patients with AP more frequently showed positive duodenal bacterial cultures than controls ($p=0.004$); positive duodenal cultures were associated with fluorescein leakage ($p=0.035$). Plasma endotoxin antibody concentrations were decreased in AP compared to controls. Multiple cytokines showed significantly increased concentrations in severe AP. DAO concentrations were significantly increased in AP patients who showed fluorescein leakage at CLE ($p=0.035$).

Conclusion: CLE identified increased IP in AP and surgically managed CP, associated with endotoxaemia, increased serum cytokines and DAO, with most marked changes in severe AP. The association of fluorescein leakage with duodenal bacterial proliferation in AP indicates major gut dysfunction that may account for bacterial translocation via epithelial gaps, contributing to AP severity. Endotoxaemia, positive duodenal bacterial cultures, and increased serum concentrations of several cytokines were associated with increasing severity of AP. The increase in positive duodenal bacterial cultures in patients who also demonstrated fluorescein leakage at CLE supports a hypothesis of bacterial translocation, suggesting that the gastrointestinal barrier is altered in AP. Evidence for increased IP was also observed in surgically managed CP patients, suggesting that the gut barrier may also be compromised in some CP patients.

Competing Interests: None

Declaration: I declare that this thesis and the research upon which it is based is the result of my own work. The contributions of others are clearly stated. This work has not previously been submitted in any substance for any degree, not is it concurrently being submitted at any other university. Parts of the study were presented at Digestive Diseases Week 2015 at the Pancreatic Disorders Distinguished Abstract Plenary and Business Meeting and at Digestive Disorders Federation 2015.

Publications:

During my period of research, I concurrently contributed to the following publications:

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LIST OF ABBREVIATIONS

AP	Acute Pancreatitis
CBP	Continuous Blood Purification
CLD	Chronic Liver Disease
CLE	Confocal Laser Endomicroscopy
CP	Chronic Pancreatitis
CRF	Case Record Form
CRP	C-reactive protein
CT	Computed Tomography
DAO	Diamine oxidase
DBC	Determinant Based Classification
EIN	Enteral Immunonutrition
EN	Enteral Nutrition
ERCP	Endoscopic Retrograde Cholangio-Pancreatography
EUS	Endoscopic Ultrasound
FSI	Fluorescence Signal Intensity
GI	Gastrointestinal
HPLC	High Pressure Liquid Chromatography
IBD	Inflammatory Bowel Disease
IEC	Intestinal Epithelial Cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intestinal permeability
L/M	Lactulose / Mannitol
LPS	Lipopolysaccharide
MRCP	Magnetic Resonance Cholangio-pancreatography

MRI	Magnetic Resonance Imaging
PAF	Platelet Activating Factor
PEG	Poly-ethylene glycol
PMN	Polymorphonuclear
SAP	Severe Acute Pancreatitis
SIRS	Systemic Inflammatory Response Syndrome
Th	T-Helper
TNF	Tumour Necrosis Factor
TPN	Total Parenteral Nutrition

CHAPTER 1

INTRODUCTION

1.1 Gastrointestinal Permeability

The function of the gastrointestinal tract in monitoring and sealing the host interior from toxins, antigens and microbes, is termed the gut barrier. [1] Intestinal epithelial cells, the gut immune system, and immunologic responses serve as layers of defence. [2] The overall maintenance of gut barrier integrity is a dynamic process expending significant energy. The cellular processes generating energy and oxidation also enable the gut to perform this role. [5] Thus, nutritional deficiencies play a key role in the development of gut barrier dysfunction. [6] Disruptions in the gut barrier result in an increase in intestinal permeability. Various mechanisms of damage have been proposed which may lead to the translocation of endotoxin and enteric bacteria. [7] There is substantial evidence from both experimental acute pancreatitis in rodent models and clinical acute pancreatitis studied in different human populations, that the intestinal permeability barrier becomes compromised in this condition. This chapter reviews the literature related to intestinal permeability in acute pancreatitis, and the correlation between this and resultant bacterial and endotoxin translocation from the gut lumen into the circulation.

Alterations in intestinal permeability (IP) have also been reported in chronic liver disease (CLD) and to a limited degree in chronic pancreatitis (CP). In acute pancreatitis, changes correlate with disease severity, multi-organ failure, mortality and outcome. [5] Increases in intestinal permeability also correlate with various disease severity markers including C-reactive protein concentration, serum cytokine concentrations, procalcitonin concentration. Apache II scoring, Atlanta criteria and the Determinant Based Classification allow classification of disease and grading of severity based on clinical parameters and also have been shown to correlate with changes in gastrointestinal permeability. Increased IP allows the passage of microbes, which are usually maintained within the gut lumen by physiological and homeostatic mechanisms, through the systemic circulation to extra-intestinal sterile sites, potentially exacerbating the severity of pancreatitis. [8] [9]

In patients with cystic fibrosis who have chronic pancreatitis, increased intestinal permeability has been reported compared with those without, and this is secondary to changes in villus and crypt tight junctions. [10] Cytokine balance has also been associated with increased pathogenic microbiota and gut permeability. [11]

Bacterial translocation and endotoxaemia are also thought to exacerbate the severity of liver disease, leading to decompensation of cirrhosis, with spontaneous bacterial peritonitis, hepatic encephalopathy, hepatorenal syndrome and variceal bleeding. [12] [13] [14] Immunomodulatory, cellular and molecular events lead to changes in the expression of intestinal tight junction proteins, representing cellular mechanisms for intestinal barrier disruption and hyper-permeability. Patients with hepatic encephalopathy have been shown to have altered intestinal flora, increased serum endotoxin levels, and an increase in the serum concentrations of various cytokines (IL-6, Tumour Necrosis Factor-alpha (TNF)-α, IL-2, and IL-13). [15] Several tests can be used to examine intestinal barrier function. [5] [16] Transport of molecules across the intestinal epithelium takes place through two major routes, trans-cellular and paracellular. Transport of solutes across the transcellular route is carrier-mediated and occurs with the help of transporters and channels whereas transport of molecules through the paracellular route across the intestinal epithelium occurs by the process of diffusion and it is not carrier-mediated. The intestinal epithelial barrier is key to this process. The disruption of this barrier system results in changes in intestinal permeability and the penetration of noxious substances. Assessment of intestinal permeability is done to assess the overall function of transport through the intestinal epithelial paracellular route. [17]

There are series of aqueous pores along the crypt villous axis of the small intestine, with smaller pores at the villus tip and slightly larger ones at the crypts. Most of the probes used to measure intestinal permeability are water-soluble, which cannot penetrate the lipid cell membrane of enterocytes and thus use the paracellular route through the tight junctions. The smaller probes can easily pass through the small,

more numerous and more accessible tight junctions of the villous tips, whereas the larger probes have to make use of the larger, less accessible and less numerous pores at the crypt base.

Intestinal permeability is measured with the help of a non-metabolizable probe molecules which pass across the mucosal barrier and are excreted in the urine after being absorbed into the systemic circulation. Quantitation of the probe in a timed urine or serum collection provides a measure of the fraction of ingested probe that has penetrated the mucosal barrier. Only a small proportion of this large probe should get through the intestinal mucosa, reach the circulation, get filtered by the kidney and get measured in the urine over a defined point of time. If there is an abnormality in the paracellular transport; the passage of the probe is enhanced and thus more is excreted in the urine which suggests abnormality in the intestinal permeability. Intestinal permeability can be assessed using a variety of marker probes such as lactulose, mannitol, rhamnose and cellobiose, polyethylene glycol (PEG) 400, PEG 1000, 51Cr-EDTA and 99mTc-DTPA. Multiple mucosal and non-mucosal factors can affect the excretion of a single dose of a test probe and thus a dual sugar probe test is used for the assessment of intestinal permeability. This involves the simultaneous oral administration of a disaccharide and a monosaccharide and measurement of their excretion in urine over a defined period of time. Intestinal permeability is measured as the ratio of percentage of excretion of disaccharides/monosaccharides in the urine or serum. Expression of these excreted sugars as a ratio overcomes the individual variations of other factors. [18] The concentration of one or more sugar probe can be measured using enzyme assay, high performance liquid chromatography (HPLC), ion-exchange chromatography in combination with mass spectrometry and liquid chromatography with mass spectrometry. If the PEG has been used as a probe, then the concentration of the PEG can be assessed using capillary column gas chromatography.

Lactulose and mannitol ratio is the most commonly used test for assessment of intestinal permeability and the most reliable method of measurement of concentration of lactulose and mannitol is HPLC. HPLC is a chromatographic technique used to separate a mixture of compounds with the purpose of identifying, quantifying and purifying the individual components of the mixture. [17]

High pressure liquid chromatography (HPLC) for the determination of lactulose and mannitol in serum, as opposed to urine, 90 minutes after ingestion of a sugar solution, is another reported method. [19]

The small intestinal epithelium is in constant equilibrium with continuous villus cell shedding and crypt cell replacement. [20] [21] A gap may form from extruding cells; this is closed by neighbouring cells forming a tight junction beneath. Lack of closure leads to gaps in the villus architecture and these have been shown to increase in various gastrointestinal disorders including inflammatory bowel disease and gastrointestinal malignancies, due to the high turnover of shedding epithelial cells. [22] [23] [24] As detailed later, these gaps have been shown to correlate with loss of intestinal barrier function. [22, 25] Confocal laser endomicroscopy (CLE) is a novel diagnostic tool which allows the evaluation of *in vivo* mucosal histology and assessment of intestinal barrier function. This technique is discussed in more detail later in this introduction.

1.2 The Gut Barrier and Intestinal Microbial Flora

The intestinal epithelium is a critical component of the gut barrier and is composed of a single layer of intestinal epithelial cells (IECs) held together by tight junctions. [26] A single contiguous layer of epithelial cells provides a dynamic mechanical barrier between the host's internal milieu and the external environment and this excludes potential pathogens. [27, 28] Rapid cell turnover and peristalsis clear adherent or entrapped microbes into the gut lumen. [29] However, multiple microbes are maintained within the gut lumen by physiological and homeostatic mechanisms.

DNA sequencing technology and computational biology advances have revolutionized the field of microbiomics. In healthy individuals, pro- and anti-inflammatory signals are balanced such that commensal organisms are recognized and tolerated, while pathogens are prevented from penetrating the mucus layer and underlying epithelium. Examples include: *E. coli, Enterobacter aerogenes, Enterococci, Pseudomonas aeruginosa, Proteus, Bacteroides, and Klebsiella.* [9] A large-scale comparative analysis of 16S rDNA sequences has better characterised the adherent mucosal and faecal microbial communities. A total of 395 bacterial phylotypes were identified of which 244 were novel. Bacterial diversity within the human colon and faeces is significant and this study demonstrated a great degree of heterogeneity between individuals. [30] More recent reports discuss the diverse consortium of bacteria, fungi, protozoa, and viruses that inhabit the gut of all mammals. Studies in humans and other mammals implicate the microbiome in a range of physiologic processes vital to host health, making the gut microbiome an active participant in host physiology. States of dysbiosis are characterised by reduced diversity in the resident microbiota. [31] The fungal microbiome, the mycobiome, is beginning to gain recognition as a fundamental part of our microbiome. Compared to bacterial communities, the human gut mycobiome is low in diversity and dominated by yeast including *Saccharomyces*, *Malassezia*, and *Candida*. [32] Another recent review discusses recent advances in sequencing technologies and bioinformatics which have enabled new insights into the genomes of our microbial symbionts, their functional capacities, and the interactions between these microbes and their human host. Metabolic conversions of dietary components and pharmaceuticals that take place in the human distal gut, as well as their implications for human health are discussed. [33] The human distal gut is home to a rich and dense microbial community with representatives of all three domains of life which are intricately connected with our physiology and health. The combined genomes of these microbes, collectively called the human microbiome, vastly

expand the metabolic capacities of our own genome, allowing us to break down and extract energy from dietary compounds that human enzymes cannot digest. The main function of the mammalian gut microbiota is to assist with food digestion and energy extraction, by degrading dietary components such as fibre and cellulose that cannot be broken down and utilized by the host's functional capabilities alone. In addition, microbial colonization of the gut educates the host's immune system and helps to shape the correct development of anatomical structures in the intestinal tract. Our gut microbes are basically small biochemical factories, expanding our body's metabolic capacities many times.

Microbiome composition not only varies between individuals but also over time. A person's individual gut microbiome composition appears to be relatively stable in the absence of disease and dietary or lifestyle changes but can quickly and dramatically respond to an altered diet, international travel, food poisoning, or antibiotic use. The fast response of the gut microbiome to such changes is thought to be the result of the rapid growth rate of many bacterial species and the regular large expulsion of gut contents. In addition to these rapid temporal responses, age has been shown to affect the composition of the gut microbiome as well, albeit at a much slower rate. While human enzymes in the small intestine break down most dietary ingredients such as proteins, starch and fatty acids into absorbable smaller molecules, such as amino acids, and monosaccharides, not all molecules present in the diet can be digested in this part of the gastrointestinal tract. The human genome does not encode for enzymes that break down complex proteins and complex carbohydrates, that is, fibres and other plant-derived polysaccharides. These molecules will therefore reach the colon largely intact, where they can be digested by the gut microbiome. The most important function of the human microbiome in the distal gut is to extract energy from these otherwise indigestible dietary components.

The cytoskeleton, intercellular tight junctions and protein kinase C, provide a paracellular pathway between intestinal epithelial cells that permits the bidirectional passage of various substances and bacteria, and also plays a role in antigen sampling and presentation. [5] [34] (Figure 1) Bacteria can also penetrate intestinal epithelial cells by a mechanism similar to phagocytosis, the transcellular pathway. [6, 35] (Figure 1)

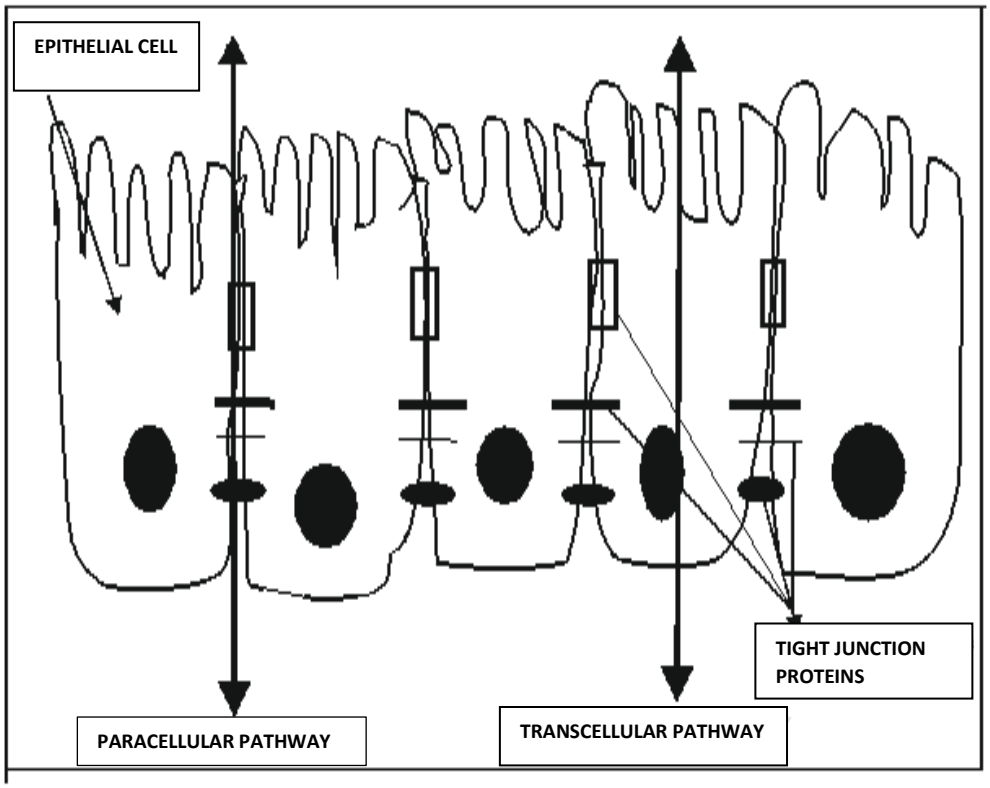


Figure 1.

Schematic diagram to demonstrate paracellular and transcellular absorption pathways across intestinal epithelial cells.

Other features also limit the entry of noxious substances. For example, goblet cells secrete mucus and crypt cells secrete chloride-rich fluid, [36] which help prevent toxins from adhering to surface receptors, reducing transfer across the epithelial barrier and assisting toxin removal with gastrointestinal motility and chemotactic activity. [5] Gastric acid is bactericidal while pancreatic enzymes can damage

bacterial cell walls. [37] Immunoglobulin A (IgA) is also secreted into the gut and binds foreign antigens in the gut lumen, as well as those that have penetrated epithelial cell walls. The immunoglobulin-antigen complex is transported through the cell cytoplasm; it is then excreted into the gut lumen and eliminated. [5] Defensins, anti-microbial peptides produced by Paneth cells, are also directly toxic and cause disruption of bacterial cell membranes. Mediators such as nitric oxide have also been shown to regulate epithelial permeability. [38] M cells are specialised epithelial cells of the follicle-associated epithelium of the gastrointestinal tract. They have a high capacity for transcytosis of a wide range of microorganisms and macromolecules. They are believed to act as an antigen sampling system with rapid uptake and presentation of particular antigens and microorganisms to the immune cells of the lymphoid follicle leading to induction of an effective immune response. The resulting immune processes result in lymphocyte migration and allow for a line of defence against invading organisms that breach the epithelium. [5] [39]

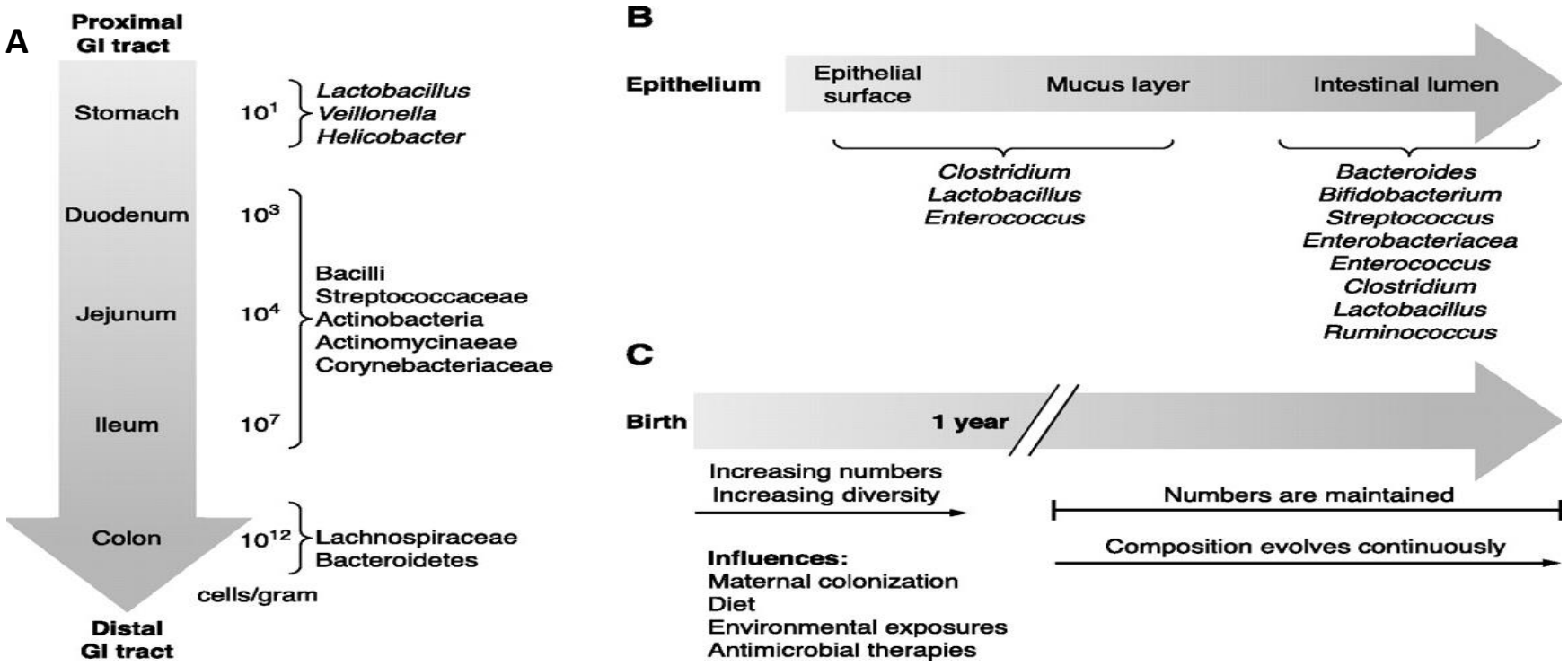


Figure 2. Three axis aspects of intestinal microbiota composition. *Adapted from Sekirov et al. [2]*

A: Composition of gut microbiota along the GI tract

B: Surface layer variations of microbiome

C: Temporal variations in microbial flora

1.3 Altered Intestinal Permeability

Proposed mechanisms of increased intestinal permeability include: mucosal ischaemia; ischaemia-reperfusion injury; impaired immune defences; and changes in the intestinal microbial population leading to bacterial overgrowth. Intracellular mechanisms including signal transduction; cellular signalling or adhesion molecule expression on endothelial and epithelial cells, may also be involved. [7]

The most common and earliest organ to fail in severe acute pancreatitis is the lungs, but the exact pathophysiological mechanisms underlying the disease are still unclear. In experimental studies, interruption of mesenteric lymphatic flow has preventive qualities for acute lung injury in the setting of critical illness with various aetiologies. Experimentally, diversion of mesenteric lymph is able to prevent acute lung injury if done before its development, whereas a later intervention partially reduces the lung damage. Thus, experimental evidence implicates mesenteric lymph as a primary route for toxic factors to gain entry into the circulation. Interruption of this flow is able to prevent acute lung injury in critical illness. [40] Morphological changes have also been demonstrated to correlate with intestinal permeability alterations. [41] Processes that lead to oxidant stress cause damage to the epithelial cell cytoskeletons that regulate tight junctions. [5] Non-steroidal anti-inflammatory drugs and tacrolimus have been demonstrated to cause this form of damage. [42, 43]

Cellular energy deprivation and related processes play a role in diseases associated with bowel injury or increased permeation. [5] The gut's vulnerability to hypoxia is related to the villi's microvascular network, which functions as a countercurrent exchanger between the arteriolar and venular endings, causing reduced oxygen tension at the villus tip. [6]

Luminal material crosses the epithelial sheet via transcellular or paracellular pathways. Tight junctions between adjacent epithelial cells are the rate-limiting factor regulating paracellular permeability. [44] Disorganisation or damage to tight junctions results in disruption of the intestinal barrier and increased paracellular

permeability. [45] Relaxation of tight junctions by protein kinase C activation increases solute flow across the junctions and epithelium by decreasing resistance. [34] A very recent study of patients with acute pancreatitis who underwent duodenal biopsies to measure the expression of tight junction proteins (claudin-2 and claudin-4) alongside lactulose-mannitol ratios, confirmed an increase in intestinal permeability in acute pancreatitis and demonstrated a reduction in claudin-4 expression. [46]

Infection with microbes results in upregulated expression of immunoregulatory genes in epithelial cells with increased production of pro-inflammatory cytokines or inadequate synthesis of anti-inflammatory cytokines. [5] [27] Intestinal epithelia bear functional receptors for a number of diverse cytokines including: IFN-γ, IL-2, IL-4, IL-10, IL-13, TNF-α, TGF-β, and hepatocyte growth factor. [47] TGF-β and IL-10 are thought to prevent or reverse impaired intestinal permeability. [27, 48] The cytokines cause changes in nitric oxide production, which results in relaxation of the cytoskeleton or oxidation / nitration of cytoskeletal proteins leading to increased antigen sampling. [49] [5] Nitric oxide may adversely contribute to cellular permeability in inflammatory conditions, by the formation of peroxynitrate, which causes DNA damage and activates poly ADP-ribose synthetase, which uses ATP. Both factors result in increased permeation. [50] Increased nitric oxide production in response to bacterial infection increases apoptosis. [28] A study involving rats challenged with lipopolysaccharide demonstrated that excessive production of nitric oxide contributed to increased gut mucosal permeability. [51] Cytokine-induced intestinal epithelial hyperpermeability is mediated by nitric oxide and interferon-γ, TNF-α and IL-1β act synergistically to induce nitric oxide synthase expression. [52] IL-13 and IL-4 have also been shown to demonstrate an increase in paracellular permeability. [47] TNF-α lowers the transepithelial resistance between tight junctions leading to increased flow of solute between cells and across the epithelium. [34] The effects of TNF-α are enhanced by IFN-γ. [53]

1.4 Epithelial Cell Shedding

The small intestinal epithelium is in constant homeostasis with continuous villus cell shedding and crypt cell replacement. [20] [21] The equilibrium between the rate of apoptosis and shedding of epithelial cells at the villus tip, and the generation of new cells in the crypt, is key to maintaining tissue homeostasis. [26] Shedding of intestinal epithelial cells (IECs) from the epithelial monolayer with lack of closure may result in the formation of transient gaps or micro-erosions within the epithelial barrier and this may result in increased intestinal permeability. Interestingly, small intestinal epithelial cells are known to have the highest turnover of any fixed cell population in the human body, with complete renewal occurring every 2 to 6 days. [54] Cell division in the crypt results in a continuous supply of epithelial cells. These epithelial cells undergo apoptosis on reaching the villus tip, or extrusion zone, and are shed into the lumen. [55] Several mechanisms for this process have been proposed. Most involve complex alterations in tight junction proteins and cytoplasmic processes underneath the shedding cell. Studies examining these processes have used techniques such as electron microscopy and confocal microscopy. [56] [57]

Creamer *et al.* confirmed the rapid turnover of intestinal epithelial cells in mice. [20] The mechanism of cell shedding remains controversial, though apoptosis has been suggested to occur. The life cycle of an intestinal epithelial cell is terminated by apoptosis and/or cell shedding. Apoptosis is initiated and cells finally lose anchorage and are shed into the lumen. They also lose their contact with the extracellular matrix. The mechanism of cell shedding is important as abundant cell shedding must be achieved without loss of intestinal barrier function. An impermeable substance has been found to plug the epithelial discontinuities that arise on the villus as a consequence of cell shedding, resulting in 3% of surface area having gaps near the villus tip. Gaps contain no cellular material, but are filled with a substance that maintains the epithelial barrier, despite discontinuities being present in the cellular

layer. The source and composition of this “plugging” substance is unknown, but it could be secreted by neighbouring epithelial cells, myofibroblasts, or the shed epithelial cells themselves. [58] Watson *et al.* emphasised that the intestinal barrier is sustained despite the high rate of cellular turnover in the epithelium. [21] Whilst investigating cell shedding and intestinal barrier function in mice, they demonstrated the presence of occasional gaps in the villus epithelium. Shed cells were largely intact, with limited evidence of caspase activity or apoptosis. [21] Cell shedding and gap formation were shown to increase in mice which had been injected intraperitoneally with TNF-α. The increased cell shedding following this treatment correlated with loss of integrity of gut barrier function. [22] Mice injected with lipopolysaccharide (LPS) also demonstrated epithelial cell shedding, endotoxaemia and subsequent intestinal leaks through gaps or paracellular spaces. Although systemic LPS administration significantly increased the leaks in gaps and paracellular spaces, it did not change the percentage of leaks in gaps and paracellular spaces compared with the control group. [59] [25]

In both localised and systemic inflammation, homeostasis may be disturbed as a result of pathological IEC shedding. The increase in epithelial cell shedding and resultant epithelial gaps have been reported in some gastrointestinal disorders due to the high turnover of shedding epithelial cells. [22] In mice injected with TNF-α, increased cell shedding has been shown to correlate with loss of intestinal barrier function. [22, 25] Pathological epithelial cell shedding refers to the increased rate of villus epithelial cell apoptosis and extrusion that is observed in several disease states. [26]

1.4.1 Diamine Oxidase

Diamine oxidase (DAO), formerly called histaminase, is found in various tissues, but is especially active in the intestinal mucosa. Its function is the oxidative deamination of several polyamines, essential substances for cell proliferation, and it

is a regulating enzyme in rapidly proliferating tissues such as the intestinal mucosa. DAO is normally present in very small amounts in the circulation and its basal plasma levels are positively correlated with the maturity and integrity of the intestinal mucosa. [60]

A study in China showed that a traditional drug, berberine, attenuated intestinal barrier function in an animal model of acute pancreatitis. Serum DAO activity and endotoxin levels were observed to be higher in the severe acute pancreatitis group than those in the Sham group and berberine pre-treatment was shown to significantly decrease serum DAO activity and endotoxin levels compared with levels in the severe acute pancreatitis group. Berberine treatment caused no significant effect on pancreatic histology, but ameliorated the intestinal mucosal barrier damage and membrane permeability that were associated with severe acute pancreatitis(SAP), as measured by serum DAO. This study showed that berberine may be associated with the inhibition of SAP-induced upregulation of myosin light chain measurements. The results from this study may lead to biased interpretation since pre-treatment in itself is likely to have resulted in amelioration of severity and resultant phemonena. A Chinese group studying the differences in the effect of enteral versus parenteral nutrition in severe acute pancreatitis also assessed gut barrier function by means of plasma DAO concentration, serum endotoxin, and urinary lactulose mannitol ratios. [61] Further clinical studies have also reported DAO as a serum marker for the same purpose. [62] [63]

Thus, DAO is a well-recognised non-invasive biomarker of gut barrier dysfunction. It is easy to measure in serum and multiple reports of its use in this context have been noted in experimental models and human conditions.

1.5 Acute Pancreatitis

The pancreas is a glandular organ which has both exocrine and endocrine functions, the latter within the pancreatic islets or islets of Langerhans and the former from acinar cells which form clusters, and are arranged in lobes separated by a thin fibrous barrier. The secretory cells of each acinus surround a small intercalated duct. Because of their secretory function, these cells contain many small granules of zymogens that are microscopically visible.

Acute pancreatitis results from acute inflammation of the pancreas and is sometimes associated with a systemic inflammatory response that can impair the function of other organs or systems. There is a wide spectrum of disease from mild (80%), where patients recover within a few days, to severe (20%) in which patients often have prolonged hospital stays and need critical care support. The diagnosis of severe acute pancreatitis depends on the presence of persistent organ failure (>48 hours) and the presence of local complications which usually become apparent later in the course of disease. [64] The two main classification systems used to stratify the severity of acute pancreatitis include the Atlanta Classification and the Determinant Based Classification. [65] [66] The latter enables classification into four categories: mild, moderate, severe and critical based on similar criteria to the Atlanta Classification with respect to the presence of transient or persistent organ failure and the presence of necrosis, sterile or infected. Opinions vary as to the efficacy of the two with comparison studies reporting summaries that both perform equally. [67] [68] The aetiology of acute pancreatitis is varied. The commonest causes in Western society are gallstones (50%) and alcohol (25%). Rarer causes (<5% each) include drugs, endoscopic retrograde cholangiopancreatography (ERCP), hypertriglyceridaemia, hypercalcaemia, pancreas divisum, and some viral infections. About 10% of patients have idiopathic pancreatitis, where no cause is identified. The disease remains a clinical challenge due to limited understanding of its pathogenesis and multi-causality. It occurs in ~50/100,000 of the population, and

when it is severe (1 in 5 patients), it can result in local necrosis that contributes to multi-organ failure, and this is associated with mortality between 20-30%. [69]

Acute pancreatitis is diagnosed by the presence of two out of three of the following clinical criteria: a clinical history of abdominal pain in keeping with acute pancreatitis; increased serum amylase activity greater than three times the upper limit of normal; radiological changes in keeping with pancreatic inflammation. [66] The spectrum of disease extends from a mild oedematous-interstitial inflammation to severe necrotising acute pancreatitis with systemic complications developing in 20-25% of affected individuals. [70] [71]

Indeed, early stratification of disease severity improves clinical outcomes and significantly reduces the length of hospital stay. [72] Improvements in imaging, such as computerised tomographic (CT) scans, have not proven superior to clinical scoring systems in early prediction of acute pancreatitis severity. [73, 74] Patients who have local complications and organ failure have a high risk of mortality and should be managed in a specialist centre or a high dependency or intensive care setting. [65] Computed tomography should be performed 3-4 days after admission in patients with persistent systemic inflammatory response syndrome or organ failure to look for local complications.

Markers of the systemic inflammatory response syndrome help to identify those patients who may develop persistent organ failure as per the determinant based classification. [75] [76] There are several different predictive scoring systems for disease severity based on physiological variables or single biochemical markers, but none has shown clear superiority. A recently published review reported a summary of the multiple acute pancreatitis scoring systems and the use of different imaging methods to predict disease severity: Ranson criteria, Glasgow criteria, Hong Kong Score, Acute Physiology and Chronic Health Evaluation II (APACHE II), computed tomography scoring systems, Bedside Index of Severity in Acute Pancreatitis (BISAP) score, Japanese Severity Score (JSS), Harmless Acute Pancreatitis Score

(HAPS), Pancreatitis Outcome Prediction (POP), Sequential Organ Failure Assessment (SOFA). CRP should also be included in this list of disease severity markers since it is actually the most effective, readily and easily available test which correlates well with disease severity. This article also described the Revised Atlanta Classification of acute pancreatitis (2012) and the correlation with computed tomography. [77] It is important to appreciate that these predictors of severity are based on clinical parameters, usually around the time of disease onset and do not allow us to assess prognosis. The assessment enables the appropriate triaging of patients and identification of those requiring higher level care. The Ranson Score is rarely utilised in the UK as the scoring includes the assessment of blood urea nitrogen which is a routine test in American practice, but not in British medicine. Glasgow criteria more favourable in the latter but again is an assessment formulated at the time of initial assessment.

A large number of clinical biomarkers for acute pancreatitis severity have been investigated over the last two decades. These include routine biomarkers, (including creatinine and haematocrit) [78] [73, 78-81] pancreas-specific enzymes, (such as amylase, lipase, trypsinogen) [80-83], acute phase proteins such as C-reactive protein (CRP) and procalcitonin, [80, 81, 84], various cytokines (including IL-6; IL-8; IL-10; IL-12; IL-15, IL-17 and TNF-α) [80, 81] immunological components (such as phospholipase A and polymorphonuclear elastase), [81] transcriptomics, [85] proteomics, [86] and others (amyloid-A, carboxypeptidase B activating peptide and blood urea nitrogen). [81] A meta-analysis of 6 studies looked at the value of carboxypeptidase-B activation peptide (CAPAP) in predicting severity of acute pancreatitis on admission. Both serum and urinary CAPAP have the potential to act as a stratification marker on admission in predicting severity of acute pancreatitis. [83] However, none of these have yet been implemented in clinical practice due primarily to the cost implications involved.

Thus, the pathophysiology of pancreatitis incorporates both the localized destruction in the pancreas and the systemic inflammatory response that is postulated to be a result of premature activation of trypsinogen to trypsin within the acinar cell. Various causes for this have been proposed and in turn, it is suggested that further enzyme activation occurs, leading to localized tissue damage and release of Damage Associated Molecular Patterns (DAMPs). The release of DAMPs causes recruitment of neutrophils and initiation of the inflammatory cascade. This inflammatory cascade is what is thought to be responsible for the systemic manifestations of acute pancreatitis and can ultimately lead to increase capillary permeability and damage of endothelium with microvascular thrombosis that causes multiorgan dysfunction syndrome (MODS), the main cause of morbidity and mortality in acute pancreatitis. [87]

Overall mortality rates in acute pancreatitis are cited to be between 2 and 6% in recent studies. [87, 88] Pancreatic necrosis leads to multiple organ dysfunction syndrome at a later stage in the disease process, [89] although this can also occur without the presence of pancreatic necrosis and often earlier. [90] A diagnosis of severe acute pancreatitis warrants admission to a critical care unit with careful supportive therapy, as treatment modalities remain limited. [69] Patients suffering from complications of infected pancreatic necrosis should be considered for early intervention. [70] Biomarkers are imprecise and despite multiple clinical trials over the last 40 years, there is no effective specific drug treatment for acute pancreatitis [91]. Microbial infection plays a key role in the pathogenesis, severity, outcome and prognosis of acute pancreatitis. Despite positive results with antibiotics in the past, recent trials have failed to confirm a role for antimicrobial prophylaxis, although antibiotic treatment using carbapenems or quinolones is still suggested by some clinicians for patients who have severe acute pancreatitis at admission. [92] Acute pancreatitis therefore remains a clinical challenge for surgeons, physicians and intensivists. Identification of severe disease is central to optimisation of treatment,

but remains a controversial clinical assessment. Clinical signs, biochemical markers, imaging methods and complex scoring systems are used in clinical practice to make the distinction between mild and severe acute pancreatitis. Many novel parameters have been investigated including interleukin-6 (IL-6) in early severity stratification and macrophage migration inhibitory factor as a marker of pancreatic necrosis. [69, 71] With evidence of the effectiveness of IL-6 rising, its surrogate marker CRP inevitably acts as an excellent alternative that is cheap to perform and readily available. Specificity remains questionable, hence the lack of consensus regarding the most effective tool, and also there is a delay in the secondary rise in CRP after the initial IL-6 response. Necrotising pancreatitis is suspected when systemic inflammation persists for over a week after the onset of pancreatitis. Intervention is not recommended for at least 14 days after the onset of symptoms and patients should only be managed in specialist units by expert teams.

More generally, the treatment of acute pancreatitis is limited to intravenous fluids and symptomatic management of pain and vomiting when the disease course is mild to moderate. In the more severe or critical courses, often there is a SIRS response with multi organ failure and intensive care support is warranted. Enteral nutrition by means of oral intake is recommended and encouraged as soon as it is feasible. [93] In cases of severe acute pancreatitis, where this is not an option, nasogastric feeding has been shown to be adequate, without the need for nasojejunal intubation. [94-96] The benefit of antibiotic therapy is questionable as previously discussed. [97] After resolution of severe acute pancreatitis, it is important to identify and treat the underlying cause of the episode and encouragement of alcohol cessation has been shown to be beneficial. [98]

1.5.1 Gut barrier dysfunction and bacterial translocation in acute pancreatitis

In severe acute pancreatitis, Gram-negative organisms infect pancreatic necrosis and intra-abdominal collections, and this results in a threefold increase in mortality.

Systemic Inflammatory Response Syndrome (SIRS) is commonly associated with morbidity, and mortality and results from a reaction to proteases, cytokines and other vasoactive mediators which are released at disease onset. [69, 70] SIRS must be differentiated from sepsis, since there is often no infective source and blood cultures are negative. The systemic reaction however involves a rise or fall in core temperature, tachycardia, tachypnoea and a rise in white cell count. A study of patients who had severe acute pancreatitis identified that an increase in intestinal permeability and higher endotoxaemia predicted poorer outcomes in patients who experienced complications. [99] Intestinal permeability, and serum concentrations of IL-6, TNF-α, and endotoxin were also reported to be significantly higher in patients with acute pancreatitis who had a high Balthazar CT severity index, indicating that these parameters are associated with severe acute pancreatitis and serious complications, and suggesting that they may potentially be reliable markers for predicting the prognosis of acute pancreatitis. [100]

Potential mechanisms for the loss of intestinal barrier function that is observed in acute pancreatitis include reduced intestinal perfusion [101] and/or reperfusion injury following initial resuscitation, [102-105] increased intestinal epithelial apoptosis secondary to circulating cytokines [106] or loss of trophic luminal enzymes. [10] Furthermore, reduced intestinal motility can lead to alterations in intestinal microflora with overgrowth of Gram negative bacteria, [41] which further contributes to local inflammation and mucosal injury. [104, 107] A review of studies demonstrated that immunological, bacteriological and morphological components seem to interact and depend on each other, further highlighting the complexities of the mechanisms underlying loss of intestinal barrier function in acute pancreatitis. [108] Figure 3 demonstrates the multiple factors and time duration involved in the progression of acute pancreatitis.

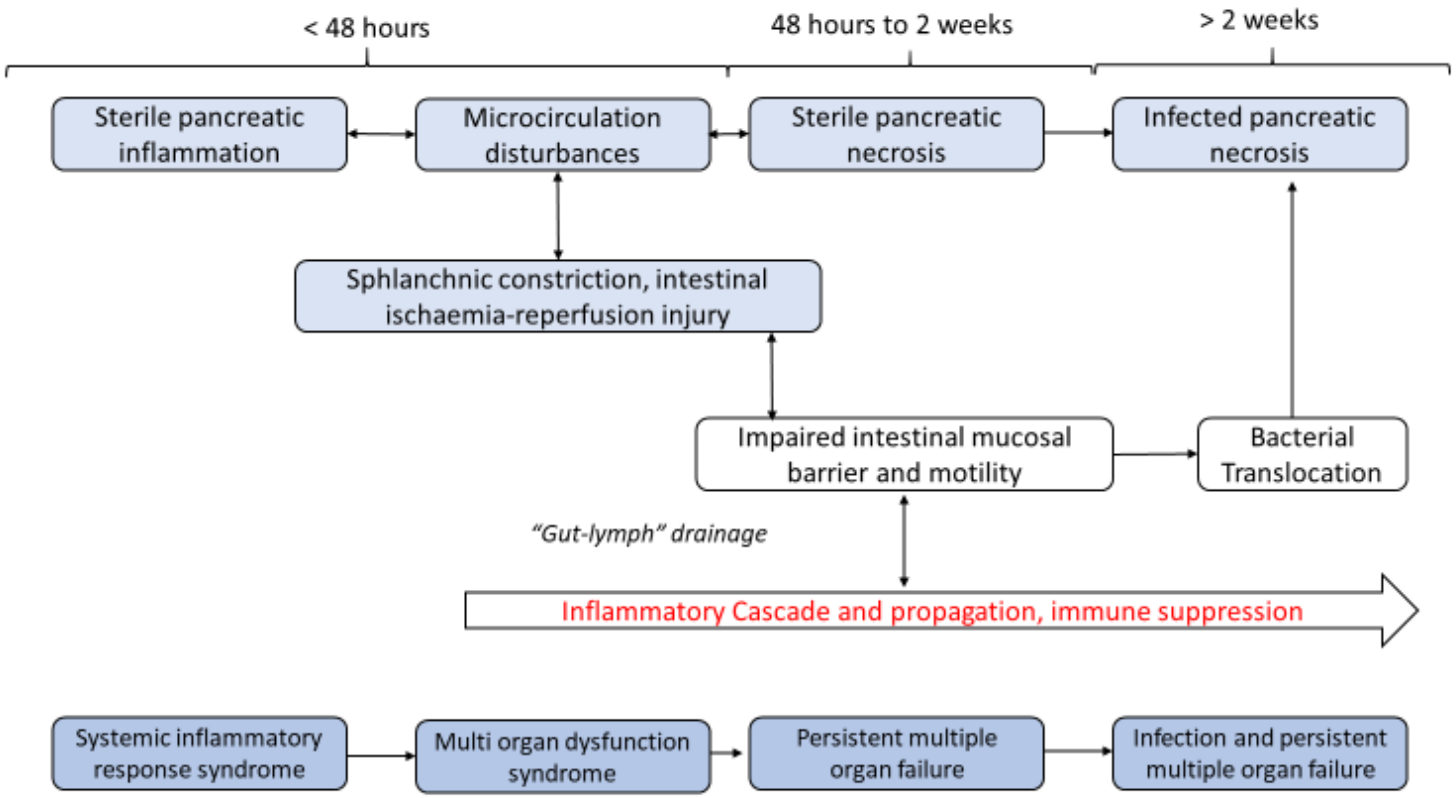


Figure 3. A representation of progression of Acute Pancreatitis.

Table 1. Gut permeability in experimental acute pancreatitis					
Study	**Model**	**Species**	**Therapeutic factors**	**IP tested**	**Findings**
Liu *et al* (1997) [109]	Sodium taurocholate (3%, 1 ml/kg, i.d.) and trypsin (3000 IU/kg, i.d.)	Rats	Human recombinant EGF (100 μg/kg, s.c.) 30 mins after operation twice daily for two days	Bacterial Translocation	• Minimised intestinal mucosa injury induced by AP • Reduced BT to blood, MLN, pancreas, spleen and liver
Kouris et al (2001) [110]	Sodium taurocholate (3%, 1 ml/kg, i.d.) and trypsin (3000 IU/kg, i.d.)	Rats	GLP-2 analog ALX-0600 (0.1 mg/kg, s.q.) 10 mins after operation twice daily for 3 days	Transepithelial electrical resistance of the terminal ileum and BT	• Reduced ileum transepithelial resistance • Reduced BT to MLN, pancreas and peritoneum • No significant effects on intestinal histopathology • No significant effects on pancreatic histopathology
Sun et al (2001) [111]	Sodium taurocholate (5%, 1 ml/kg, i.d.) and trypsin (1.67×10^5 U/kg, i.d.)	Rats	None	Plasma D-lactate and endotoxin	• Increased gut permeability
Wang et al (2002) [112]	Sodium taurocholate (5%, 1.5 ml/kg, i.d.)	Rats	Human recombinant GH (0.75 U/kg, s.c.) immediately after operation	None	• Down-regulated intestinal epithelial cell apoptosis
Leveau et al (2005) [113]	Sodium taurodeoxycholate (5%, 0.2 ml, i.d.)	Rats	PAF inhibitor lexipafant (5 mg/kg, i.p.) 30 mins and 6 h after operation	Bilateral measurement of ^{131}I-labeled human serum albumin from blood to intestinal lumen	• Improvement in intestinal barrier dysfunction in AP • Reduction of intestinal IL-1 levels and local leukocyte recruitment
Alhan et al (2006) [114]	Caerulein (5 μg/kg/h for 6 h, i.v.), + Glycodeox. acid (10 mM, 1.2 ml/kg, i.d.)	Rats	ω-3FA* (0.8 ml/kg/h, i.v.) and saline (5.2 ml/kg/h, i.v.) for 42 h	24 h excretion of urine phenol sulfophthalein and BT	• Reduced gut permeability • Reduced BT to pancreas, liver and spleen • Reduced pancreatic and lung inflammation • Reduced mortality
Yasuda et al (2007) [115]	Sodium deoxycholate (3%, 0.1 ml, i.d.)	Rats	Caspase inhibitor Z-VAD-fmk (500 μg/rat, i.p.) during induction of disease	Leaked amount of FITC-dextran outside of the intestinal pouch, endotoxin and BT	• Locked intestinal mucosa caspase-3 activation • Reduced intestinal permeability and improved intestinal mucosa histopathology • No reduction in BT but reduced serum endotoxin levels • No significant effect on pancreatic histopathology
Luan et al (2010) [116]	Sodium taurocholate (5%, 1.5 ml/kg, i.d.)	Rats	EP (40 mg/kg, i.v.) 24 h after disease induction, every 6 h for 24 h	Plasma DAO and endotoxin	• Down-regulated intestinal mucosa HMGB1 • Reduced plasma amylase, endotoxin, DAO and activity of intestinal mucosa MPO • Reduced plasma amylase and pancreatic histopathology
Zhang et al (2010) [117]	Sodium taurocholate (3.5%, 1 ml/kg, i.d.)	Rats	Danshen (10 ml, 15 g/rat, i.p.) 15 mins after disease induction, i.p.	None	• Reduced intestinal mucosa apoptosis and NF-kB activation in the treatment group
Zou et al (2010) [118]	Sodium taurocholate (5%, 1 ml/kg, i.d.) and trypsin (7500 BAEF units/ml, i.d.)	Pigs	Enteral immunonutrition 24 h after disease induction*	L/M, serum endotoxin and BT	• Decreased gut permeability, serum endotoxin and BT • Improved intestinal mucosa histopathology • No significant effects on pancreatic histopathology
Lu et al (2011) [119]		Rats		Evans blue assay for intestinal capillary leakage	• Reduced intestinal capillary leakage was observed • Decreased elevated intestinal VASP and MMP-9

Zhongkai et al (2012) [120]	Sodium taurocholate (2%, 1 ml/kg, i.d.)	Rats	VIP (5 nmol, i.p.) 5 mins after disease induction	Serum endotoxin	• Serum endotoxin levels decreased in the treatment group • Intestinal mucosa mRNA levels of TNF-α, IL-6 and IL-10, and TLR4 expression were reduced in the treatment group • Improved intestinal mucosa histopathology
Chen et al (2012) [121]	Sodium taurocholate (3%, 1 ml/kg, i.d.)	Rats	GLP-2 (0.1 mg/kg) immediately after operation twice a day for 3 days, i.p.	Serum DAO activity	• Serum DAO was decreased • Intestinal epithelial cell apoptosis was partly prevented • Reduced histopathological score of ileal mucosa
Dang et al (2012) [122]	Sodium taurocholate (5%, 1 ml/kg, i.d.)	Rats	TREM-1 inhibitor LP17 (1.0 mg, i.v.), just before disease induction	L/M	• Reduced gut permeability • Reduced levels of serum DAO and D-lactose and BT • Reduced intestinal TREM-1, TNF-α, and IL-1β mRNA
Lu et al (2013) [123]	Sodium taurocholate (1 ml/kg, 5%)	Rabbits	Saizen (0.15 IU/kg, s.c.) 1 h after disease induction and 24 h later after the first injection; Stilamin (3.5 μg/kg/h, i.v.) 1 h after disease induction for 48 h	Serum D-lactate	• Serum D-lactate was significantly lower • Reduced intestinal mucosa epithelial cell apoptosis • Improved intestinal mucosa histopathology • Improved mortality
Li et al (2013) [124]	Sodium taurocholate (5%, 0.2 ml, i.d.)	Rats	Infliximab (8 mg/kg, i.v.); or infliximab (8 mg/kg) and octreotide (10 μg/kg, i.v.), at 6 h after modelling	Serum DAO and D-lactate	• Significantly reduced serum amylase, DAO and D-lactate; • Improved intestinal motility and reduced intestinal injury • Combination of infliximab and octreotide had better effects than infliximab
Tian et al (2013) [125]	Caerulein (50 μg/kg/h × 6, i.p.) followed by LPS (10 mg/kg, i.p.)	Mice	None	Serum DAO	• Increased serum amylase, TNF-α and DAO • Increased oxidative stress markers in gut mucosa • Increased gut mucosa apoptosis
Han et al (2013) [126]	Sodium taurocholate (3.8%, 1 ml/kg, i.d. via pancreatic tail)	Rats	PN; EN; EN + Gln; PN + Gln	None	• Reduced severity of pancreatic histopathology • Reduced intestinal epithelial apoptosis
Li et al (2013) [127]	Sodium taurocholate (4.5%, 1 ml/kg, i.d.)	Rats	None	None	• IAP was 2-3 times higher than the control group • Amylase, TNF-α, creatinine, bilirubin, DAO and D-lactate gradually increased over time
Sun et al (2013) [128]	Sodium taurocholate (1 ml/kg, 4 %), i.d.	Rats	Melatonin (50 mg/kg, i.v.) 30 mins before modelling	BT (Bacterial DNA)	• Serum and ileum DAO were significantly reduced • E. Coli DNA was lower in the treatment group • Mortality lower (21.43% vs 55.56%)
Shen et al (2013) [129]		Rats	Continuous blood purification	Intestinal occludin and ZO-1 levels	• Intestinal permeability was reduced • Intestinal proteins occludin and ZO-1 were decreased
Tian et al (2013) [130]	Caerulein (50 μg/kg/h × 6, i.p.) followed by LPS (10 mg/kg, i.p.)	Mice	None	Serum DAO, ZO-1, E-cadherin	• Increased serum amylase, TNF-α and DAO; • Over-expressed miR-155 • Under-expressed proteins of RhoA, ZO-1 and E-cadherin
Liang et al (2014) [131]	Sodium taurocholate (1 ml/kg, 2.5 or 5%), i.d.	Rats	None	Serum endotoxin, DAO, D-lactate and BT	• Gut permeability increased over time and it was correlated with taurocholate dose • Intestinal mucosal injury correlated taurocholate dose

Tu et al (2014) [132]	Sodium taurocholate (1 ml/kg)	Rats	MSCs were injected via the dorsal penile vein 1 h later	Serum DAO	• Reduced gut permeability • Decreased intestinal mucosa histopathology score • Reduced systemic inflammation
Tang et al (2014) [133]	Sodium taurocholate (1 ml/kg, 3%)	Rats	Berberine 50mg/kg in 1ml N/Saline / 200g body weight i.g. od for 5 days	Serum DAO, ET, ZO-1, occludin mRNA, Bacterial culture of MLNs	• No significant changes to histology • Decrease in serum DAO and ET • Decrease in bacterial translocation • No influence on ZO-1 and occludin mRNA expression in SAP
Deitch et al (2014) [134]	Sodium taurocholate (5%, 1 ml/kg, i.d.) with simultaneous intraperitoneal deposition of Caerulein.	Rats and Mice	None	Plasma FD-4 (fluorinated Dextran) further to intraluminal ileal injection, Villi injury and mucus coverage, mucus protein carbonyl and nitrated tyrosine residues	• The intestinal mucus layer is lost after AP • Loss of unstirred mucus layer is associated with: ○ gut barrier failure; ○ with ROS- and RNI- mediated structural changes to the mucus layer ○ occurring before microscopic morphological evidence of damage to the underlying mucosa
Ling et al (2014) [135]	Sodium taurocholate (0.5% for mild AP; 3.8% for severe AP)	Rats	None	Cultured epithelial cells were infected with αSNAP shRNA Cell apoptosis by flow cytometry- Occludin	• In SAP, Intestinal villi showed oedema, atrophy, necrosis and shedding of IEC • IP in rats with AP increased significantly • Increased TNF-α and endotoxin in SAP • Down-regulated expression of αSNAP and IEC occluding in SAP
Machado et al (2016) [136]	Sodium taurocholate into biliopancreatic duct	Rats	None	COX-2 and TJ protein and gene expression	• Significantly higher number of bacterial colonies in pancreas of elderly • Intestinal damage in AP is exacerbated by aging • Increase in bacterial colonies was associated with a more severe intestinal inflammatory response, associated increase in expression of COX-2 and decrease in TJ proteins • COX-2 inhibition could potentially be a therapeutic target in the elderly
Feng et al (2018)	Caerulein (50 μg/kg/h × 4, i.p.) followed by LPS (7.5 mg/kg x 2, i.p.)	Rats	Dachengqi Decoction (DCQD)	Serum amylase, TNF-α, IL-1β, IL-2, and IL-6 by ELISA Intestinal tissue by histology Capillary permeability by Evans blue extravasation assay AQP-1, MMP9, and JAM-C	• DCQD can reduce capillary endothelial damage in acute pancreatitis-associated intestinal injury and the mechanism may be associated with the regulation of endothelial barrier function-associated proteins AQP-1, MMP9, and JAM-C • DCQD attenuated SAP intestinal injury and lowered the levels of serum amylase, TNF-α, IL-1β, IL-2, and IL-6 effectively[137]
Bai et al (2017) [138]	5% sodium taurocholate injection (1ml/kg body mass) i.d.	Rats	Dai-Huang-Fu-Zi-Tang (DHFZT)		• DHFZT inhibited the openness of the mitochondrial permeability transition pore (MPTP) • It reduced the contents of serum D-lactic acid and activity of diamine oxidase activity • It relieved histopathological manifestations and epithelial cells injury of intestine
Cao et al (2018) [139]	Sodium taurocholate (350 mg/kg, 3.5 %), i.d	Rats	Free total rhubarb anthraquinones (FTRAs) (36 / 72 mg/kg) 12 hours later	ET, IL-1β, TNF-α, and NO in the blood and TNF-α, IL-1β in the intestinal tissues	• Decreased levels of ET, IL-1β, TNF-α, and NO in the blood and TNF-α, IL-1β, and protein extravasation in the intestinal tissues in SAP rats • FTRAs could protect intestinal injury and improve intestinal mucosal barrier function through regulating immune function of SAP rats • FTRAs may have the potential to be developed as the novel agent for the treatment of SAP clinically

Table 2. Summary of clinical studies evaluating intestinal permeability in severe acute pancreatitis						
Authors	**Year**	**Patient Cohort**	**Classification Tool**	**Key Methods**	**Key Results**	**Further Conclusions**
Ammori et al [140]	1999	• 85 AP • 56 MAP • 29 SAP • 25 Controls	Atlanta Criteria	• Urinary excretion of PEG-3350:PEG-400	• Increased IP and endotoxaemia as AP severity increased and relative to controls • IP highest in SAP with MOF or death	• Gut barrier dysfunction plays a role in sepsis development, MOF and resultant mortality
Juvonen et al [141]	2000	• 23 AP • 15 MAP • 8 SAP • 20 Controls	Atlanta Criteria	• Lactulose / Rhamnose test on day 2 and on recovery	• Increased IP and decreased absorption capacity in AP • Findings exacerbated in SAP • All indices normalised during recovery	• Gut permeability increased in AP, and increased further in SAP
McNaught et al [142]	2002	• 59 AP • 24 SAP • 35 MAP	Modified Glasgow Criteria	• Lactulose / Rhamnose test • Culture of nasogastric aspirate	• Colonisation with enteric bacteria in SAP • Differences in IP were not seen	• AP is associated with BT and gut origin sepsis
Rahman et al [101]	2003	• 61 AP • 19 SAP • 42 MAP • 12 Controls	Atlanta Criteria	• Urinary excretion of PEG-3350 and PEG-400	• A strong correlation between IFABP levels and gut macromolecular permeability • IgM anti-endotoxin antibodies decreased as IFABP levels increased	• IFABP is a marker of intestinal ischaemia • Loss of intestinal mucosal integrity is related to SAP
Ammori et al [143]	2003	• 60 AP • 48 MAP • 12 SAP	Atlanta Criteria	• PEG-3350	• Correlative increase in IP, endotoxaemia and calcitonin precursor (CTpr) levels as AP severity increased • Weak positive correlation between CTpr and CRP levels	• In AP, CTpr levels were reflective of IP
Rahman et al [7]	2003	• 65 AP • 20 SAP • 45 MAP • 20 Controls	Atlanta Criteria	• Urinary PEG 3350/4000 ratio	• Urinary nitrite excretion correlated with increasing IP in AP, and with endotoxaemia	• Endotoxaemia is central to the mechanism of septic complications in SAP

Penalva et al [144]	2004	• 68AP • 51MAP • 17 SAP • 13 Controls	Atlanta Criteria	• Lactulose: Mannitol ratio on admission and day 15	• Increase in IP as severity of AP increased • Increase in endotoxaemia as severity and course increased • No significant correlation with cytokines	• IP and inflammatory response may be independent of each other
Nagpal et al [145]	2006	• 52 AP • 16 SAP • 30 MAP • 46 Controls	Atlanta Criteria	• Lactulose: Mannitol urinary ratio on days 1, 4 and 7	• Increased IP in AP patients compared with controls • IP increased as AP severity increased	• IP also increases with the time course of disease
Liu et al [104]	2008	• 68 AP • 18 SAP • 26 MAP • 20 Controls	Atlanta Criteria	• Urinary lactulose: • Mannitol ratio	• Increase in IP early in AP and in correlation with severity and MOF • Increase in IP correlated with increase in endotoxaemia and TNF-α	• Patients with severe mucosal dysfunction had a higher rate of septic complications
Pan et al [146]	2010	• 32 AP • 25 SAP • 7MAP	Atlanta Criteria	• Urinary Lactulose: Mannitol ratio	• Increase in IP, CRP, IFABP as severity of AP increased	• IFABP indicates gut dysfunction and predicts prognosis and clinical AP course
Amicucci et al [147]	2016	• 63 AP • 21 SAP • 42 MAP	Atlanta Criteria	• Lactulose: Mannitol ratio on days 0, 1, 3, 7, 9, and 11	• Increase in intestinal barrier dysfunction and endotoxaemia in SAP compared with MAP	• Severe AP can lead to systemic endotoxaemia contributing to SIRS and MOF
Du et al [148]	2017	• AP • Controls	Continued Blood Purification	• DAO, ET, Rho-kinase, Protein F-actin and Claudin 1, TNFα	• Increased levels of serum DAO, endotoxin and intestinal epithelial monolayer permeability when compared with normal controls • ROCK mRNA expression and serum TNF-α level were increased • After CBP treatment, levels of serum DAO, endotoxin and intestinal epithelial monolayer permeability decreased	• CBP can effectively improve intestinal mucosal barrier dysfunction • The beneficial effect is associated with the improvement of cytoskeleton and tight junction proteins

One of the most serious consequences of gut barrier dysfunction is bacterial translocation, or passage of viable indigenous bacteria from the mucosal surface to extraintestinal sterile sites. Changes in intestinal flora, relative mucosal ischaemia and impaired immune surveillance are all thought to contribute to this phenomenon. [149] Most bacteria associated with pancreatic and peri-pancreatic infections are indigenous enteric species, implicating the gut as their origin. [8, 41, 107, 150] Indeed as early as 1976, a study by Holden *et al.* reported that the enteric organisms *E. coli* and *Klebsiella spp*. were responsible for infections during acute pancreatitis. [151] Suggested routes of spread include direct transmural migration to the peritoneal cavity or retroperitoneum and pancreas; lymphatic spread; haematogenous spread and transmission via contaminated bile. Indeed, recent experimental reports have reported inactivation of antimicrobial agents by bile, leading to colonisation of the biliary tree with enteric organisms, excretion in bile and re-infection of the host via the entero-hepatic circulation. As well as contributing significantly to morbidity and mortality in human disease, bacterial infection of pancreatic and extra-pancreatic sites has been shown to increase disease severity, morbidity and mortality in experimental models of acute pancreatitis. [152] Experimental models and controlled clinical studies of acute pancreatitis also provide ample evidence for an association between intestinal barrier dysfunction and acute pancreatitis. Acute pancreatitis has been shown to lead to increased mucosal permeability to macromolecules. [7, 153] Experimental acute pancreatitis in rodents (rats and mice), induced by intraperitoneal caerulein or ductal infusion of taurocholate is associated with increased ileal villus cell shedding, raised serum endotoxin levels as well as *E. coli* DNA in postcaval blood.[127, 128, 130-132] (See table 1) Apoptosis of gut epithelial cells has also been observed to increase during experimental acute pancreatitis, thus contributing to increased mucosal permeability to endotoxin and viable bacteria. [45, 106, 112, 154] Pancreatitis-associated ascitic fluid has also been found to contain factors which accelerate the apoptosis of

intestinal epithelial cells in culture, [45] a phenomenon mediated through caspase activation. [115] Bacterial translocation itself has been reported in a number of studies, [41, 155] and indeed it has been found to be increased following laparotomy in humans, proposing a possible mechanism for the high mortality observed in patients who undergo surgery for acute pancreatitis. [41, 156] Traditionally, open necrosectomy was the only tool available for surgical treatment of pancreatic necrosis. This was found to be associated with high mortality rates up to 40%. A more recent study of a step-up approach in treatment and minimally invasive necrosectomy, showed an overall mortality of 10.38%. [157] In a study of patients undergoing open necrosectomy, more than a quarter were found to develop early infection of pancreatic necrosis within the first week of illness. [158, 159] In an analysis of long-term outcomes of trial participants, the step-up approach for necrotizing pancreatitis was found to be superior to open necrosectomy, without increased risk of reinterventions. [160] A published review of local practice examined the outcomes from minimal access retroperitoneal pancreatic necrosectomy and open pancreatic necrosectomy for severe necrotizing pancreatitis in a single centre. Intensive care admission was required less frequently in the former cohort and the mortality rate was reported as 15.3% compared with 23.3% in the open necrosectomy group. Mortality and complication rates improved with time and experience. [161]

A study detailed in Table 2, supported the theory of bacterial translocation and gut origin of sepsis, but not that these factors resulted from intestinal barrier dysfunction. [142] Reports of microcirculatory changes in the pancreas, colon, liver and lungs, at different stages during severe acute pancreatitis also suggest that these contribute to the multiple organ dysfunction syndrome in acute pancreatitis. [162] Fluid depletion contributes to relative intestinal ischaemia and alters the pancreatic microcirculation, increasing pancreatic ischaemia and exacerbating the systemic inflammatory response syndrome (SIRS) leading to multisystem organ failure. [103] Ammori *et*

al. conducted a clinical study involving examination of the terminal ileum of three patients with severe acute necrotising pancreatitis who underwent pancreatic debridement and ileocolic resection, and seven control patients. They demonstrated significant atrophy of mucosal villi with reduction in mast cell numbers in patients who had severe necrotising pancreatitis compared with controls, and this correlated with an increase in intestinal permeability and bacterial translocation. [163]

There are, therefore, several experimental and clinical studies that have demonstrated a clear association between the severity of acute pancreatitis and gut barrier dysfunction (tables 1 and 2), together with a plausible biological mechanism (bacterial translocation) that may lead to an exacerbation of the disease.

1.5.2 Effect of nutrition on gut barrier function in acute pancreatitis

Studies demonstrating a benefit of correcting gut barrier dysfunction mainly relate to the use of enteral nutrition. The historical management of severe acute pancreatitis involved allowing the pancreas and gut to rest as enteral nutrition (EN) was thought to stimulate the production and release of pancreatic enzymes and further aggravate retroperitoneal inflammation. [69] Extensive research has however recently shown that enteral feeding is safe, [164] [165] [166] and even beneficial as it protects against intestinal mucosal atrophy and bacterial translocation. [118] Prevention of gastrointestinal dysfunction is based on the recovery and maintenance of mucosal integrity, which requires adequate provision of oxygen and nutrients. [6] A systematic review of eight trials reported that in patients with acute pancreatitis, enteral nutrition significantly reduced mortality, multiple organ failure, systemic infections, and the need for operative interventions compared to patients who received total parenteral nutrition (TPN). [167] Kotani *et al.* similarly reported significantly less bacterial translocation and a lower blood endotoxin concentration in rodents treated with enteral nutrition rather than TPN. A difference in survival was however not seen. [168] A randomised prospective trial of patients with severe

acute pancreatitis who were treated with enteral nutrition demonstrated a reduction in septic complications. [165] Windsor *et al.* reported a reduction in septic complications, APACHE-II scores and CRP levels when patients were treated with enteral nutrition as opposed to TPN. An increase in serum IgM anti-endotoxin concentrations was found in the parenterally fed patients, but remained unchanged in those who had been fed enterally. [166] Early enteral feeding through the nasogastric route was found not to be inferior to the nasojejunal route in patients with severe acute pancreatitis. [169] One study of patients with severe acute pancreatitis who were treated with either enteral or parenteral nutrition, reached a contradictory conclusion when no significant differences were found between the two groups with respect to intestinal permeability, endotoxin levels or cytokine responses. A greater number of overall complications with enteral nutrition was however reported in this study. [170] A very recent published guideline for intensivists looked at various uses of enteral nutrition in critically unwell patients and concluded that they suggest using EN in patients with severe acute pancreatitis. [171]

Combined nutritional therapy, consisting of enteral *and* parenteral nutrition, was found not to reduce intestinal permeability, infection rates or mortality. Hyperglycaemia and deranged liver enzymes also occurred more frequently. [172] A recent published review reports that the goal of nutritional support in acute pancreatitis is to reduce inflammation, prevent nutritional depletion, correct a negative nitrogen balance, and improve outcomes. Nutrition maintains the integrity of the gut barrier, decreases intestinal permeability, downregulates the systemic inflammatory response, maintains intestinal microbiota equilibrium, and reduces the complications of the early phase of SAP, improving morbidity and possibly improving mortality. [173]

A study of 70 patients with severe acute pancreatitis randomly divided subjects into two groups including a group of patients having parenteral nutrition and a group that

had enteral nutrition. Changes in IP were subsequently assessed. Interestingly, before the intervention, both groups had similar levels of serum endotoxin and lactulose/mannitol ratios. One and two weeks after the intervention, the serum endotoxin level and the lactulose/mannitol excretion rate of urine of the group with PN were significantly higher than the group EN. [174]

1.5.3 Effect of fluid resuscitation on gut barrier function in acute pancreatitis

Fluid replacement is crucial in severe acute pancreatitis, as patients may develop a vascular leak syndrome which results in hypovolaemia and hypotension that can in turn lead to renal complications. Early fluid resuscitation is associated with a reduced incidence of SIRS and organ failure at 72 hours. [103] A study comparing the effects of different resuscitation fluids demonstrated that a combination of normal saline, hydroxyethyl starch and glutamine was more efficient in resuscitating patients with severe acute pancreatitis (SAP) (by relieving inflammation and sustaining the intestinal barrier) than normal saline alone. [175] However, in a separate study looking at the effect of glutamine on gut permeability, no significant effect was identified with respect to inflammation, endotoxemia or infectious complications in severe acute pancreatitis. [176] A recent review of AP substantiates that the foundation of management remains early aggressive fluid resuscitation. Lactated Ringer's solution is the recommended fluid. The fluid resuscitation is monitored with a combination of blood urea nitrogen, haematocrit, and urine output, monitoring every 4 to 6 hours in first 24 hours of resuscitation to adjust the fluid rate. Continued non-response indicates a high likelihood of ensuing multi-organ failure and is grounds for upgrading the level of care. [87]

1.5.4 Effect of antibiotic therapy on gut barrier function in acute pancreatitis

Many clinicians have advocated the use of prophylactic antibiotic therapy in patients with acute pancreatitis in whom there is necrosis or proven infection. [69] The choice of antibiotic seems most frequently to be imipenem or a quinolone, due to good pancreatic tissue penetration and a broad spectrum of activity against enteric organisms. [177] In one study of patients who had severe acute pancreatitis, subjects randomly received either standard therapy or selective decontamination with oral amphotericin or norfloxacin. Pancreatic infection with necrosis and mortality were significantly decreased in the treated group. [178]

Significant controversy remains with regards to antibiotic use however, and a number of large multi-centre randomised, controlled trials have found no evidence to support their use. The use of ciprofloxacin and metronidazole in patients with pancreatic necrosis resulted in no differences in the rates of infected or systemic complications, or mortality, in patients who received antibiotic prophylaxis compared to those who did not. [179] A recent review confirmed no clinical benefit or reduction in morbidity or mortality from administering prophylactic antibiotics to patients who had severe acute pancreatitis. The use of antibiotics was however recommended in patients who had severe acute pancreatitis and multi-organ failure at admission; in patients with shock; for patients who developed acute respiratory distress syndrome, pancreatic necrosis or clinical signs of sepsis during the course of their disease; and for patients with positive cultures from any source. [92] False negative blood cultures in septic patients make the latter more difficult. [180]

Early evidence from animal studies and a small placebo-controlled trial suggested that probiotic prophylaxis stabilised intestinal barrier dysfunction, minimised bacterial translocation and reduced infectious complications. [181] [182] A major randomised placebo-controlled trial of probiotic prophylaxis in severe acute pancreatitis however contradicted these findings and demonstrated a significant increase in mortality associated with probiotic administration (due to small bowel

ischaemia and jejunal necrosis), and showed no reduction in infection rates. [35] The role of probiotics was also in the process of being evaluated in a smaller scale study, but this trial was terminated prematurely due to the findings of the prior probiotic trial. However, from the 50 patients who were included prior to termination, no significant benefit of probiotics was seen on gut permeability or endotoxaemia in acute pancreatitis. [183] Several recent reports have shown that probiotics can reverse the leaky gut by enhancing the production of tight junction proteins; however, additional and longer-term studies are still required. Conversely, pathogenic bacteria that can facilitate a leaky gut and induce autoimmune symptoms can be ameliorated with the use of antibiotic treatment. Therefore, it is hypothesized that modulating the gut microbiota can serve as a potential method for regulating intestinal permeability and may help to alter the course of autoimmune diseases in susceptible individuals. [184]

Selective bacterial decontamination of the gut and early enteral feeding have therefore failed to make a significant impact on the clinical course of severe acute pancreatitis and do not appear to prevent bacterial translocation. [185]

Thus, on review of the evidence and with particular focus on the PROPATRIA trial, probiotics are not recommended for use in patients with acute pancreatitis, though it is arguable that an alteration of the gut bacterial load with ‘healthy bacteria’ should enhance. The outcomes of the studies have to be considered carefully as the patients affected are likely to have been acutely unwell individuals with multiple factors contributing to morbidity and mortality and an element of chance may be contributory to the reported outcomes.

1.5.5 Effect of immunonutrition on gut barrier function in acute pancreatitis

Enterocytes are major consumers of glutamine as an energy source and the exogenous administration of this amino acid is associated with increased villus height and DNA content, improved intestinal perfusion and decreased bacterial translocation. [6] [186] Arginine is an antecedent of nitric oxide and targets intestinal blood vessels improving gut ischaemic conditions, restoring intestinal mucosal integrity and also promoting immune function. [118] Zou *et al.* therefore investigated the use of enteral immunonutrition (EIN) in experimental pancreatitis in pigs, by adding glutamine and arginine to the conventional enteral feeding regimen along with probiotics. [118] Pigs with severe acute pancreatitis which were treated with TPN demonstrated clear changes in villus architecture and disruption of the gut barrier. The villi of pigs which were treated with EN or EIN however showed normal architecture. EIN was seen to improve villus height and mucosal thickness, suggesting that it can prevent mucosal atrophy, extend absorptive capacity and maintain villus integrity. [118] Those pigs which were treated with EN and EIN showed a decrease in intestinal permeability after a plateau phase. This was particularly enhanced in the EIN group. [118] A prospective, randomised clinical trial has also demonstrated favourable responses following the administration of EIN. The diet in this study included arginine, omega-3 fatty acid, and an RNA-enriched enteral diet. [187] Qin *et al.* also reported positive outcomes related to gut permeability and reduced septic complications with EIN. Their adjunctive treatment was *Lactobacillus plantarum*, a bacterial species which has been documented to have strong anti-inflammatory and immunomodulatory effects. [188]

EN therefore enhances intestinal perfusion, preserves mucosal mass and intestinal microbial ecology, and has been shown to reduce septic morbidity in high-risk patients. [189] Addition of immunonutritional factors seems to add significant benefit. Its administration in patients with severe acute pancreatitis is safe and

feasible, and has been shown to significantly reduce systemic inflammation and septic complications. [107]

1.5.6 Potential therapeutic targets of gut barrier function in acute pancreatitis

Preservation or restoration of gut barrier function may have a beneficial effect on sepsis related morbidity in acute pancreatitis and may reduce mortality. [107] Several factors which are thought to maintain or reverse integrity of the gut barrier have therefore been investigated for their therapeutic potential in experimental models. These are summarised in table 2. Few human studies have however been carried out to date. A randomised double blind, placebo-controlled study in 1995, assessed the effects of Lexipafant, a platelet activating factor (PAF) inhibitor and demonstrated a reduction in the incidence of organ failure and in serum IL-8 and IL-6 concentrations. [190] These findings were replicated in Scotland in a similar study that was undertaken in eleven hospitals. [191] A subsequent larger randomised, double blind, placebo controlled, multicentre trial investigated this same mediator, but no effect on organ failure or mortality was demonstrated. More rapid decreases in serum IL-8 and E-selectin levels were seen, but no differences in absolute levels were observed. [192] Continuous blood purification (CBP) has been shown to improve gut barrier dysfunction through improvement of cytoskeletal instability and by downregulating inducible nitric oxide synthase, through the removal of excess pro-inflammatory factors. [62] A prospective study of 40 patients with sustained severe acute pancreatitis who were given traditional Chinese medicine (Dachengqi decoction) has also been reported. The Chinese herbal medicine was given to half of the cohort and, when compared to controls, treated patients showed a significant improvement in intestinal permeability and a significantly lower incidence of multiple organ dysfunction syndrome and pancreatic infection. [193]

1.5.7 Interventional Treatments in acute pancreatitis

Local complications of pancreatitis include early peripancreatic fluid collection and pancreatitic /peripancreatic necrosis (less than 4 weeks), and late pancreatic pseudocyst and walled-off necrosis (more than 4 weeks). Acute peripancreatic fluid collection resolves spontaneously in most cases, and less than 10% result in pancreatic pseudocysts. Most of these pseudocysts resolve with observation alone with periodic radiological follow-up. Drainage is only recommended in symptomatic, infected or rapidly enlarging pseudocysts. The drainage modalities include endoscopic or open, the endoscopic approach being the preferred modality. [87]

The management of necrotic collections remains challenging. If sterile, intervention becomes necessitated if it causes symptoms such as persistent abdominal pain, nausea, vomiting, gastric outlet obstruction, bowel obstruction or disrupted pancreatic duct. A third of this necrosis become infected. Infection results in clinical deterioration, lengthens the recovery and is associated with high mortality. Antibiotics are required when infected necrosis develops. Diagnosis of infected necrosis is established in the presence of gas bubbles within on imaging and CT-guided percutaneous aspiration culture. Surgical necrosectomy is needed if patients continue to deteriorate clinically despite antibiotics. In stable patients, antibiotics are continued for 4 to 6 weeks and necrosectomy performed after the wall matures. The initial approach includes less invasive modalities which include endoscopic and percutaneous drainage and surgical debridement is reserved for unsuccessful cases.

Further management depends upon the aetiology of pancreatitis. In gallstone pancreatitis, early cholecystectomy is strongly recommended. Early ERCP (within 24 hours of presentation) is of benefit in cases of concurrent cholangitis and obvious biliary obstruction.

1.5.8 Intestinal permeability and severity of acute pancreatitis

Detection of an increase in endotoxaemia has been documented in patients with severe pancreatitis. Serum tumour necrosis factor concentrations are also correlated with disease severity and endotoxaemia and this cytokine has been proposed as a potential prognostic marker. [194] [140] Ammori *et al.* demonstrated that plasma levels of calcitonin precursors on the day of admission may predict disease severity more accurately than the APACHE II scoring system. [195] Cytokines, endotoxin, mast cells and mediators such as platelet-activating factor, oxygen free radicals, adhesion molecules and polymorphonuclear enzymes are all produced and liberated from pancreatic tissue in response to acinar cell necrosis. They contribute to early pancreatitis-induced systemic epithelial barrier dysfunction. [104] [196] Endotoxin also penetrates the epithelial mucosa when intestinal permeability increases. In response to reports of the correlation between endotoxin and the development of multi-organ failure, Windsor reported a series of 33 patients and showed that the initial trend in Acute Physiology Score and the concentration of endogenous IgG antiendotoxin core antibody, though not direct endotoxin, provided a means of identifying patients who had acute severe pancreatitis and were at high risk of developing multi-organ failure. [197] A similar study demonstrated that the presence of endotoxaemia accompanied by a fall in antiendotoxin antibody titres predicted poor outcome in patients with acute pancreatitis. [198] Gianotti postulated that once enteric bacteria reach pancreatic tissue, they can trigger an infection that can worsen the course of the disease. The worsening of pancreatitis might promote further passage of bacteria and endotoxin from the gut lumen and promote a self-perpetuating septic state. [199] Acute pancreatitis also resulted in increased intestinal permeation and the magnitude of this permeation correlated with disease severity in an experimental animal model. [200] Another similar study involving experimental pancreatitis reported increases in intestinal permeability which correlated with increases in plasma endotoxin concentrations and bacterial

translocation. Electron microscopy of ileal mucosa showed shortened microvilli and discontinuous tight junctions when compared to the normal villus cells of control rats. [201] Luan *et al.* demonstrated a significant increase in intestinal tissue high-mobility group box 1 (HMGB1) concentrations in rats which had severe acute pancreatitis. [116] Hotz *et al.* demonstrated that impairment of colonic capillary blood flow correlated with increased intestinal permeability, occurred early in acute pancreatitis and correlated with disease severity.

A number of clinical studies have also demonstrated a relationship between increased intestinal permeability and the severity of acute pancreatitis. Some of these studies have also identified potential markers that correlate with the severity of disease. To date none of these have been brought into routine clinical practice. These studies have been summarised in Table 2.

1.5.9 Cytokines in Acute Pancreatitis

Inflammatory cytokines are thought to play a major role in the pathogenesis of pancreatitis and the inflammatory response. A number of studies have therefore investigated the roles of individual cytokines as potential biomarkers in acute pancreatitis, but there are fewer reports in which more global changes in cytokine expression in acute pancreatitis have been assessed. The measurement of cytokine concentrations appears to provide a more accurate and objective method for the assessment of the severity of acute pancreatitis. In cases of acute pancreatitis with limited extent of injury, spontaneous resolution of the inflammation occurs. When inflammation persists, mediators are released into the circulation leading to the malfunction of various organs and a systemic inflammatory response.

After the initial acinar cell injury, local and systemic inflammatory responses are triggered. Inflammatory cells migrate into the interstitium by adhering to the endothelium and escaping from the microcirculation. The mechanisms by which inflammatory cells adhere to endothelial cells are determined by a variety of mediators or cytokines which are released at sites of tissue damage. Cytokines hold the key to both the local and the systemic inflammatory responses in acute pancreatitis. The inflammatory process results in excessive leukocyte activation and increased migration of neutrophils to the inflamed area, with a consequent release of pro-inflammatory mediators including interleukins (IL-1b, IL-6, IL-8, IL-10, IL-18) and TNF-α. (Figure 4) These mediators have been shown to influence the progression of a pancreatic infection to necrosis and consequently to SIRS and multi-organ failure. [202] Monocytes produce interleukin (IL)-6, a pro-inflammatory cytokine which is known to be involved in the pathogenesis of acute pancreatitis. Through the subsequent release of CRP, it is one of the most useful parameters for predicting the severity of an episode of acute pancreatitis.

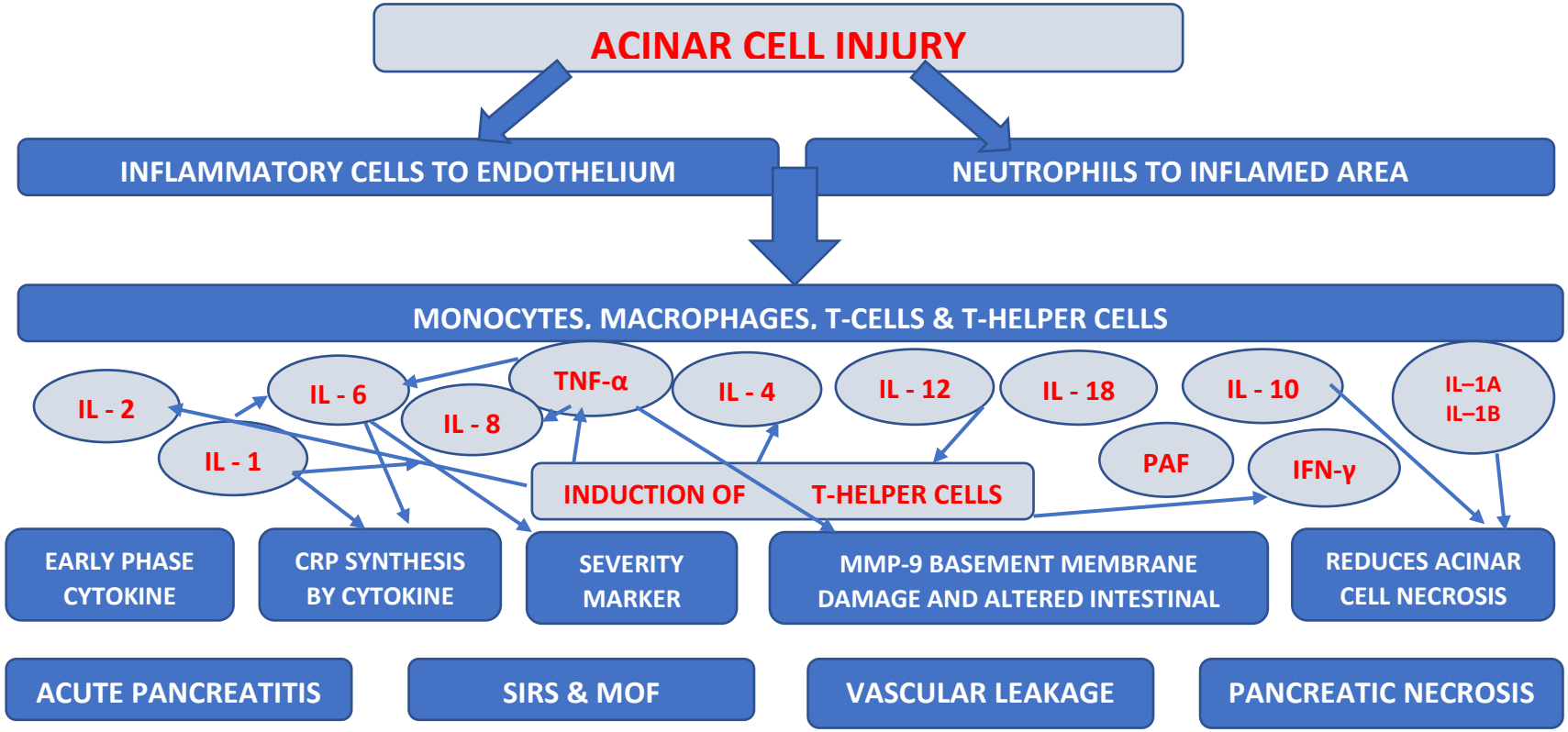


Figure 4. Schematic diagram to summarise the involvement of various cytokines in acute pancreatitis.

Interleukin-12 is also produced by monocytes and is a well-known inducer of the T-helper (Th) 1 cell immune response whereby T cells are induced to secrete interferon (IFN)-γ, IL-2 and TNF-α. The T lymphocytes secrete immunomodulatory cytokines that regulate the development and effector functions of their subset Th1 and Th2 cells. One study of immunological parameters in acute pancreatitis showed that the T-cell population is affected in pancreatitis and that CD4+ and CD8+ T-cell numbers are significantly reduced. The percentage of IL-6-producing monocytes was significantly higher in patients with acute pancreatitis. [203] Interleukin 6 is the principal mediator of the acute phase protein response and may be a natural host defence mechanism. It is an excellent marker of the severity of acute pancreatitis with peak concentrations (three to six times baseline) occurring at 48 hours and preceding rises in serum concentrations of the acute phase protein C-reactive protein. Values correlate with disease severity and predict outcome. [204] [205] A further study also showed that IL-6 had the highest significance as a predictor of acute pancreatitis severity. [206]

A study investigating serum TNF-α concentrations in acute pancreatitis did not find a significant increase between patients who had acute pancreatitis and controls, but did report increased expression of this cytokine when compared with patients with CP. Elsewhere it has been reported that tumour necrosis factor-alpha is detectable in the serum of 10-40% of acute pancreatitis patients and it is an appropriate target for anti-cytokine therapy in acute pancreatitis. TNF-α is a pleiotropic predominantly macrophage-derived cytokine that is thought to be involved in pathophysiological responses in cases of severe injury or sepsis, providing an autocrine and paracrine signal. [194]

TNF- α is among one of the inflammatory cytokines that is produced once necrosis develops. Nuclear factor –κB (NF-κB), a transcription factor associated with early gene activation, plays a critical role in the development of necrosis and, TNF-α is a cytokine primarily derived from macrophages which plays a major role in formation

of many pathophysiological responses of the organism to injury or disease. Systemic symptoms in septic shock, which is associated with increased TNF-α level, shows a close resemblance in patients with severe acute pancreatitis. [207]All of this kind of complications may be due to cellular response such as excessive neutrophil activation, increased capillary permeability and direct cellular toxicity. Current research is working towards TNF as a potential target for therapy in AP since blocking the production and the effects of this cytokines is a strategy that makes sense in its treatment. A recent case report however reports a case of AP induced by the very agent to be considered in its treatment, infliximab, a monoclonal anti-TNF-α antibody. [208]

It is considered that TNF-α increases the vascular permeability by a direct effect on vascular endothelium and also by indirect mechanisms involving leukocytes. In this way it increases the collection and activation of leukocytes in inflammation area. It is known that activated leukocytes secrete various mediators under the effect of TNF-α which is known to increase the vascular permeability. [209]

Innate immune cells and mediators are intrinsically linked to the pathogenesis of AP. Cathelicidins are innate immunity-derived antimicrobial peptides that exert immunomodulatory effects on various host cells. How cathelicidins are involved and modulate the severity and inflammatory responses of AP remains unclear, but a recent study in experimental acute pancreatitis using CRAMP gene-deficient cnlp-/- mice and their wild-type C57BL/6J littermates demonstrated that cnlp-/- mice exhibited a more severe phenotype and inflammatory response following AP induction compared with the wild-type mice, as evidenced by increased serum amylase levels, pancreatic myeloperoxidase release, and early inflammatory mediator TNF-α production. Histological examination confirmed that CRAMP deficiency worsened the pancreatic inflammatory condition. These results indicate that CRAMP may be considered a novel modulatory mediator in mouse experimental AP. [210]

Platelet activating factor (PAF) is a potent biological mediator that exerts its effects in a variety of cells and tissues. PAF primes polymorphonuclear (PMN) white cells and acts as a mediator for the interaction between PMN cells and endothelial cells, facilitating the migration of activated white cells into tissue spaces. [204] As described above, a platelet activating factor (PAF) inhibitor, Lexipafant, demonstrated a reduction in the incidence of organ failure in patients with severe acute pancreatitis. Seven of 12 patients with severe acute pancreatitis who received Lexipafant recovered from organ failure compared with only two of 11 patients with severe acute pancreatitis who had placebo. The study suggested a rationale for further clinical trials with the potent PAF antagonist Lexipafant in human acute pancreatitis. [190] Interleukin 1 is regarded as an early inducer of many of the systemic and acute phase responses to injury. It is difficult to measure in serum and its natural antagonist, interleukin 1 receptor antagonist may more accurately reflect the degree to which IL-1 stimulation has occurred. IL-1 and TNF-α are thought to induce the release of IL-6 and IL-8. [211]

IL-8 has been reported to show increased expression in the early phase of acute pancreatitis. It is released by many cell lines in the presence of activated neutrophils and is considered to be one of the main secondary mediators of TNF-α induced neutrophil activation. It is a polymorphonuclear cell activating peptide and seems to be the earliest cytokine to appear in the serum of patients with acute pancreatitis and is reported to correlate with severity of disease. [212]

Interleukin-10 is a naturally occurring anti-cytokine that inhibits several functions of macrophages including tumour necrosis factor and IL-1 production and the release of oxygen free radicals. Like IL-1ra, it is a naturally occurring inhibitor. In experimental acute pancreatitis in mice, recombinant IL-10 reduced acinar cell necrosis and tumour necrosis factor mRNA expression. [204] It has an anti-inflammatory role inhibiting the synthesis and release of other pro-inflammatory cytokines and free oxygen radicals from macrophages and T-helper lymphocytes.

On the other hand, it shows a positive effect on the proliferation and differentiation of B lymphocytes and promotes the production of immunoglobulins. [206]

IL-1 and IL-6 are thought to induce the synthesis of C-Reactive Protein (CRP) by hepatocytes in acute pancreatitis. CRP is currently the most clinically used non-specific inflammatory marker in acute pancreatitis patients, but a delay is often seen in its increase, and this can be explained by this secondary release. [202] TNF-α, IL-1b and IL-8 can stimulate the release of MMP-9, which damages basement membranes and alters intestinal permeability. [211] Other mechanisms for the alteration in epithelial permeability have also been investigated and proposed. One such study has reported that IFN-γ acts synergistically with IL-1b and TNF-α to induce the functional expression of NO synthase and therein provide a mechanism to induce intestinal epithelial hyperpermeability. [52] A more recent study reports a T-helper 1 profile that was associated with severe AP and a T-helper 2 profile with mild or moderately severe AP. The same authors also report an IL13/IFNγ ratio of potential value to predict the prognosis in AP. [213]

Our own research group undertook a meta-analysis on the value of carboxypeptidase-B activation peptide in predicting severity of acute pancreatitis on admission. We concluded that both serum and urinary CAPAP have the potential to act as a stratification marker on admission in predicting severity of acute pancreatitis. [83]

This brief overview of cytokines expressed in acute pancreatitis demonstrates that both pro-inflammatory and anti-inflammatory cytokines are released during the disease process. Pro-inflammatory mediators in acute pancreatitis include TNF-α, IL-1, IL-2, IL-6, and IL-18, and IL-8, platelet-activating factor, and reactive-oxygen and reactive-nitrogen species. With the release of these pro-inflammatory mediators, anti-inflammatory cytokines are concomitantly produced, leading to a compensatory response syndrome. High circulating concentrations of the anti-inflammatory mediators such as TNF-α receptors, IL-10, and IL-1ra have also been documented

in acute pancreatitis. [214] Thus, a number of inflammatory mediators have been shown to be involved in the development of acute pancreatitis. The pathways of damage are similar, whatever the aetiology of pancreatitis, with three phases of progression: local acinar injury, systemic response, and generalised sepsis. The pro-inflammatory response is countered by an anti-inflammatory response, and an imbalance between these two systems leads to localised tissue destruction and distant organ damage. [215] Cytokines are involved in all aspects of the cascade leading to systemic inflammatory response syndrome and multiple organ dysfunction syndrome and indeed also in the release of mediators that are likely to exacerbate alterations in intestinal permeability.

1.6 Chronic Pancreatitis

To support a study looking at acutely unwell patients, we were required to establish protocols in more stable patients with reported changes in intestinal barrier function. We thus undertook a pilot study looking at patients with CP and CLD, both with literature reporting alterations in gut permeability.

Chronic pancreatitis is a disease with an incompletely understood pathogenesis that features progressive inflammatory change in the pancreas, resulting in progressive, permanent structural damage. Chronic pancreatitis should be in the differential diagnosis of a patient with typical features of epigastric pain with radiation to the back, steatorrhea, weight loss, or recurrent acute pancreatitis. It is characterized by atrophy and fibrosis of the exocrine tissue with or without chronic inflammation. [216]

As expressed in the guidelines and in a recent retrospective study, there needs to be more stringent application of the systems used for diagnosing chronic pancreatitis with revision of the current terminology 'indeterminate', 'suggestive', 'possible', and 'early' chronic pancreatitis. A number of patients with chronic abdominal pain syndrome find themselves with a label of chronic pancreatitis despite minimal changes on EUS. [216]

Intestinal permeability changes have been reported, though limited, and will be described in the following section. The disease is associated with increasing exocrine insufficiency (steatorrhoea) and often later endocrine dysfunction (diabetes), with progressive fibrosis and atrophy. [217] Prevalence has been reported to be approximately 30 per 100,000 and is higher in populations which have a high alcohol intake. The incidence varies from 5-11 per 100,000 in men to 2-3 per 100,000 in women. [218-221] As a result of increased alcohol consumption, the disease has an increasing incidence. The commonest cause is long-standing excessive ethanol consumption, [222] [223] followed by hypertriglyceridemia, autoimmune pancreatitis, and disease caused by genetic abnormalities (e.g.

hereditary pancreatitis caused by mutations of the cationic trypsinogen gene, PRSS1) or in association with genetic abnormalities (e.g. mutations of the cystic fibrosis transmembrane conductance regulator, CFTR, or serine protease inhibitor Kazal type 1, SPINK-1). [224] About 10% of cases are idiopathic. [219] Morphologically, chronic pancreatitis is characterised by loss of tissue parenchyma, especially acinar cells, an inflammatory infiltrate and varying amounts of fibrosis that hardens and distorts the gland and its surroundings. [91] Pancreatic stellate cells are the mediators of fibrosis in the pancreas, and lead to the formation of extracellular matrix in interstitial spaces. [225] This process leads to progressive loss of the lobular morphology and structure of the pancreas, deforming the large ducts and leading to changes in the islets. This pancreatic fibrosis is initiated by injury to the pancreas, the mechanism of which depends on the underlying disease aetiology. The exact pathogenesis of chronic pancreatitis is unknown, but several hypotheses have emerged as to its mechanism, reflecting the different processes that may apply due to the variety of different aetiological factors implicated in the disease. [226] The diagnosis of chronic pancreatitis is often difficult because symptoms are frequently non-specific, there is no reliable means to identify early disease and more advanced disease can often only be confirmed by complex imaging. Due to the deep-seated retroperitoneal nature of the organ, histological diagnosis is awkward and requires specialist input. [91] Diagnostic imaging studies include endoscopic ultrasound (EUS), more recently with endoscopic ultrasound elastography with or without contrast, endoscopic retrograde cholangiopancreatography (ERCP), computerised tomography (CT) and magnetic resonance imaging (MRI) with MR cholangiopancreatography (MRCP). [227] [228] Ultrasound and CT are best for the late findings of chronic pancreatitis but are limited in the diagnosis of early or mild pancreatitis. Intraductal pancreatic calcifications are the most specific and reliable sonographic and CT signs of chronic pancreatitis. [229] CT is helpful for the diagnosis of complications of chronic pancreatitis and for other conditions that can

mimic chronic pancreatitis. [216] Compared to US and CT, MRI is a more sensitive imaging tool for the diagnosis of chronic pancreatitis. Ductal abnormalities are very specific and reliable MRI signs of chronic pancreatitis. Signal intensity changes in the pancreas, seen on MRI, may precede ductal abnormalities and suggest early chronic pancreatitis. Stimulation of the pancreas using IV secretin may improve the diagnostic accuracy in the detection of ductal and parenchymal abnormalities seen in chronic pancreatitis. [216] MRCP is increasingly being used, as it is a safe, non-invasive modality, does not involve radiation, and does not cause acute pancreatitis. [230] After secretin injection, duodenal fluid volume can be calculated and used as a marker of secretory function. Endoscopic Ultrasound (EUS) has an increasing role in the diagnosis and management of chronic pancreatitis. EUS in some studies has been shown to be as effective as ERCP in diagnosing CP. [231] EUS can also demonstrate abnormalities in many individuals without definite ERCP features. [232] It allows high resolution imaging of both the parenchyma and pancreatic ducts. Experienced clinicians can detect early stage CP with good inter-observer agreement (92%). [232] Recent guidance has been given in the diagnosis of CP using EUS; this is referred to as the Rosemont classification.[233] The gold standard for diagnosis of chronic pancreatitis is histopathology, but this is rarely available unless surgery has been undertaken. [234] The diagnosis of advanced CP may be more straightforward if there are calcifications within the gland. These may be picked up with a plain abdominal X-ray, although calcifications are not completely exclusive to CP. [235, 236] Calcifications typically develop years after the onset of symptoms in alcoholics (median 8.7 years) and even longer in early onset idiopathic pancreatitis (24.9 years). [237] The main diagnostic challenge is detecting early stage disease.

Indirect pancreatic function tests generally are sensitive for steatorrhea, and useful in quantifying the degree of exocrine insufficiency. They are moderately sensitive and specific for diagnosing advanced chronic pancreatitis, but are less so for

diagnosing early chronic pancreatitis. The faecal elastase assay, can be limited in specificity. Faecal chymotrypsin may be useful in detecting compliance with exogenous pancreatic enzyme supplementation. Faecal fat assays are sensitive for steatorrhea but are of limited utility due to the cumbersome nature of patient collection and lab handling of samples. In addition, strict adherence to dietary recommendations for several days is required. Direct pancreatic function tests have high sensitivity for detecting late chronic pancreatitis, but lower sensitivity for early chronic pancreatitis. The traditional secretin and CCK tests performed with the oro-duodenal tube pancreas fluid collection are highly accurate, but require fluoroscopy for confirmation of tube placement and are not widely utilized. An endoscopic pancreatic function test can also be considered. As structural severity worsens in CP, exocrine function declines and pancreatic function tests correlate accordingly as do EUS findings. [216]

Direct pancreatic exocrine function tests have been acknowledged as reliable confirmatory methods to diagnose or exclude chronic pancreatitis. Tube-based versions of these tests measure the organ's secretory capacity (enzymes and/or bicarbonate) in response to hormonal stimulants (cholecystokinin [CCK] and/or secretin). Measurement of the concentrations of cytokines and other pro-inflammatory molecules in pancreatic secretions has also been proposed, but has not yet been substantiated. The sensitivities of pancreatic function tests depending on duodenal intubation to sample pancreatic juice range from 72-94%, with specificity of 80-90% when compared with histology. Unfortunately, due to the prolonged duodenal intubation times required and technical demands of tube-based pancreatic function testing, this approach has fallen out of favour and is no longer available in the UK. [227] Elsewhere, notably in the USA, endoscopic sampling of duodenal juice collected after pancreatic stimulation has been developed and is now available in several centres, but this approach samples for shorter periods than the original tube-based tests (15 min compared to over two hours), is labour intensive and more

invasive, and has yet to be adopted in the UK. In the UK and elsewhere, tubeless pancreatic function tests are used such as faecal elastase activity and the Pancreolauryl test. However, these have lower sensitivities and specificities, and although they are more widely used, are less reliable. Thus a gold standard alternative test to those currently available would be an invaluable addition to the diagnostic algorithm for patients with suspected chronic pancreatitis. Endocrine testing does not feature as part of the diagnostic pathway in Chronic Pancreatitis. However diabetes is a well-recognised late feature of the disease. Little is known about pancreatic endocrine function before the development of DM in CP. A study of patients CP patients had higher fasting and random glucose levels, without a compensatory increase in insulin secretion suggesting subtle early islet dysfunction. Better understanding of the progression from endocrine dysfunction to diabetes mellitus is critical to develop screening tools for early detection and intervention. [216]

Autoimmune pancreatitis accounts for about 5% of all pancreatitis, and it is associated with an increase in serum immunoglobulin G4 which is a diagnostic criterion. Interestingly, the gastric Helicobacter pylori infection has been shown to be associated with AIP. This bacterium is known to trigger immune responses against host tissues via several molecular mimicry pathways. Other suggestions link bacterial infections with the development of AIP. In a mouse model, Escherichia coli induces a severe pancreatic inflammation and fibrosis similar to the human AIP. Numerous studies have reported that specific microbial antigens may trigger the development of AIP activating immune responses. [238]

1.6.1 Gut barrier dysfunction and bacterial translocation in chronic pancreatitis

Reports of alterations in intestinal permeability, bacterial translocation and endotoxaemia in chronic pancreatitis are limited. In CP, increased IP has been reported in cystic fibrosis patients who have pancreatic insufficiency compared to those without, secondary to changes in intestinal epithelial tight junctions. [10] The concentrations of circulating cytokines have been associated with increased concentrations of pathogenic microbiota in the gut, and gut permeability. [11]

Alterations of gut microbiota have been reported in pancreatic diseases. In particular, the gut microbiota may be affected by malnutrition or impaired exocrine pancreas function that is associated with pancreatic diseases. [239] One recent study conducted a comprehensive analysis of gut microbiota in patients with type 1 autoimmune pancreatitis, a pancreatic manifestation of the systemic IgG4-related disease, and chronic pancreatitis to enable a comparison between altered immune reactions in autoimmune pancreatitis and/ or long-standing malnutrition in CP and to assess the influence on gut microbiota. Gut microbiota profiles were different between patients with AIP and those with CP; namely, the proportions of Bacteroides, Streptococcus and Clostridium species were higher in patients with CP, for reasons yet unknown. Pancreatic diseases such as pancreatic cancer, chronic pancreatitis and autoimmune pancreatitis are often accompanied by pancreatic exocrine insufficiency which could affect the gut microenvironment and microbiota. It is reasonable to assume that altered immune reactions in AIP and long-standing malnutrition in CP modify the dysbiosis. [240]

1.7 Chronic Liver Disease

Intestinal permeability changes in CLD have been reported and will be discussed in the forthcoming section. Cirrhosis is a consequence of chronic liver disease and is characterised by replacement of liver tissue by fibrosis, scar tissue and regenerative nodules leading to loss of hepatic function. Uncorrected long-term, this is likely to lead to portal hypertension and death from liver failure, variceal haemorrhage or malignant transformation (hepatoma / hepatocellular carcinoma). Cirrhosis is most commonly caused by excess alcohol consumption, hepatitis B or C infection, and non-alcoholic fatty liver disease (NAFLD), but also has many other autoimmune and metabolic causes. Some cases are idiopathic. It is difficult to estimate the exact prevalence of cirrhosis as there is often undiagnosed disease and previously undiagnosed cirrhosis is often found at post mortem examinations. The incidence of cirrhosis is estimated at approximately 7000 per year in the UK. An analysis published in the Lancet showed that between 1960 and 2002, total recorded alcohol consumption in Britain doubled. [241] Mortality in women from cirrhosis has also increased significantly.

Patients with cirrhosis of the liver may present in a variety of ways and with various clinical findings. The history and physical examination can provide clues as to the presence of cirrhosis and suggest its aetiology. Routine laboratory tests are generally not diagnostic, but can suggest the presence of liver disease by means of an elevated prothrombin time and total bilirubin, and reduced platelet count and serum albumin with abnormalities in serum aminotransferase levels. Radiographic findings can also suggest the presence of cirrhosis by detecting the presence of complications such as ascites, hepatocellular carcinoma and hepatic or portal vein thrombosis, but are not adequately sensitive or specific to use as a primary diagnostic modality. A review of the treatment of liver disease and cirrhosis is outside the remit of this chapter.

1.7.1 Gut barrier dysfunction and bacterial translocation in chronic liver disease

Gut barrier dysfunction, endotoxaemia and bacterial translocation also compound liver disease severity, and lead to decompensation of cirrhosis as a result of spontaneous bacterial peritonitis, hepatic encephalopathy, hepatorenal syndrome and variceal bleeding. [12] [13] [14] Immunomodulatory, cellular and molecular events lead to changes in intestinal tight junction proteins, cellular mechanisms which may lead to intestinal barrier disruption and hyper-permeability. Patients with hepatic encephalopathy show an altered intestinal microbiome, increased serum endotoxin levels, and increases in serum cytokine concentrations (particularly IL-6, TNF-α, IL-2, and IL-13). [15] There is considerable evidence to demonstrate that intestinal permeability increases in patients with liver cirrhosis, and that the impairment in intestinal barrier function may contribute to severe septic complications in these patients. Gut-derived complications in liver cirrhosis and altered intestinal permeability result in bacterial or endotoxin translocation-related systemic disorders such as spontaneous bacterial peritonitis, hyperdynamic state, portal hypertension, hepatorenal syndrome, hepatic encephalopathy, and multiple organ failure. [242] [243] This is further supported by studies which have shown significantly increased intestinal permeability in patients with ascites compared to healthy controls, as opposed to patients without ascites when compared with healthy controls. [244]

Increased intestinal permeability is also thought to be a major risk factor for the development of acute on chronic liver failure and has been linked to disease progression and to infectious complications. However, it has not yet been systematically evaluated in this cohort of patients. A recent study evaluating urinary lactulose:mannitol ratios in such patients showed significantly higher intestinal permeability in patients with acute on chronic liver failure when compared to normal controls. This difference normalised post treatment, suggesting that gut permeability

improved in patients with chronic liver failure once the acute illness had resolved. [245]

Different aetiologies of liver cirrhosis, including alcohol and obesity, are associated with different gut microbiota profiles and differing mechanisms of developing liver fibrosis. [246] [247] Alcohol abuse impairs the function of the intestinal barrier, which might enhance the translocation of bacterial toxins, thereby contributing to inflammatory processes in alcoholic liver disease. [248] Chronic endotoxin exposure, suggesting intestinal permeability alterations, has also been identified in patients with non-alcoholic steatohepatitis. [249] Cirrhosis per se has been shown to be associated with increased intestinal permeability as assessed by lactulose:mannitol ratios. [250] [251]

Dysfunction of the intestinal epithelial barrier may therefore enhance the risk of translocation of bacteria and bacterial products into the systemic circulation, and thereby contribute to the pathogenesis of chronic liver diseases and the development of complications. [244]

1.8 Gut barrier dysfunction and bacterial translocation in patients with a history of gastrointestinal surgery

A few reports of gut barrier dysfunction post-surgery have been published, though those studies that have been reported tend to be limited to a period soon after an operation rather than after much time has elapsed. One such study investigated 63 patients undergoing elective abdominal surgery and assessed bacterial culture, microbial DNA, plasma D-lactate and plasma endotoxin up to 2 days post-surgery. All patients were observed for a subsequent period of 30 days for infectious complications. The investigators concluded that increased intestinal permeability was closely related with bacterial translocation and that the latter occurred most commonly early after surgery. It was also postulated that postoperative SIRS and infection might bear a close relationship with bacterial translocation. [252] Increases

in intestinal permeability and endotoxaemia have also been reported during open cholecystectomy and in the peri-operative period. This study was also limited to 48 hours post-surgery. [253] An operation directly involving the stomach and gastrointestinal tract, the Roux-en-Y gastric bypass has also been found to cause increased intestinal permeability in 16 morbidly obese patients at varying times, but this study also, demonstrated similar findings after 6 months. [254]

Interestingly, cardiac surgery also appears to cause an increase in intestinal permeability and endotoxaemia. Findings were more pronounced in patients who became unwell and developed multiple organ dysfunction and, once again, long term follow up data were not reported. [255] [256] In the case of cardiac surgery, a further study did postulate a hypothesis for the observed alterations in gut permeability. Mean arterial pressure is reduced during hypothermic cardiopulmonary bypass, whilst maintaining generally acceptable levels of haemodynamic performance. This intestinal hypoperfusion may be responsible for the altered intestinal permeability that was observed. [257] Similarly, a significant increase in intestinal permeability has been reported in patients after elective and emergency major vascular surgery. It has been suggested that this was due to reperfusion injury rather than the ischaemic period of the intestine itself. [258] Again, lactulose:mannitol ratios were assessed up to 48 hours after the operation.

It would be interesting to understand the mechanisms involved in altered intestinal permeability in patients who have undergone surgery of the gastrointestinal tract as well as the indirect effects from other forms of surgery. At present, the long-term effects of surgery on gut barrier function remain largely unknown and it would be useful to test whether the documented short-term alterations persist, increase or return to normal.

1.9 Techniques used to measure intestinal permeability

Several tests have been developed to examine intestinal barrier function and these are summarised in Table 3. These tests take into account the epithelial barrier and intestinal permeability. [5] Other factors that need to be accounted for include: the concentration gradient; epithelial surface area; permeation time and the barrier's permeability properties. [16] Most of the tests are non-invasive and involve measurements of changes in absorption and subsequent urinary excretion of specific non-metabolised compounds which have different sizes and routes of absorption. A small and water-soluble test substance is used as a probe and is administered orally. This substance must be quantitatively cleared by the kidney and excreted in the urine and a breach in the mucosal barrier causes an increase in uptake of the substance in the bowel and a subsequent increase in urinary excretion. [16] Due to pre and post mucosal influences on probes, a single test substance has been deemed an inaccurate indicator of permeability. The ratio of two substances with varying molecular weights is therefore now used instead. [18] Four major classes of probes are used: ethylene glycol polymers (PEG-400), oligosaccharides (lactulose), monosaccharides (mannitol), and radiolabelled chelates (Cr-EDTA). The probes cross the epithelial barrier by means of paracellular, transcellular aqueous, and transcellular lipid routes. The excretion ratio is expressed as a function of the two probes used. The smaller sugar is absorbed via the transcellular route, by active transport or simple diffusion, whereas the larger sugar diffuses passively via the paracellular route. Quantitation of the probe in a timed urine or serum collection provides a measure of the fraction of ingested probe that has penetrated the mucosal barrier. Only a small proportion of this large probe should get through the intestinal mucosa, reach the circulation, get filtered by the kidney and get measured in the urine over a defined point of time. If there is an abnormality in the paracellular transport; the passage of the probe is enhanced and thus more is excreted in the urine which suggests abnormality in the intestinal permeability. Intestinal permeability can be assessed using a variety of

marker probes such as lactulose, mannitol, rhamnose and cellobiose, polyethylene glycol (PEG) 400, PEG 1000, 51Cr-EDTA and 99mTc-DTPA. Multiple mucosal and non-mucosal factors can affect the excretion of a single dose of a test probe and thus a dual sugar probe test is used for the assessment of intestinal permeability. This involves the simultaneous oral administration of a disaccharide and a monosaccharide and measurement of their excretion in urine over a defined period of time. Intestinal permeability is measured as the ratio of percentage of excretion of disaccharides/monosaccharides in the urine or serum. Expression of these excreted sugars as a ratio overcomes the individual variations of other factors. [18]

The concentration of one or more sugar probe can be measured using enzyme assay, high performance liquid chromatography (HPLC), ion-exchange chromatography in combination with mass spectrometry and liquid chromatography with mass spectrometry. If the PEG has been used as a probe, then the concentration of the PEG can be assessed using capillary column gas chromatography.

Lactulose and mannitol ratio is the most commonly used test for assessment of intestinal permeability and the most reliable method of measurement of concentration of lactulose and mannitol is HPLC. HPLC is a chromatographic technique used to separate a mixture of compounds with the purpose of identifying, quantifying and purifying the individual components of the mixture. [17]

High pressure liquid chromatography (HPLC) for the determination of lactulose and mannitol in serum, as opposed to urine, 90 minutes after ingestion of a sugar solution, is another reported method. [19]

In rodent models, intestinal permeability can also be measured by assessing the *in vivo* leakage of radio-labelled albumin or by the *in vitro* passage of smaller molecular markers using Ussing chambers. [259]

More recently, the assessment of cell shedding from the epithelium of the villus tip has been proposed as a marker of intestinal permeability. Small intestinal epithelial gaps have also been identified in humans by means of confocal endomicroscopy. [4]

[26] An increase in the number and density of gaps has been demonstrated in patients with certain gastrointestinal conditions, as detailed below. The confocal endomicroscopy features associated with increased intestinal permeability can be rapidly identified with high inter- and intra-observer agreement. [260]

1.10 Confocal Laser Endomicroscopy

Confocal microscopy is a method used to obtain cross sectional images at a chosen microscopic level – a 'slice' – that is too small to be seen with the naked eye and shows the features of individual cells. The method uses chemicals that are loaded into cells and when these are excited with light of certain wavelengths, they emit light of different wavelengths, a phenomenon termed fluorescence. This fluorescent light is then used to generate an image which provides information about the nature of cells and their specific states, for example whether they are undergoing apoptosis or necrosis. The technique has been used for many years in laboratories around the world, and recently has begun to be used directly in patients for the diagnosis and treatment of disease.

Confocal probes have now been put into endoscopes (confocal endomicroscopy) so that microscopic examination of the intestinal mucosa can be made. Work conducted using the technique of confocal endomicroscopy has identified epithelial gaps in the intestinal mucosa of mice and humans and their number increases dramatically in various inflammatory disease states. (Figure 5) [21] Administration of TNF-α greatly increases the appearance of epithelial gaps, most likely through the induction of epithelial apoptosis. This process is reversed by the administration of anti-TNF-α antibody, which prevents bacterial translocation in cirrhosis. [261] Trophic factors also reduce this process. [110, 154, 262, 263] Intestinal epithelial apoptosis has been demonstrated to be markedly increased in conditions associated with elevated cytokine levels, and this leads to increased intestinal permeability and barrier

dysfunction. [45, 106, 115, 264] Bacterial overgrowth [265] and loss of tight junctions [45] have been ruled out as explanations for this process.

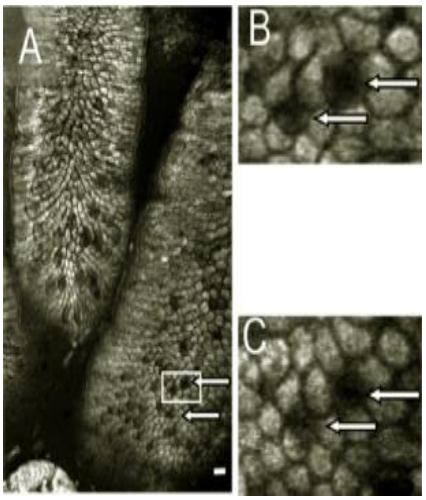
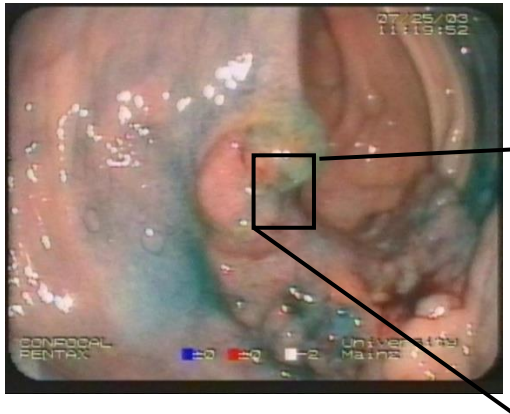


Figure 5. Confocal imaging of intact small intestine and effect of TNF-α seen with acriflavine stain: **(A)** Human ileal villi showing epithelial gaps; (**B)** Villi at higher power; **(C)** Villi at deeper tissue plane. *Figure courtesy of Keisslich et al. [4]*

Table 3. Gut permeability tests			
Gut epithelial barrier integrity	**Mechanisms**	**Specificity**	**Notes**
I-FABP	I-FABP is a small cytosolic protein (14-15 kDa) exclusively expressed on the tops of villi in mature enterocytes and is rapidly released into circulation upon enterocyte damage	Enterocyte necrosis	Non-invasive (blood/urine); Gut specific
Serum DAO	DAO is an enzyme that catalyses the oxidation of diamines, in human and rodents, DAO specifically located at mature villous cells in intestinal mucosa	Integrity and maturity of small intestine mucosa	Non-invasive (blood); Gut specific
αGST	αGST is predominately expressed in liver, kidney and intestine. It belongs to GSTs families which conjugate a range of toxic and foreign compounds within the cell to glutathione executing cell protection, antioxidation and detoxification activities	Intestinal epithelial cell damage	Non-invasive (blood); Not gut specific
Tight junction proteins (ZO proteins, claudin proteins and F-actin)	Tight junction proteins are components that form paracellular intestinal barrier	Paracellular intestinal barrier damage	Non-invasive (blood); Despite predominately gut origin but may involve other sources
Functional assessment			
L/M ratio; PEGs; D-Xylase; L/R ratio; Multisugar probes; ^{51}Cr-EDTA	Large size molecules would traverse the epithelium by paracellular pathways via tight junction if it is impaired	Paracellular intestinal barrier damage	Non-invasive (urine); Consider pre-/post-mucosal, mucosal conditions for test used
L-citrulline	L-citrulline is a nonprotein amino acid almost entirely generated by enterocyte in small intestine from glutamine	Enterocyte mass loss or dysfunction	Non-invasive (blood); Gut specific; Circulating levels confounded by renal function
Gastric mucosal $PiCO_2$	Increase in gastric CO_2 production is associated with anaerobic metabolism in the regional splanchnic perfusion	Gut mucosal perfusion	Reflecting regional splanchnic perfusion status; Invasive; Not organ specific
Intestinal epithelial cell shedding	Intestinal epithelial cells are constantly shed leaving gaps in the epithelial monolayer. In inflamed mucosa, the sealing mechanism fails with loss of barrier function	CLE: fluorescein leakage by CLE	Invasive procedure requiring trained endoscopist
Bacterial translocation (BT)			
Endotoxins	Bacterial components such as lipopolysaccharides are increased when commensal bacteria translocate into circulation	Direct evidence of BT	Non-invasive (blood); Relative low sensitivity
DNA/RNA	Bacterial breakdown products	Direct evidence of BT	Non-invasive (blood); Technique demanding
EndoCAb	Antibodies to endotoxins are consumed and reflect circulation endotoxin levels	Indirect evidence of BT	Non-invasive (blood); Technique demanding
D-lactate	Bacterial fermenting product	Indirect evidence of BT	Non-invasive (blood); Not organ specific

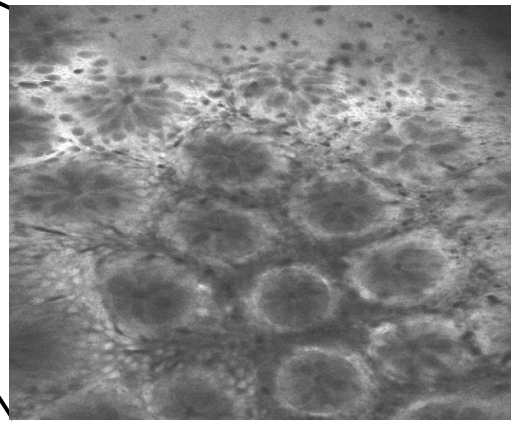
Confocal laser endomicroscopy (CLE) is a novel diagnostic tool which enables assessment of *in vivo* mucosal histology, and assessment of intestinal barrier function, during endoscopy. It allows for the performance of real time histology during endoscopy by observation of the mucosal layer of the gastrointestinal tract at the cellular level. CLE uses a fibre-optic cable to convey blue laser light to a miniaturised confocal microscope integrated into the 12-mm-diameter tip of an endoscope. [266] The laser light is focused on an area of interest and back-scattered light is then refocused onto the detection system by the lens. The back-scattered light passes through a pinhole aperture which increases the resolution of the image. Laser light collected from the tissue in focus by the confocal microscope is transferred to the photodetector. The intensity of the photo signal is measured to create a two-dimensional image. The image depth can be adjusted by moving the microscope optics. CLE provides subcellular resolution with 1000-fold magnification. (Figure 6) Multiple images at varying depths can be taken at the same point of mucosa. (Figure 7)

Patient preparation for confocal endomicroscopy is the same as the preparation for conventional endoscopy. The procedure is performed under conscious sedation or general anaesthesia depending on the clinical indications. The endoscope is fragile due to the multiple components within it and, in particular, the laser element must be handled with care. Two screens are required to ensure simultaneous visualisation of the mucosa macroscopically and the fluorescence. (Figure 8)



Normal Endoscopy
- **~30X**
- **No microscopic details**

Zoom Endoscopy
- **~100X**
- **Crypts visible**



CLE
- **~1000X**
- **Cellular resolution of crypts**
- ***In vivo* histology**

Figure 6. Demonstration of level of magnification with normal endoscopy to zoom endoscopy compared with confocal laser endomicroscopy (CLE) - *Images adapted with courtesy from Pentax – Best of 2nd International Endomicroscopy Meeting*

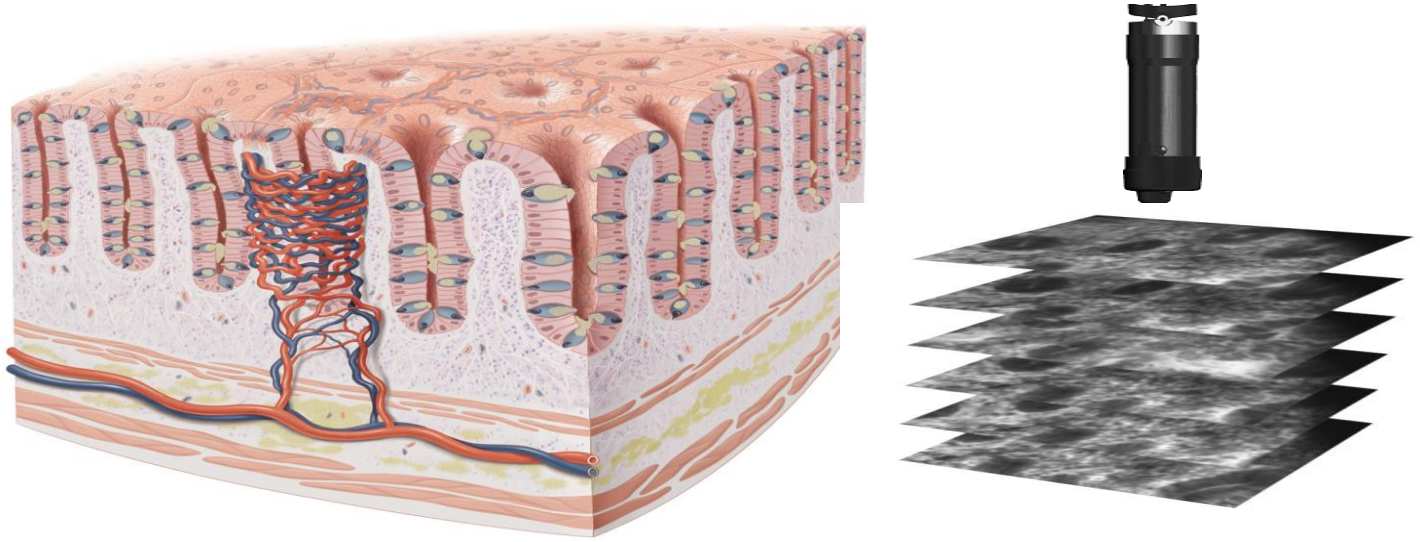


Figure 7. The scope tip is held in a stable position with contact with the mucosa. Images are taken to a set depth as per the above magnification producing a z-stack of images of increasing depth.

Images adapted with courtesy from Pentax – Best of 2nd International Endomicroscopy Meeting

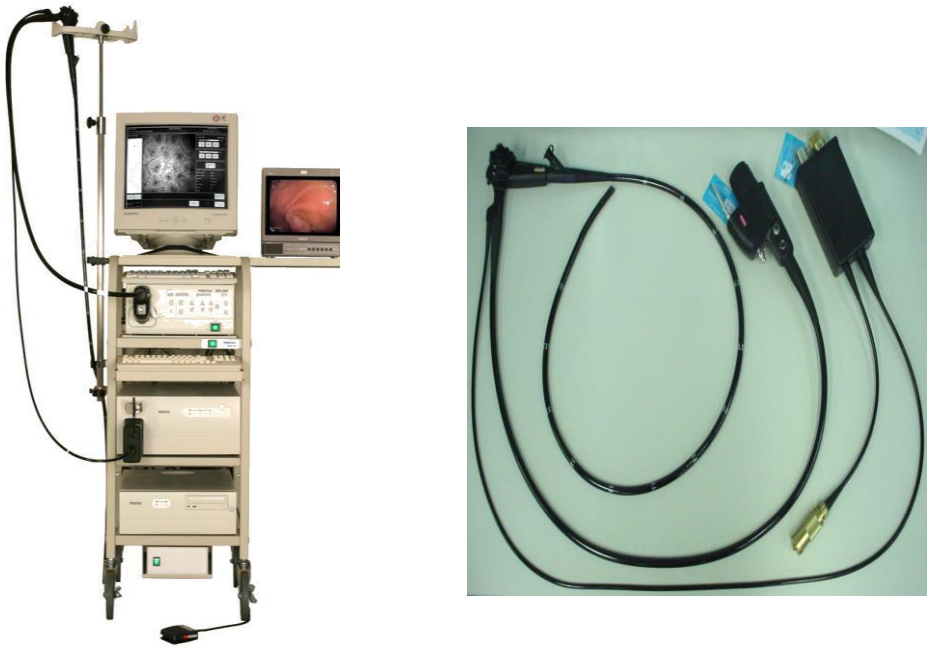


Figure 8. Confocal laser endomicroscopy system with 2 screens and image analyser. *Images adapted with courtesy from Pentax – Best of 2nd International Endomicroscopy Meeting*

Acriflavine hydrochloride, cresyl violet and fluorescein sodium are the fluorescent dyes that can be used during CLE. Fluorescein is FDA (Food and Drug Administration authority) - approved for diagnostic angiography of the retina and a further study has looked specifically at its safety during CLE. [267] Of 2272 procedures undertaken in which 2.5-5ml of 10% sodium fluorescein was injected intravenously in each subject, mild reactions were noted in only 1.4% of individuals, including nausea/vomiting, transient hypotension without shock, injection site erythema, diffuse rash and mild epigastric pain. Thus, fluorescein use was reported to be safe for use for gastrointestinal CLE with few acute complications. Intravenous fluorescein has very rapid distribution in the vasculature, and can be visualised by confocal imaging within seconds of administration. Acriflavine however is not FDA approved as it is potentially mutagenic due to binding to nucleic acids. It also has limited penetration and gives uneven staining. Cresyl violet has limitations due to

the fact that it does not demonstrate the vascular structure of the tissue being studied well. Hence, fluorescein is the most widely used dye in CLE. Once the fluorescein dye has been absorbed by the vasculature, it is taken up by the intervillous capillaries resulting in a horizontal cross section on the endomicroscopy screen, comparable to H & E staining on conventional histology, as demonstrated in figure 9. These images can be taken and stored on the hard drive provided with the CLE system.

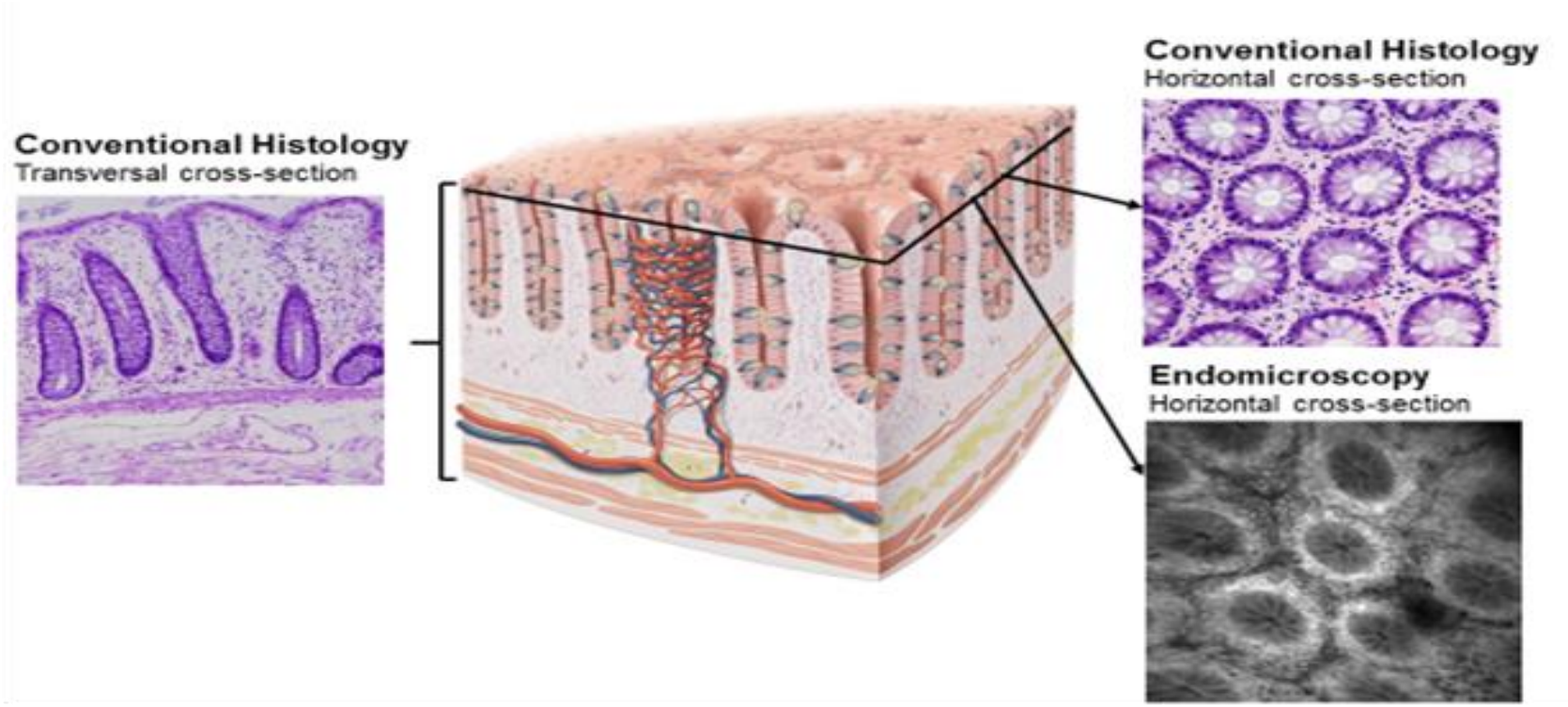


Figure 9. A demonstration of *in vivo* histology as per confocal laser endomicroscopy compared to conventional histology – horizontal cross-section. *Images adapted with courtesy from Pentax – Best of 2nd International Endoscopy Meeting.*

The technique of confocal endomicroscopy has been used to evaluate various upper gastrointestinal disorders such as Barrett's oesophagus, gastric carcinoma and coeliac disease. It also has applications in the lower gastrointestinal and biliary tracts and can assist in the diagnosis of conditions such as colonic polyps, ulcerative colitis and pancreaticobiliary strictures. The use of CLE in IBD, polyps and Barrett's oesophagus is extensive and the technique is now used for clinical evaluation and therapy. [268] A very recent report of the use of CLE for the evaluation of pancreaticobiliary strictures and pancreatic cystic lesions showed that CLE has the ability to determine the malignant potential of such lesions that are so difficult to characterise. The technique therefore also has the potential to increase the diagnostic yield of endoscopic retrograde cholangiopancreatography and endoscopic ultrasound. [269, 270]

CLE has also been used for the assessment of small intestinal epithelial gaps, but no single approach for their assessment and interpretation has yet been validated. [271] Various methods of interpretation of gut barrier function by CLE have been reported. [271] Features suggestive of increased IP can be determined with high inter-/intra-observer agreement from confocal endomicroscopy images and without previous pathology training. [272] One study which reported mucosal barrier defects in gastric intestinal metaplasia demonstrated an impaired paracellular barrier and confirmed the findings using electron microscopy. [273] CLE has previously identified epithelial gaps in the ileal and duodenal mucosa of patients who had inflammatory bowel disease (IBD) and this correlated with increased IP and clinical relapse. [274] [275] [1] [4] The findings in the duodenum, an area usually unaffected in IBD, suggest that increased cell shedding and barrier loss may be underlying pathogenic mechanisms. [23] CLE has also demonstrated the presence of intramucosal bacteria *in vivo* in patients with IBD, [276] and increases in epithelial gaps and IP in patients with irritable bowel syndrome. [277] Increased gaps have

also been demonstrated by CLE in indomethacin-induced enteropathy. [277] [278] [279] It is therefore reasonable to hypothesise that cell shedding is increased in inflammatory diseases where there is evidence of involvement of the intestine directly or indirectly. On the basis that epithelial cells destined for extrusion from the intestinal surface should stain positive for activated caspase-1 or -3 on mucosal biopsy samples, investigators compared stained terminal ileum biopsy samples with CLE images from the same site in patients with IBD, IBS and healthy controls. Intestinal epithelial gap density via CLE correlated strongly with quantitative analysis of immune-histochemical staining of mucosal biopsy samples. [280]

Studies reporting intestinal permeability changes as evidenced by CLE, have each used the standard principle of fluorescein leakage into the extravascular space, but have had subtle differences in terms of specific methodology. One such study which analysed mucosal healing in patients with ulcerative colitis for example used 10 CLE images obtained from each colon for analysis. To examine intestinal permeability, they measured fluorescence signal intensity (FSI) inside the vessel and outside the vessel lamina propria in six standardised fields per image, and then mean values were calculated for each patient. The FSI gradient between vessels and lamina propria was calculated. Figure 10 shows a representational image od a duodenal villus as seen by CLE and by conventional histology.

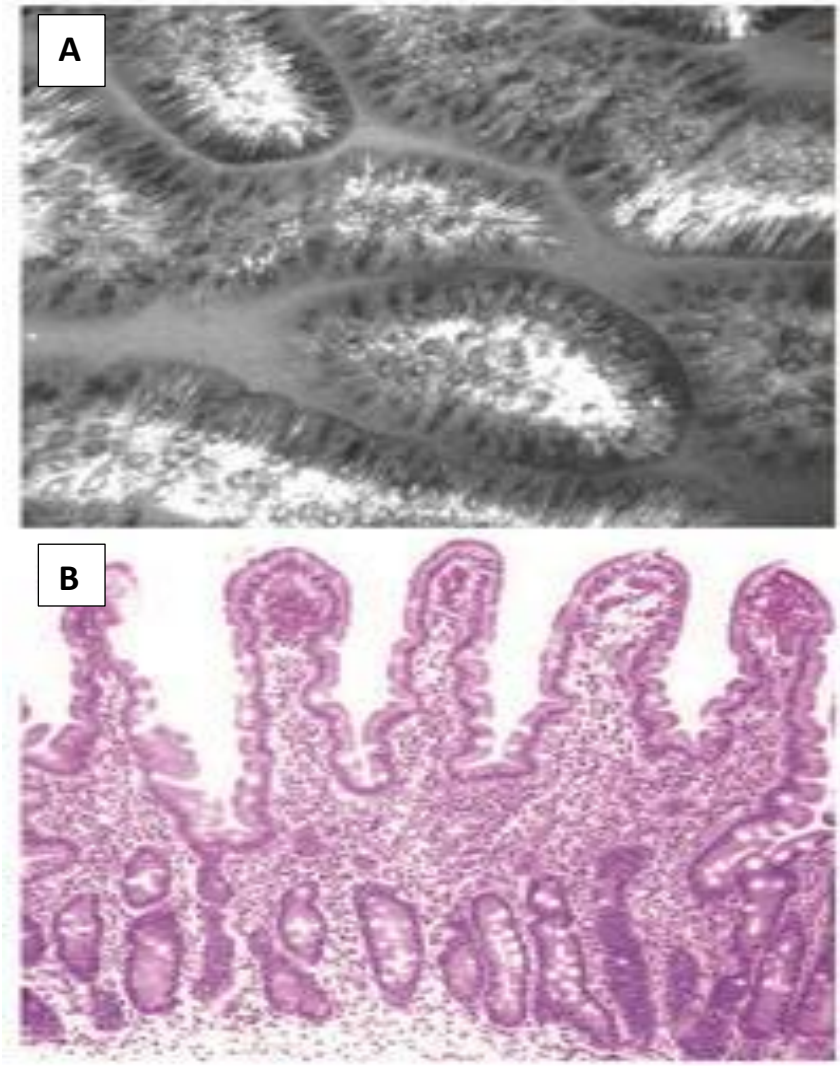


Figure 10. Comparison of confocal images with conventional histological images of duodenal villi. A: Confocal image delineating the fine slender finger-like projections of the duodenal villi; B: Histological image of duodenum. [3]

A lower FSI gradient indicated increased fluorescein leakage into the extra vascular space and reflected increased vascular permeability. [281] In a second study, again looking at patients with ulcerative colitis, segmental optical (by CLE) and tissue biopsies were taken. In order to avoid motion artefacts and acquire better orientation of the imaged area, an image reconstruction algorithm using a video mosaicing technique was employed. This group measured fluorescence intensity, related to the

fluorescein leakage in a region of interest, by using special dedicated software. [282] In a further study of inflammatory bowel disease, images stacks (Z-stacks) at a given area were collected from the surface of the epithelium down to the lamina propria (optical biopsy). The endomicroscopic images of the terminal ileum were analysed for the presence of gaps, micro-erosions and fluorescein leakage. The overall perimeter of the villi in each image was measured using Image J and the proportion of affected cells per perimeter was calculated (in percentage per optical analysis, which represented a z-stack of images in a given mucosal area). This detailed analysis was entitled the Watson grading system. Grading was based on the amount of cell shedding and the intensity of the luminal fluorescein signal on single good quality images from four separate fields of view within the terminal ileum. [1] (Figure 11) A similar concept was employed by another group in patients who had irritable bowel syndrome. The primary outcome was the density of epithelial gaps (gaps/cells counted) in adequately imaged villi using CLE. Images were reviewed by two independent blinded reviewers. [277]

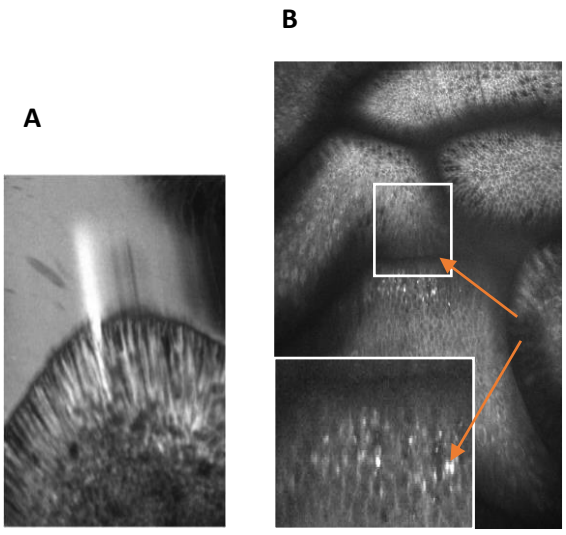


Figure 11. Confocal imaging of small intestine with intravenous fluorescein:

(A) 'Plume' of fluorescein entering intestinal lumen through epithelial gap. **(B)** Demonstration of bacteria within a villus using fluorescein stain. Arrows represent intensely fluorescent shedding cells seen en face. Images courtesy of Watson *et al*. [1]

Confocal Laser Endomicroscopy is therefore proving to be a rapidly advancing field of research. In the study described in this thesis, CLE was used to assess epithelial barrier integrity and intestinal permeability in patients with chronic liver disease, chronic pancreatitis and acute pancreatitis. It is the first such study to examine such parameters using this modality. Detailed methods and results will follow in the forthcoming chapters.

1.11 Summary

Alterations in intestinal permeability, bacterial translocation and endotoxaemia have been reported in acute pancreatitis and chronic liver disease, and to a limited degree in chronic pancreatitis. In acute pancreatitis, changes have been shown to correlate with disease severity, multi-organ failure, mortality and outcome.

Several tests can be used to examine intestinal barrier function and most commonly the ratio of two substances is used. D-amine oxidase is another test that can be used and there are multiple reports of its use in experimental models of acute pancreatitis and a few in human subjects.

Novel methods to investigate intestinal permeability have been more recently reported. CLE is one such diagnostic tool. It enables *in vivo* mucosal histology and the assessment of intestinal barrier function, during endoscopy. We have therefore investigated whether CLE can be used to assess intestinal permeability in patients with acute and chronic pancreatitis and have compared the results with those obtained from non-invasive measurements of intestinal permeability in the same subjects.

CHAPTER 2

HYPOTHESES, AIMS AND OBJECTIVES

2.1 Hypotheses

At the start of this thesis, I set out to conduct an investigation into intestinal permeability, bacterial translocation and gut barrier dysfunction in patients with acute pancreatitis. To facilitate this, a decision was made to investigate patients with stable chronic disease in the first instance. The following hypotheses were proposed to be tested:

1. Intestinal permeability alterations can be seen in patients with chronic pancreatitis, chronic liver disease and acute pancreatitis.
2. Confocal laser endomicroscopy can be used as a tool to assess alterations in intestinal permeability in pancreatic disease and in chronic liver disease.
3. Pro-inflammatory cytokines are involved in the loss of barrier function in acute and chronic disease, and correlate with severity of disease in acute pancreatitis.
4. Alterations in gastrointestinal permeability are associated with gut barrier dysfunction and alterations in the gut microbiome.

2.2 Aims

To determine whether CLE can be used to assess intestinal permeability, bacterial translocation and endotoxaemia in patients with chronic pancreatitis, chronic liver disease and acute pancreatitis.

To assess the responses of immunology and infection in patients with acute pancreatitis whilst also assessing changes related to increasing severity of disease in acute pancreatitis.

2.3 Objectives

In order to test the identified hypotheses and aims, we designed a study to be able to examine each of the required parameters. We set out to test the hypothesis that intestinal permeability alters in patients with chronic pancreatitis, chronic liver disease and acute pancreatitis and investigated whether these changes are associated with systemic endotoxin concentrations and bacterial translocation.

A measure of intestinal permeability was investigated with the novel tool, CLE, alongside the gold standard lactulose:mannitol test, whilst endotoxaemia was also measured. Further analyses to enable the differentiation of severity of acute pancreatitis in relation to systemic cytokine responses whilst also examining duodenal microbial growth in an attempt to assess bacterial translocation and the gut microbiome were carried out.

In summary, we aimed to determine whether *in vivo* CLE has a clinical role for assessing the integrity of the intestinal permeability barrier in inflammatory pancreatic and liver disorders, whilst assessing immunological and infection responses also.

CHAPTER 3

METHODS

3.1 Study population

We conducted a two tier study, initially investigating patients with CP and CLD then patients with AP.

CP patients were recruited from outpatient clinics and had a diagnosis based on clinical and imaging diagnostic criteria. They were subcategorised into surgically managed CP and conservatively managed CP. Aetiology was variable. CLD patients, classified with cirrhosis according to the Child's Pugh Score, were approached following referral to the endoscopy service for variceal screening or from the wards. [283] Aetiology was again variable. Control subjects were referred for endoscopy to investigate dyspepsia, but did not have alarm features or pre-existing comorbidities. Subsequent selection was based on these subjects having a normal gastroscopy and negative *Helicobacter pylori (H. pylori)* tests, rendering a likely diagnosis of non-ulcer (functional) dyspepsia.

Patients with a diagnosis of AP with serum amylase activity >450 U/l and consistent symptoms, fulfilling Revised Atlanta Classification diagnostic criteria, were recruited 24-48 hours after admission at the Royal Liverpool University Hospital. [66] Subsequent classification was as mild, moderate, severe or critical AP using the determinant based classification (DBC) system. [65]

3.2 Study Design

A proof of principle study was initially undertaken in which we investigated stable patients who had chronic disease: CP and CLD. Once experimental protocols had been established, a study of patients with acute illness (AP), was subsequently carried out. A further study of patients with severe acute pancreatitis admitted to ITU was proposed, but an application / amendment for ethical approval to acquire assent from next of kin was unsuccessful.

3.3 Ethics

This study was granted approval by the North West Research Ethics Committee under two study codes: 11/H1005/6 and 12/NW/0420. An amendment to the study 12/NW/0420 was proposed to enable recruitment of ITU patients unable to consent through the process of assent by next of kin, but this was declined by the regional ethics committee due to the invasive nature of the primary procedure. Identified patients were invited to participate in the study and informed consent was obtained.

3.4 Documentation

Patient information leaflets, consent forms and case record forms (CRFs) were designed and prepared for recruitment and for recording all data collected during the study (see Appendices). All documents were scrutinised and approved by the local ethics committee, prior to ethical approval being granted. CRFs were completed in a clear, legible manner by investigating team members during the course of the study. Records identifying the subject were kept confidential. All participants were allocated a study ID number at the time of recruitment with a prefix – CEM.

3.5 Safety Considerations

The study was conducted with close attention to patient safety. Consenting patients who were recruited underwent upper gastrointestinal tract endoscopy at the Endoscopy Unit in the Department of Gastroenterology at the Royal Liverpool and Broadgreen University Hospitals NHS Trust. British Society of Gastroenterology guidelines for safety and sedation in endoscopy were adhered to as is routine practice in this unit. All procedures were carried out by trained, competent endoscopists.

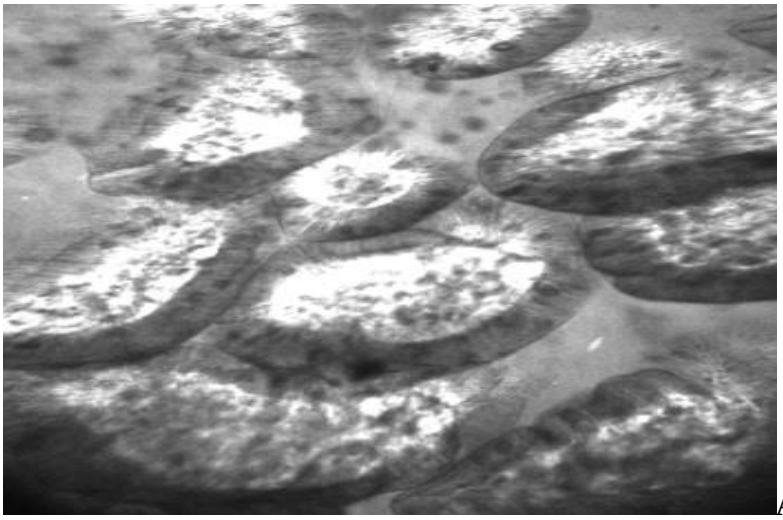
3.6 Assessment of Intestinal Permeability

3.6.1 Confocal laser endomicroscopy

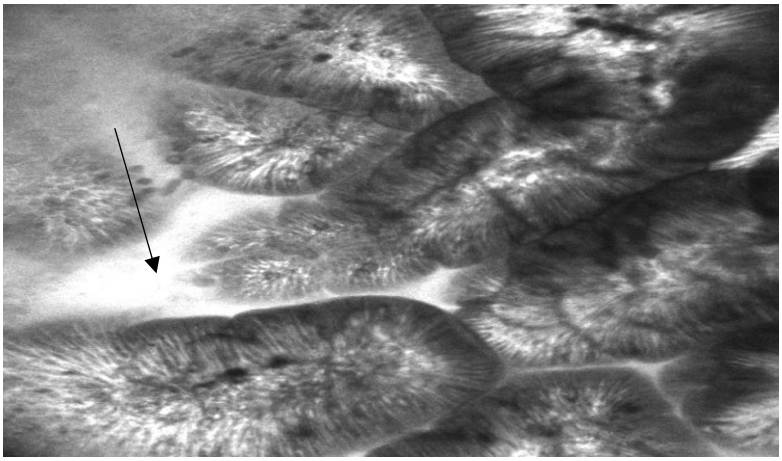
CLE was performed under conscious sedation with midazolam and pethidine by one of two fully trained endoscopists (SB and JG) using endoscope EC-3870CILK (Pentax, Tokyo, Japan.) After completion of the macroscopic examination, 5ml 10% fluorescein was administered intravenously and serial confocal images were acquired from the second part of the duodenum. The duodenal wall was then flushed with 10mls sterile water and duodenal fluid was aspirated for microbiology (see below). Two gastric antral mucosal biopsies were taken and a rapid urease test conducted to assess for the presence of *H. pylori*. All recorded images were re-evaluated by two co-authors (SB and JA) and selected for analysis based on defined inclusion and exclusion criteria. (Table 4) Selected images were analysed and scored by two independent, blinded collaborators/authors (CD and DL) for the presence or absence of fluorescein, the white element of each image, outside of the villous capillary network. Images were scored as 1 - 'positive for leakage' or 0 - 'negative for leakage'. (Figure 12) An average score based on the total score over the total number of images was calculated and the information populated onto a scoring sheet as seen in figure 13.

Table 4. Image Selection Criteria

Inclusion Criteria	Exclusion Criteria
• **Still image / absence of movement artefact** • **Image in focus** • **Uniform / near uniform perfusion** • **Full contact across whole or majority of image** • **Balanced exposure (brightness and contrast)**	• Movement artefact within image • Out of focus image • Areas of image under or over-perfused • Image too bright • Image too dark despite manipulation • Repeated image



A



B

Figure 12. Fluorescein leakage or ‘score 1’ positive image demonstrated in B and a negative or ‘score 0’ image as per A.

CEM NO: _____ TOTAL NO OF IMAGES =

	LOCAL BARRIER DYSFUNCTION	IMAGES
1 NO LEAKAGE	None	(0 point per image)
2 LEAKAGE	Fluorescein visible signal visible in the intestinal lumen	(1 point per image)
TOTAL		Total Points
ANY EVIDENCE OF LEAKAGE/DEFECT WITHIN SERIES Y=YES : N=NO		

AVERAGE SCORE = TOTAL SCORE / TOTAL NO OF IMAGES

Figure 13. Scoring Sheet

3.6.2 Sugar absorption tests

A dual sugar absorption test was used to assess IP. A solution of 5g lactulose and 2g mannitol, dissolved in 100ml of water, was prepared by the clinical trials pharmacy department, and this was further diluted to a 300ml solution at the time of administration, following a 6 hour fast. The solution was administered orally or via a nasogastric tube, immediately after collection of a 3.5ml blood sample for serum (Gold BD Vacutainer® SST™) to determine baseline levels. After a further 90-minute fasting period, a further 2ml serum sample was taken. Samples were centrifuged at 1500g/min for 10 minutes (Heraeus Megafuge 16R) and serum was aspirated, aliquoted and stored at -80°C. Batches were later analysed using HPLC (Dionex DX-500 BiolC HPLC system, California) as described by Fleming et al. [19] The ratio of the serum concentrations of lactulose and mannitol was recorded as the index marker for IP.

3.7 Venous Blood Sampling

A total of 20ml of blood was taken from each patient under aseptic conditions: 2ml whole blood in a lavender BD Vacutainer® K_2 EDTA Tube; 4.5 ml blood in a blue BD Vacutainer® K_2 Na Citrate Tube; 3.5 ml serum in a gold BD Vacutainer® SST™ Tube and 5ml blood in a blue culture bottle and 5ml in a purple blood culture bottle. Each tube was inverted ten times and transported to the PBRU laboratory as soon as possible. Sample processing and storage is detailed below.

3.7.1 Blood Cultures

Blood cultures were loaded onto a BacT/ALERT® 3D automated microbial detection system (bioMerieux, Marcy l'Etoile, France) and incubated for an extended period of ten days. Positive blood cultures were to be cultured onto Columbia blood agar, MacConkey agar, fastidious anaerobic agar and fastidious anaerobic agar with neomycin (Oxoid, Basingstoke, UK) and incubated for 48 hours. Blood cultures remaining negative for the ten-day incubation period were subcultured onto the above media and plates were incubated for five further days.

3.7.2 Measurement of diamine oxidase

A 3.5ml blood sample was taken from each subject (Gold BD Vacutainer® SST™), centrifuged at 1500g/min for 10 minutes at 20°C (Heraeus Megafuge 16R) and serum was carefully aspirated, aliquoted and stored at -80°C. Diamine oxidase (DAO) levels were measured in the serum of all patients with AP and controls who underwent CLE and whose images were subsequently included in the study for analysis and scoring. Identified serum samples were assayed using an ELISA kit with an antibody specific to DAO (USCN Life Science Inc.) according to the manufacturer's instructions. The concentration of DAO was determined by comparison to a standard curve.

3.7.3 Measurement of Endotoxin

3.7.3.1 Measurement of anti-endotoxin antibodies

A 4.5 ml peripheral venous blood sample was taken from each subject for plasma sampling (Blue BD Vacutainer® K_2 Na Citrate Tube) and this was centrifuged at 1600rcf for 15 minutes at 20°C. The plasma supernatant was carefully extracted, aliquoted and stored at -80°C. Plasma samples were later used for the measurement of antibody to endotoxin core (EndoCAb; Hycult Biotechnology, Netherlands) by measurement of levels of endogenous IgG and IgM EndoCAb by a direct ELISA. The test was conducted as per the manufacturer's instructions, and a drop in antibody levels was reported directly proportional to the severity of endotoxaemia.

3.7.3.2 Measurement of direct endotoxin

Limulus amoebocyte lysate assay was measured in the serum of selected patients, by Charles Rivers Laboratories, according to the manufacturer's instructions.

3.7.4 Measurement of cytokines

The collection and preparation of serum has been described above. Bio Rad Bio-Plex Human Cytokine, Chemokine and Growth Factor Multiplex Assay 27 Plex Panel Kits (Bio-Rad Laboratories Inc.) were used to measure the concentrations of multiple cytokines. Data were read on the Bio Rad Bio-Plex 200 instrument and associated Bio-Plex Manager Software version 5.0. Concentrations of cytokines were calculated using standard curves.

3.7.5 Helicobacter pylori testing by serology

In the pilot study, *H. pylori* testing by rapid urease testing was not routinely performed unless clinically indicated, and so all patients recruited had retrospective testing of serum IgG using stored serum samples. Frozen serum samples were removed from the freezer and thawed for analysis. Samples were processed by the microbiology laboratory team and involved ELISA testing for *H. pylori* IgG antibodies.

3.8 Duodenal Fluid Collection

Whilst the second part of the duodenum was intubated, after images had been obtained, and prior to biopsies being taken, duodenal fluid was aspirated from the lumen using a suction trap. Approximately 1ml of fluid was transferred using a sterile syringe and needle into a non-pyrogenic cryovial for microbiology and labelled accordingly.

3.8.1 Duodenal fluid culture

1ml of duodenal fluid was transferred using a sterile syringe and needle into a non-pyrogenic cryovial for microbiological analysis. Samples were centrifuged at 2500 rpm for 10 minutes and the supernatant was removed, ensuring 250 µl of the sample remained in the tube. The pellet was re-suspended and 25 µl was inoculated onto the following media: Columbia blood agar, MacConkey agar, fastidious anaerobic agar and fastidious anaerobic agar with neomycin and incubated for 10 days (plates were examined at 5 and 10 days). A cooked meat-BHI overlay broth (EandO laboratories, Bonnybridge, UK) was also inoculated and incubated for

ten days prior to subculture onto Columbia blood agar, fastidious anaerobic agar and fastidious anaerobic agar with neomycin. These plates were incubated for five days. Significant positive isolates were identified by Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (Bruker Corporation, Bremen, Germany) and stored on PROTECT beads (Technical Services Consultants Ltd, Heywood, UK) at -70°C.

3.9 Tissue Acquisition

Once duodenal intubation had been achieved, and after image acquisition and fluid aspiration, 6 mucosal biopsies were taken from the second part of the duodenum. 2 samples were transferred to formalin and taken to the pathology lab for processing of slides and cassettes of duodenal villi. This was in preparation for immune-histochemical analysis, however due to the limitations of time, we were unable to perform immunohistochemistry. A further two tissue samples were immersed into PBS for a minute then transferred and into 4% PFA as per the electron microscopy protocol detailed below. Two tissue samples from the gastric antrum were also taken. These were transferred to a slide for *H. pylori* analysis, as detailed below. The final two were immersed into blue cryovials containing isopentane, and were transferred to the laboratory in a carrier of liquid nitrogen for the possibility of tissue microbiological analyses. Samples were transferred into the GCLP -150^{0}C Freezer. Due to the limitations of time, such analyses were not performed. At a late stage in the study, 2 tissue samples were sent direct to the laboratory in formalin, for routine histological analysis instead of electron microscopy.

3.9.1 Helicobacter pylori testing by rapid urease test

In the cohort of patients recruited in the second or main part of the study, all patients underwent rapid urease testing for *Helicobacter pylori*. Two mucosal biopsies from the antrum were used to detect the presence of *H. pylori* by means of a rapid urease test (Pronto Dry, MIC, Brignais, France). The rapid urease test slides were read one hour after sample collection and the results were recorded.

3.10 Statistical analysis

A database was designed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA) and data were entered and stored in this program. Statistical analysis was performed using the statistical software package GraphPad PRISM version 6 (GraphPad Software, Inc., San Diego, CA, USA). A kappa statistic was calculated to determine agreement on image analysis between scorers. Normality was assessed using the Shapiro Wilk test. Data were compared using the Mann-Whitney U test in the case of quantitative and continuous data, with two tailed p values and $p<0.05$ indicating statistical significance. In the case of categorical or binary data, differences were analysed using the χ^2 test applying Yates' correction when required. Spearman's R Correlation Coefficient was used to assess for an association between DAO concentration and fluorescein leakage. In all analyses, $p<0.05$ was considered indicative of statistical significance.

CHAPTER 4

RESULTS I

METHOD DEVELOPMENT TO ESTABLISH USE OF A NOVEL TECHNIQUE TO ASSESS INTESTINAL PERMEABILITY THROUGH A PILOT STUDY

4.1 Introduction

Recent work conducted in our institution using the new technique of CLE on mouse and human small and large intestine has identified epithelial gaps in the intestinal mucosa that appear to increase in number dramatically during inflammatory disease states. [21] Identification of localised barrier loss in the intestinal epithelium is both novel and highly relevant to this project. Intravenous fluorescein was seen using confocal endomicroscopy to leak through the epithelium into the intestinal lumen at sites of barrier loss. [279] To our knowledge there is no other method of identifying such localised areas of increased IP *in vivo*. The identification of areas of barrier loss at a cellular level accurately complements sugar absorption studies that give an integrated measure of IP over a long segment of gut.

The Royal Liverpool University Hospital has an excellent, fully resourced endoscopy unit and is one of three National Endoscopy Training Centres. In 2010, 5,907 gastroscopies were carried out; of these, 245 patients had chronic liver disease and at least 47 had CP. There are also 150 new referrals with CP to the Liverpool Pancreas Clinics per year. A significant proportion of both of these cohorts are stable outpatient referrals who undergo their endoscopic procedures as elective out-patients. With this in mind, and considering that there are documented intestinal permeability disturbances in CP and CLD, it was thought to be a reasonable proposition to study these two disease cohorts, for their own merit, but also to enable the setup of a robust protocol in stable, clinically well, patients prior to studying the more unwell group of AP subjects.

Formal power calculations could not be performed since samples from pancreatitis or liver disease patients undergoing CLE have not previously been assessed and therefore not all the required variables for an accurate sample size calculation were known. However, based on previous endoscopic investigations involving analysis of tissue biopsies, group sizes of at least 20 patients were deemed reasonable to allow detection of significant differences in outcomes. The study was deemed entirely feasible as the patient population was available, the necessary endoscopic skills were acquired through training by two endoscopists, and all the necessary equipment, reagents, image analysis tools, expertise and infrastructure were already available

and validated. Confocal microscopy (*in vivo* and *in vitro*) is routinely performed in our laboratories as is the analysis of cell shedding and assessment of epithelial gaps and so a support network was available when interpreting the results of this study. With respect to CLE in human subjects, Professor Watson and Professor Kiesslich were important advisors, having utilised the modality in subjects with inflammatory bowel disease and reported on intestinal permeability assessments in such individuals. [1, 4, 279] Professor Kiesslich provided training in the CLE technique at his centre at Mainz University Hospital. Professor Watson explained the development and evolution of the Watson Score used to score fluorescein leakage by CLE, during meetings in Liverpool and Norwich and by further correspondence thereafter.

4.2 Study Design

A 2-tier study was conducted due to the nature of the main cohort under consideration. An initial pilot study was designed involving two separate groups of patients with chronic illness:

1. Patients with chronic liver disease – due to the extensive literature reporting alterations in intestinal permeability in this condition.
2. Patients with chronic pancreatitis – as this was also an inflammatory process of the pancreas, but in which patients were more stable and the disease was more chronic, allowing greater flexibility in scheduling investigations.

Ethical approval was also sought in a 2-tier process and hence, the initial application was to study intestinal permeability using confocal laser endomicroscopy in patients who had chronic liver disease and chronic pancreatitis.

4.3 Patient Recruitment

Patients with chronic liver disease (CLD) or cirrhosis were recruited via the endoscopy unit. Patients with chronic liver disease who were referred for screening for oesophageal and/or gastric varices or those with known varices who were on a surveillance programme were contacted by telephone and invited to participate in the study. A small number of inpatients with decompensated liver disease were also recruited following direct approach on the wards, once the protocol had been established. Due to the nature of this disease and the need for endoscopic evaluation and assessment, recruitment was relatively straightforward as the invasive element of the study was already being undertaken by patient. This was also the case for the potential healthy control subjects. We approached patients who had dyspeptic symptoms without alarm symptoms as the reason for endoscopy referral and selected those in whom the endoscopy did not reveal an organic cause such as peptic ulcer, thereby suggesting an underlying diagnosis of non-ulcer dyspepsia. Patients were contacted by telephone and invited to participate in the study. The confocal laser endomicroscopy was a small extension to a procedure they were already anticipating, and the other aspects were less invasive than that which was already being undertaken.

Patients with chronic pancreatitis were approached by two methods. Initially, it was felt that patients who required an endoscopic procedure for a clinical indication (e.g. assessment of abdominal pain or vomiting) should be recruited, as described above for CLD and healthy control subjects. However, due to the nature of the disease, diagnostic gastroscopy is not a common requirement for most patients with chronic pancreatitis. Such patients do however sometimes require endoscopic ultrasound examinations, so the initial few patients recruited were offered back to back procedures. It however became rapidly apparent that this was too much for subjects to tolerate and so we re-visited the recruitment strategy for this cohort. Subsequently therefore, patients with stable CP were approached in the out-patient setting following their routine clinic review. They were invited to participate in the study solely for research purposes.

All patients were initially informed about the study and were provided with the relevant information leaflet explaining the study. They were then asked if they agreed to take part and written consent was obtained. At this time, initial blood sampling was also undertaken. They were then given two appointments – one for the endoscopic procedure and another for the sugar absorption test.

4.4 Study Population

A total of 128 patients were recruited in this initial pilot study. Baseline demographics of these patients are detailed in tables 7-8. Of these, not all participants underwent all aspects of the study due to a range of reasons including withdrawal of consent, missed appointment, technical difficulties, delay in acquisition of test solution. All 128 recruits did however undergo blood sampling and subsequent analyses of plasma and serum. This included cytokine analysis, endotoxin, and blood cultures.

For healthy controls, 48 patients who had been referred for endoscopy to investigate symptoms of dyspepsia, but who did not have alarm symptoms, were recruited. Medical history was taken at the time of initial consultation. 13 *H. pylori* negative patients who had no major co-morbidities and who also had a macroscopically normal endoscopy were subsequently selected as 'healthy controls'. A co-morbidity was defined as any long-standing health problem requiring medication such as hypertension or diabetes mellitus, or any history of previous surgery except ENT or ophthalmic surgery. Of the 48 potential controls referred for gastroscopy, 39 underwent confocal laser endomicroscopy and 24 of these had normal endoscopic findings. Eleven of these were however subsequently excluded from the healthy control group based on positive *H. pylori* status or pre-existing co-morbidities. Thus leaving a total of 13 healthy controls.

Patients with CLD and CP were recruited as described above. Not all subjects however attended all their appointments; this explains the different numbers undergoing the various different elements of the study. The recruitment cohort for the pilot study is summarised in the Prisma diagram below. (Figure 14)

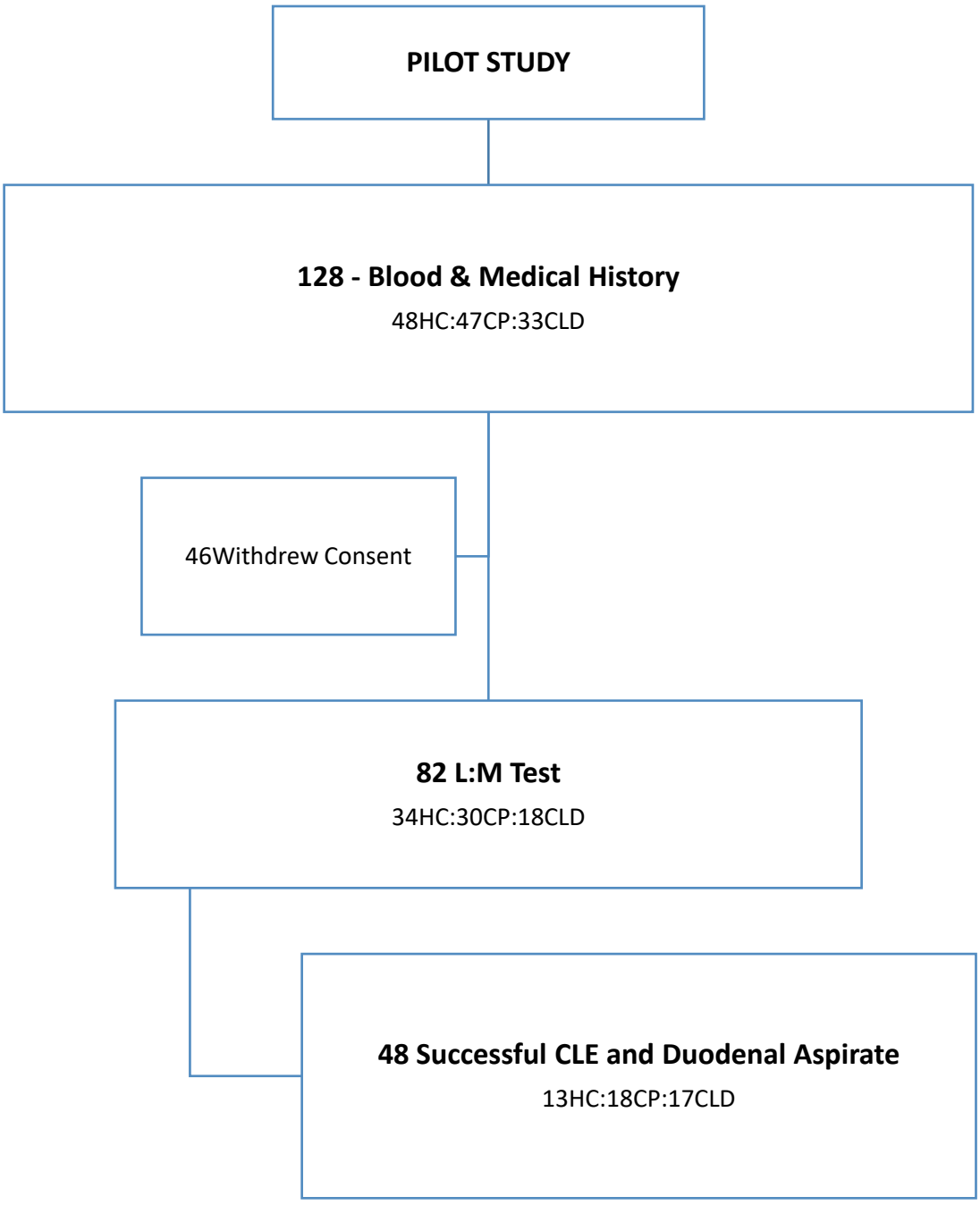


Figure 14. PRISMA diagram to summarise key components of recruitment and analysis during pilot study.

Key: n:m:p:q = Healthy Controls (HC) : Acute Pancreatitis (AP) : Chronic Pancreatitis (CP) : Chronic Liver Disease (CLD)

4.5 Study Cohorts

Details of the healthy control cohort have been described above. Patients recruited with chronic liver disease comprised a range of aetiologies including alcoholic liver disease (16); non-alcoholic steatohepatitis (3); hepatitis B or C virus (5); primary biliary cirrhosis (3); primary sclerosing cholangitis (1); hereditary haemochromatosis (2); unknown / idiopathic (3). Of these, 26 patients were classified as Child's Pugh A at the time of recruitment; 4 subjects as Child's Pugh B and 3 as Child's Pugh C. (Table 5)

Table 5. Characteristics of Patients with Chronic Liver Disease

Characteristic	**Chronic Liver Disease (n=33)**
Sex M:F	23:10
Median age (range), yrs	53 (22-53)
Aetiology, n (%)	
Alcohol	16 (48)
Idiopathic	3 (9)
Hepatitis B/C virus	5 (15)
Other	9 (27)
Child's Pugh Score, n (%)	
Child's Pugh A	26 (79)
Child's Pugh B	4 (12)
Child's Pugh C	3 (9)

The chronic pancreatitis cohort comprised 20 patients who had excess alcohol consumption as the primary cause; 20 patients had idiopathic chronic pancreatitis; 4 patients had a genetic aetiology; 2 had autoimmune pancreatitis and 1 had chronic pancreatitis secondary to trauma. Pancreatic exocrine insufficiency was common in this cohort, with endocrine insufficiency being less commonly reported. 57% of patients who had chronic pancreatitis in our study had undergone surgery involving the pancreas or biliary tract during their disease course or for prior acute pancreatitis: 9 Whipple's procedure; 3 Hepaticojejunostomy / cyst resection; 1 Berne's procedure; 1 Beger's procedure; 1 trauma surgery; and 12 necrosectomy for previous AP, or other. (table 6)

Table 6. Characteristics of Patients with Chronic Pancreatitis

Characteristic	**Chronic Pancreatitis (n=47)**
Sex M:F	29:18
Median age (range), yrs	47 (19-75)
Aetiology, n (%)	
Alcohol	20 (43)
Idiopathic	20 (43)
Genetic	4 (8)
Other	3 (6)
Manifestations of Disease Progression, n (%)	
Exocrine Insufficiency	43 (91)
No Exocrine Insufficiency	4 (9)
Endocrine Insufficiency	12 (26)
No Endocrine Insufficiency	35 (74)
Patient Management	
Surgically Managed, n (%)	27 (57)
Conservatively Managed, n (%)	20 (43)
Tertiary Referral Patients, n (%)	0 (0)
Time since diagnosis to recruitment, n (%)	
<12 months	3 (6)
1 – 5 years	10 (21)
5 -10 years	14 (30)
>10 years	20 (43)

4.6 Assessment of Intestinal Permeability by CLE

4.6.1 Method development for image interpretation

The process of image selection was consistent throughout the study. Images of poor quality were discarded according to the exclusion criteria detailed previously (section 3.6.1, table 4). Initial assessment of pilot study data was based on the Watson Grade (Table 7). [1] Image acquisition, selection and interpretation were initially all performed by the same investigator. Two further blinded investigators also scored the images. However, due to variable interpretation of specific elements of this scoring system, concordance rates between the scorers were poor and the process was also potentially biased by one investigator (the author) both selecting and scoring images.

	Cell shedding	Local barrier dysfunction
I. Normal	Cell shedding confined to single cells per shedding site	None
II. Functional defect	Cell shedding confined to single cells per shedding site	Fluorescein signal visible in the intestinal lumen with an intensity the same or brighter than the epithelium or fluorescein plumes out of the epithelium into the lumen
III. Structural defect	Microerosions in any field. Microerosion is defined when the lamina propria is exposed to the lumen with multiple cells being shed per site	Fluorescein signal visible in the intestinal lumen with an intensity the same or brighter than the epithelium or fluorescein plumes out of the epithelium into the lumen

Table 7. Watson Endomicroscopic Grade for *in vivo* identification of local barrier dysfunction. [1]

After much consideration and discussion, it was decided to simplify the scoring process to reduce bias and the possibility of variable image interpretation. A major factor leading to this process was the inability to confirm the presence of true z-stacks in the images acquired as even slight movement of the confocal probe during the endoscopic procedure led to images being acquired from different locations. It was therefore deemed to be more accurate to identify images from different stacks and not to analyse more than one image from the same 'z' stack. Interpretation of the level of fluorescence and signal intensity was also felt to be subjective, whereas detection of its presence or absence alone was more objective. Hence, a more simplified scoring system, as detailed in chapter 3, was introduced and separate blinded investigators were recruited to select and score the confocal images. Two objective scores were acquired – one rendering binary data and the other continuous. Each image was scored as either '1' or '0' using the method detailed previously. A numeric value was therefore acquired and this was divisible by the number of images examined for that patient leading to an 'average score' – a continuous variable. The presence of any positive score suggested the presence of fluorescein leakage into the intestinal lumen and hence generated the binary score. (Figure 15) Images of poor quality were discarded based on the exclusion criteria detailed in chapter 3. Examples of discarded images are shown below. (Figure 16)

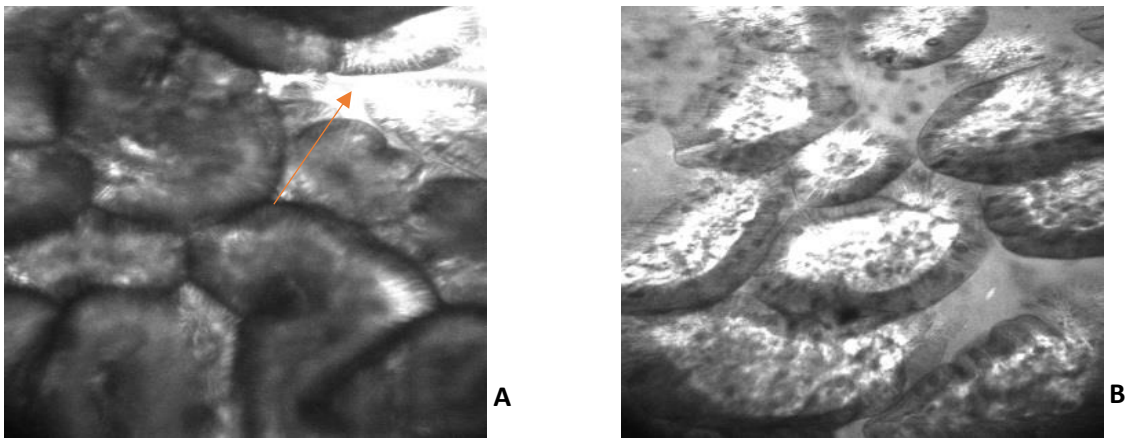


Figure 15. Confocal imaging of small intestine with intravenous fluorescein from pilot study: (A) 'Plume' of fluorescein entering intestinal lumen through epithelial gap in a patient with chronic liver disease. See arrow. **(B)** Normal villi with no evidence of fluorescein leakage in a control patient.

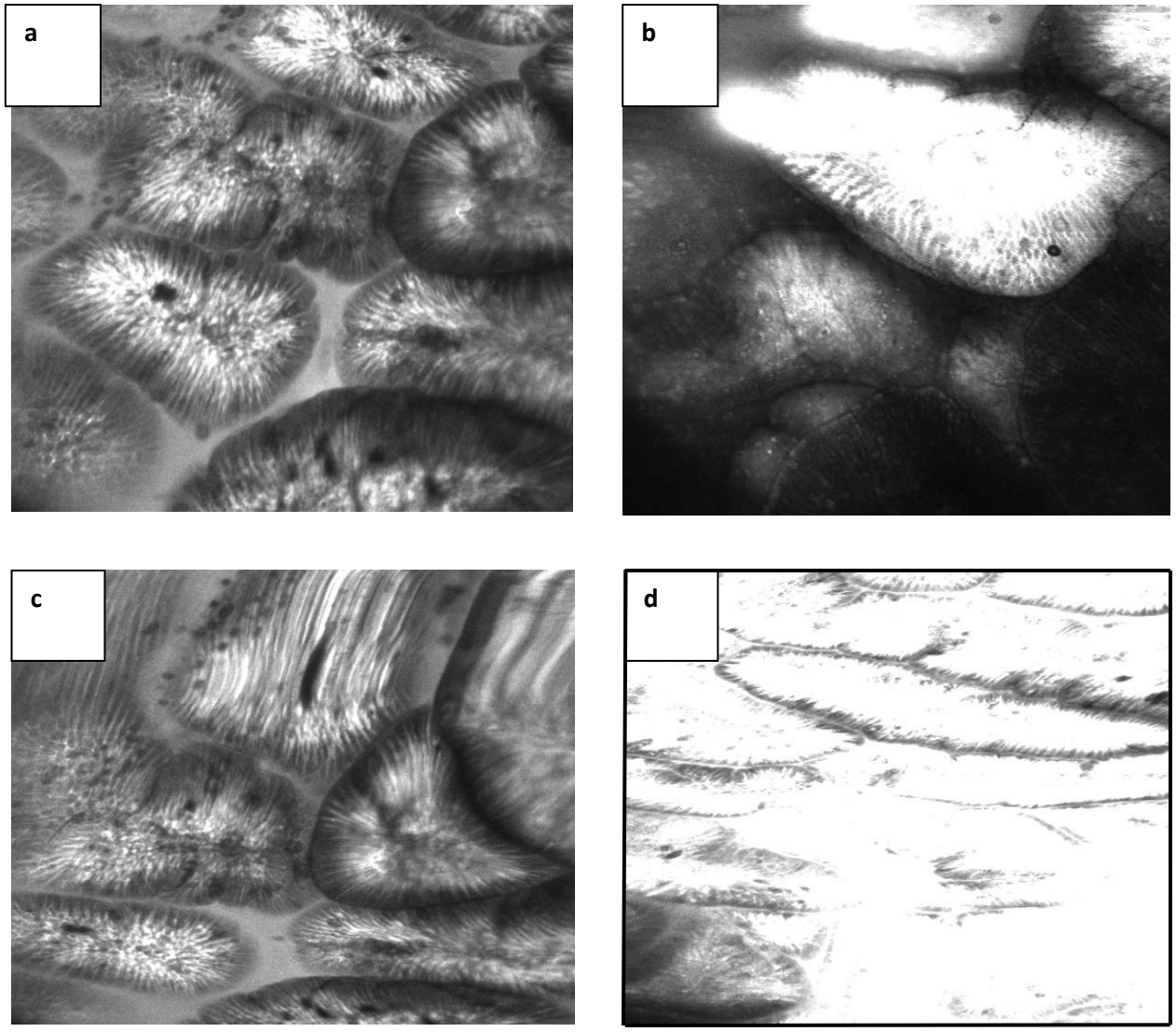


Figure 16. Examples of discarded confocal images.

a. Lack of full contact across image;

b. Too dark;

c. Movement artefact;

d. Too bright.

4.7 Discussion

The methods of confocal microscopy and *in vivo* (mice) confocal probe microscopy have previously been established in our institution. Fluorescein is a dye that has been deemed safe and efficacious in human experimentation and is utilised as a spray once the focal point is seen endoscopically. [267]

The Watson score described a method of analysing a z-stack of images 7 µm apart that had been taken from villi in the *en face* orientation up to a depth of 70 µm. [1] Whilst this formed the initial method used for image analysis, it was later amended and developed so that it was workable for the clinical and laboratory teams and so that the degree of inter-observer variability was minimised. In the early stages of the study, control subjects and volunteers were recruited to master the technique of confocal laser endomicroscopy and to consolidate training. Multiple images were taken from the second part of the duodenum (D2), prior to any other intervention, to prevent interference. To begin with, a stack of images from one focal point in D2 were acquired up to a depth of approximately 70 µm, or until the images became faint, by enabling contact between the tissue and the confocal window. Images were then acquired as the direction of image capture was changed into reverse to return to the top of the z-stack, maintaining the same stable position. Image numbers were noted and the method was repeated for up to 6 sites, the aim being to acquire a minimum of 3 stacks of good quality images. The images from each site were separated and then images were selected as per previously detailed criteria. 3 sets of images from 3 sites were selected for interpretation and scoring. A very large number of images were acquired in this way and it was found that due to invariable peristaltic movement, stacks became inconsistent and images thought to be from a single stack, were actually independent of each other.

In these initial stages of the study, the Watson score was performed and attempts were made to score images identifying the point and extent of leakage, and scoring where an actual defect or ‘gap’ could be seen. This method resulted in a continuous scale for analysis, but led to considerable variability between scorers and it was found to be a subjective method of assessment. A variable number of images were scored from each stack and an average

score was calculated. There was also considerable variation in the numbers of images analysed per subject using this method. Once the pilot study recruitment was completed, the method was fully evaluated and a decision was made that it was too cumbersome and not the best method for analysing the images acquired in this study.

A review of the method was therefore undertaken. Image numbers were consolidated so that only one image per duodenal site was taken and all good quality images were analysed as long as they had been definitively acquired from independent positions. The scoring was also changed from a subjective, continuous variable to a discrete value of positive or negative. This significantly improved inter-observer variability. This method did not however allow for the identification of gaps, but proved to be far more accurate for assessment of fluorescein leakage. Hence, this method was utilised for the remainder of the study and the details have been outlined in chapter 3.

Whilst the study protocols were being established, the numbers of subjects who successfully completed the various aspects varied due to a number of factors. This improved by the time the main study was established. Some of the problems faced and the reasons for them are briefly outlined here:

Electron microscopy was undertaken using duodenal tissue samples obtained from the initial recruits in the pilot study, but it was later abandoned due to difficulties in interpreting the images and the complexity of acquiring them.

Helicobacter pylori status was not initially tested by urease test on a gastric biopsy. Urease testing was done during the main study but not during the pilot study. Thus, the patients from the pilot study were tested for *H. pylori* by serology instead.

Blood samples were taken at the time of recruitment and hence, serum, plasma and blood culture samples were available from all subjects. The endoscopic procedure and sugar absorption tests were undertaken at separate appointments and so this was dependant on the subject's attendance. There was a significant withdrawal rate for one or both of these tests.

Duodenal biopsies were only sent for routine histology later in the study. This analysis was therefore only undertaken for a small number of patients who had acute pancreatitis and a few control subjects who were recruited at a late stage in the study.

Images were excluded based on exclusion criteria as detailed previously. In the first few subjects who underwent confocal laser endomicroscopy, the endoscopists were acquiring new skills in this procedure. In some of these subjects no interpretable images were obtained. In a small number of patients who underwent gastroscopy, the procedure had to be abandoned prematurely due to patient intolerance, a technical fault with CLE or anatomical variation causing a difficulty in duodenal intubation. This resulted in CLE not being performed or lack of duodenal tissue or fluid acquisition in these subjects.

The pilot study also served its second purpose, and generated invaluable results from two very important patient cohorts, namely patients with chronic pancreatitis and chronic liver disease. These findings were combined with those from the main study of AP patients and all analyses were undertaken once recruitment was completed.

CHAPTER 5

RESULTS II

METHOD ADAPTED TO INVESTIGATE PATIENTS WITH ACUTE PANCREATITIS

5.1 Introduction

This study was thus designed to examine the role of CLE in identifying alterations in the intestinal barrier in patients with AP, and to compare these findings with a well-recognised gold-standard test, the lactulose:mannitol test. Due to the aggressive clinical course and severity of AP, patients are often acutely unwell in-patients. It was therefore imperative to establish a robust experimental protocol before embarking on an AP study that employed this relatively invasive test protocol (CLE).

Over a hundred patients a year are admitted to the Royal Liverpool University Hospital, either as direct admissions or tertiary centre referrals. This rendered it an excellent centre to investigate this cohort of patients.

5.2 Study Design and Recruitment

Ethical approval was also sought in a 2-tier process and so once a protocol and standard operating procedure had been firmly established from the pilot study, we sought ethical approval to enrol patients who had acute pancreatitis and thereafter, patients who had severe acute pancreatitis and who were on the intensive care unit.

In the second phase of the study, patients with acute pancreatitis were approached at the time of hospital admission and were invited to participate. It was however soon evident that subjects with severe disease were not being recruited and numbers in this subgroup were small. We established that those patients who had severe acute pancreatitis tended to be admitted directly to the intensive care unit from other centres, as tertiary referrals. A large number of these patients were intubated and sedated and therefore we submitted a separate ethics application to request approval to recruit such subjects with assent from a relative or next of kin. This application was however declined as it was deemed by the local ethics committee that the study participant themselves must be able to give consent for such a study and that a relative's assent was inadequate. On this basis, potential subject numbers in the severe acute pancreatitis group were limited. All patients were then recruited by the same method as used during the pilot study.

5.3 Study Population

A total of 54 patients were recruited during the second part of the study. Baseline demographics of these patients are detailed in table 8. All patients who had been admitted directly to the Royal Liverpool University Hospital with AP were recruited within 4 days of symptom onset and 48 hours of admission. Two patients with moderate disease and 6 with severe were recruited at a later stage in their illness due to different referral pathways.

Table 8. Characteristics of Patients with Acute Pancreatitis

Characteristic	Mild Acute Pancreatitis	Moderate Acute Pancreatitis	Severe Acute Pancreatitis
Sex M:F	17:18	10:1	5:3
Median age (range), yrs	49.5 (17-82)	49 (22-76)	58.5 (37-80)
Aetiology, n (%)			
Gallstones	14 (40)	6 (55)	3 (38)
Alcohol	6 (17)	4 (36)	4 (50)
Idiopathic	2 (6)	0 (0)	0 (0)
Other (structural, post-ERCP, unknown)	13 (37)	1 (9)	1 (12)
Classification* (DBC), n (%)			
Sterile Local Complications	0 (0)	3 (27)	4 (50)
Sterile Peripancreatic Necrosis	0 (0)	5 (45)	2 (25)
Infected Peripancreatic Necrosis	0 (0)	0 (0)	2 (25)
Persistent Organ Failure	0 (0)	0 (0)	8 (100)
Transient Organ Failure	0 (0)	3 (27)	0 (0)
Patients requiring necrosectomy, n (%)	0 (0)	5 (45)	5 (63)
Tertiary Referral Patients, n (%)	0 (0)	3 (27)	6 (75)
Time since onset of symptoms to recruitment, n (%)			
12-24 hrs	21 (60)	5 (46)	1 (12.5)
>24 hrs - 4 days	14 (40)	4 (36)	1 (12.5)
>4 - 59 days	0 (0)	2 (18)	6 (75)

*** Nil with Critical Acute Pancreatitis recruited in patient cohort**

5.4 Study Cohorts

Of the patients who had acute pancreatitis, gallstones were the most common aetiological factor in those who had mild and moderate disease, whereas excess alcohol consumption was most prevalent in the severe group. Causes included gallstones (23 subjects), alcohol (14 subjects), unknown / idiopathic (13 subjects), structural (2 subjects), genetic (1 subjects), and post-ERCP (1 subject). Patients were diagnosed with clinical symptoms consistent with acute pancreatitis and had raised serum amylase activity. C-reactive protein levels were documented to range between 5 mg/l and 450 mg/l over the course of patients' illnesses. White cell counts ranged from 5 x 10^9/l to 32.8 x 10^9/l.

Of the patients with mild AP, none developed organ failure. 3 of 11 patients with moderate disease had transient organ failure and all 8 patients with severe AP had documented evidence of organ failure. Most of the 35 patients with mild AP were recruited within 48 hours of direct admission to the Royal Liverpool University Hospital with a slight delay up to 4 days in a few cases due to logistical reasons. Of the moderate and severe AP cohorts, 3 of 11 and 6 of 8 patients respectively were tertiary referrals from other centres. These patients were frequently enrolled later in their disease course and were recruited up to 59 days post presentation in the most extreme case. All patients underwent radiological or endoscopic evaluation at some stage during their admission and 41% (22 of 54) patients underwent a surgical procedure. These investigations and the surgical procedures undertaken are summarised in tables 9 and 10 below.

	Number of Patients					
	US	**CT**	**EUS**	**MRCP**	**ERCP**	**Total**
Mild Acute Pancreatitis	28	13	7	4	10	35
Moderate Acute Pancreatitis	4	11	2	1	2	11
Severe Acute Pancreatitis	3	8	0	0	2	8

Table 9. Representation of patients with acute pancreatitis in study cohort undergoing radiological or endoscopic intervention.

Key: US - ultrasound; CT – computed tomography; EUS – endoscopic ultrasound; MRCP – magnetic resonance cholangiopancreatography; ERCP – endoscopic retrograde cholangio-pancreatography

	Number of Patients	
	Laparoscopic Cholecystectomy	**Pancreatic Necrosectomy**
Mild Acute Pancreatitis	10	0
Moderate Acute Pancreatitis	2	5
Severe Acute Pancreatitis	0	5

Table 10. Representation of patients with acute pancreatitis in study cohort undergoing surgical intervention within the time-frame of this study.

5.5 Discussion

The pilot study enabled us to identify potential problems with recruitment and data interpretation so that the protocol was fine tuned for this second main stage of the study. Patients with acute pancreatitis were intuitively a more unwell group and thus the recruitment process more challenging.

The problems identified were addressed during the pilot study and prior to the ethics proposal submission for the recruitment of AP patients. It confirmed our suspicions that there would be a number of issues requiring resolution and confirmed the value of the pilot study in stable patients.

The findings from this part of the study were combined with those from the pilot study of CP and CLD patients and all analyses were undertaken once recruitment was completed.

CHAPTER 6

RESULTS III

STUDY COHORTS COMBINED FOR ANALYSIS

6.1 Introduction

Once recruitment was complete, data from both arms of the study were combined and analysed using established methods. The general cohorts and numbers evaluated are described in this chapter.

6.2 Study Population

A total of 182 patients were recruited in this prospective study between 2011 and 2014. All patients had negative blood cultures at the time of recruitment and *H. pylori* status was consistent across all cohorts with 25% positivity overall. 61 subjects withdrew consent after initial assessment and prior to undergoing a lactulose:mannitol test and CLE, and a further 20 did not attend for CLE following the lactulose:mannitol test. In these cases, only serum and plasma samples were available for analysis. Thus, a total of 121 subjects underwent the lactulose:mannitol test and 84 attempted CLE due to a further 17 withdrawing consent.

Of the 182 subjects, a total of 62 underwent successful CLE with images suitable for analysis, and aspiration of duodenal fluid following technical difficulties with 17 patients who underwent the procedure. Duodenal aspirate was retrieved in a total of 62 patients also. The study population and summary of recruitment and analysis is depicted below in figures 17 and 18.

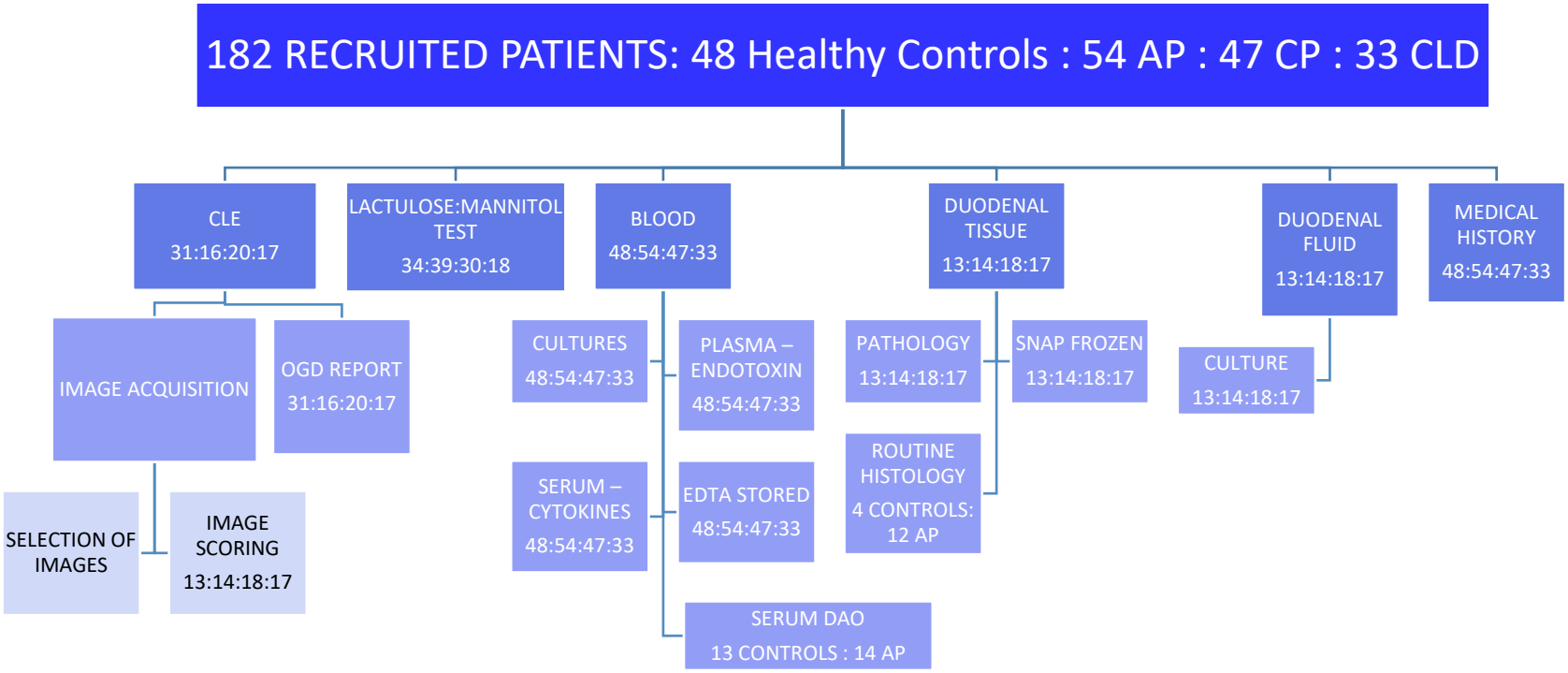


Figure 17. Summary flow diagram to demonstrate recruitment cohort and all variables tested.

Key: n:m:p:q = Healthy Controls : Acute Pancreatitis (AP) : Chronic Pancreatitis (CP) : Chronic Liver Disease (CLD)

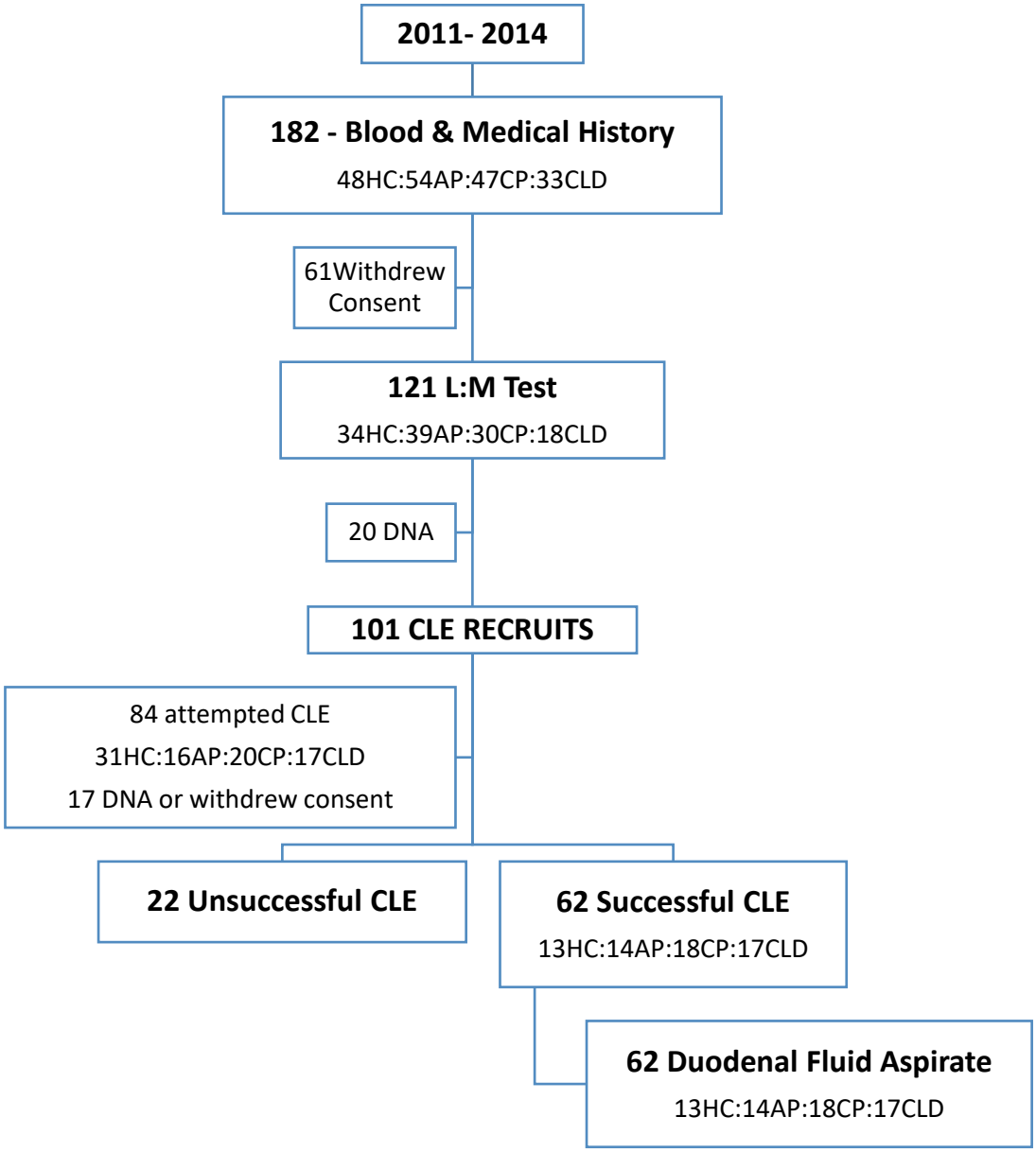


Figure 18. PRISMA diagram to summarise key components of recruitment and analysis.

Key: n:m:p:q = Healthy Controls (HC) : Acute Pancreatitis (AP) : Chronic Pancreatitis (CP) : Chronic Liver Disease (CLD)

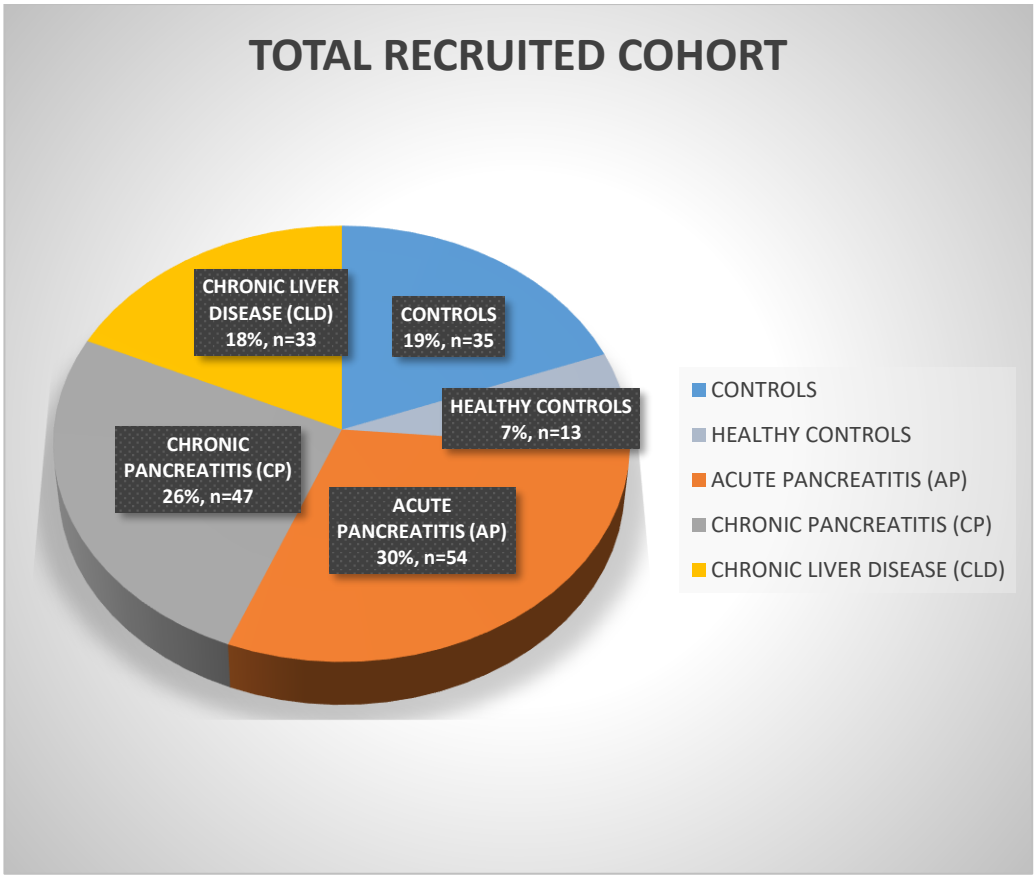


Figure 19. Representation of recruited cohort of patients.

6.3 Image analysis

A total of 498 confocal images were analysed; these were taken from macroscopically normal duodenal mucosa from 62 subjects (10 mild / moderate AP; 4 severe AP; 18 CP; 17 CLD and 13 controls). A mean of 8 ($4<n<17$) images per patient, from distinctly separate mucosal points, were analysed by 2 independent scorers. Scoring was undertaken as previously detailed. Figure 20 is an example of a set of images analysed from a subject with acute pancreatitis, whilst figure 21 is an example of a set of images from a healthy control subject with no evidence of fluorescein leakage. Following the exclusion of images of poor quality, images from 62 patients were analysed by the 2 independent scorers using consistent criteria. To assess concordance, Cohen's kappa coefficient was performed as a statistical measure of inter-rater agreement. Binary data of each scorer's total assessment of subjects with leakage and non-leakage were tabulated in a 2x2 table and the calculation was performed using a statistical calculator. Cohen's kappa coefficient was calculated at 0.603, consistent with substantial agreement and concordance as demonstrated in Table 11.

		Scorer A		
		Leakage	**No Leakage**	**Total**
Scorer B	**Leakage**	42	9	51
	No Leakage	6	23	29
	Total	48	32	80
Number of observed agreements: 65 (81.25% of the observations) Number of agreements expected by chance: 42.2 (52.75% of the observations) 95% confidence interval: From 0.424 to 0.783 The strength of agreement is considered to be 'good'.				

Table 11. Cohen's Kappa co-efficient to assess inter-rater concordance between the two image scorers.

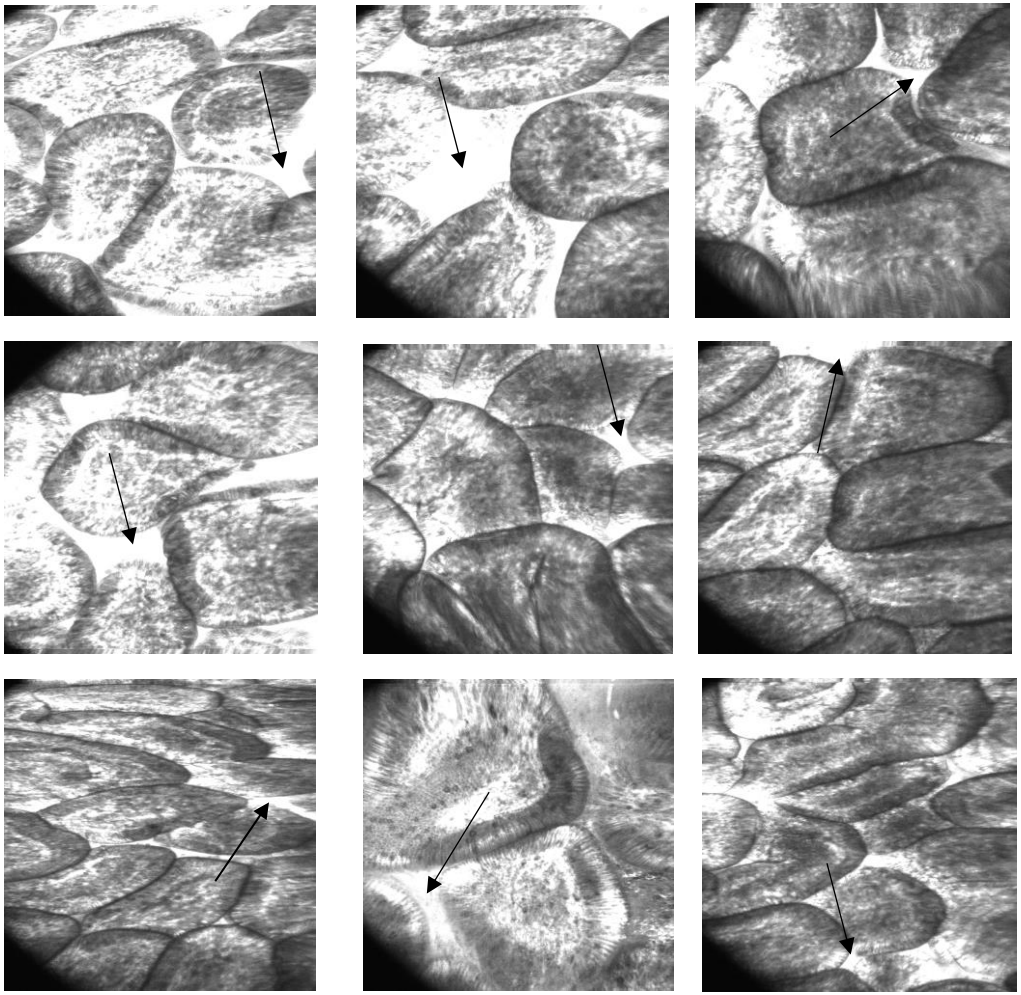


Figure 20. A set of images from a subject with acute pancreatitis, post-selection, ready for scoring by separate blinded investigators. Each image was selected based on being of good quality without movement artefact and with adequate fluorescence. Images were acquired from distinctly separate points on the duodenal mucosa. The arrows demonstrate sites of fluorescein leakage rendering a positive score for each image.

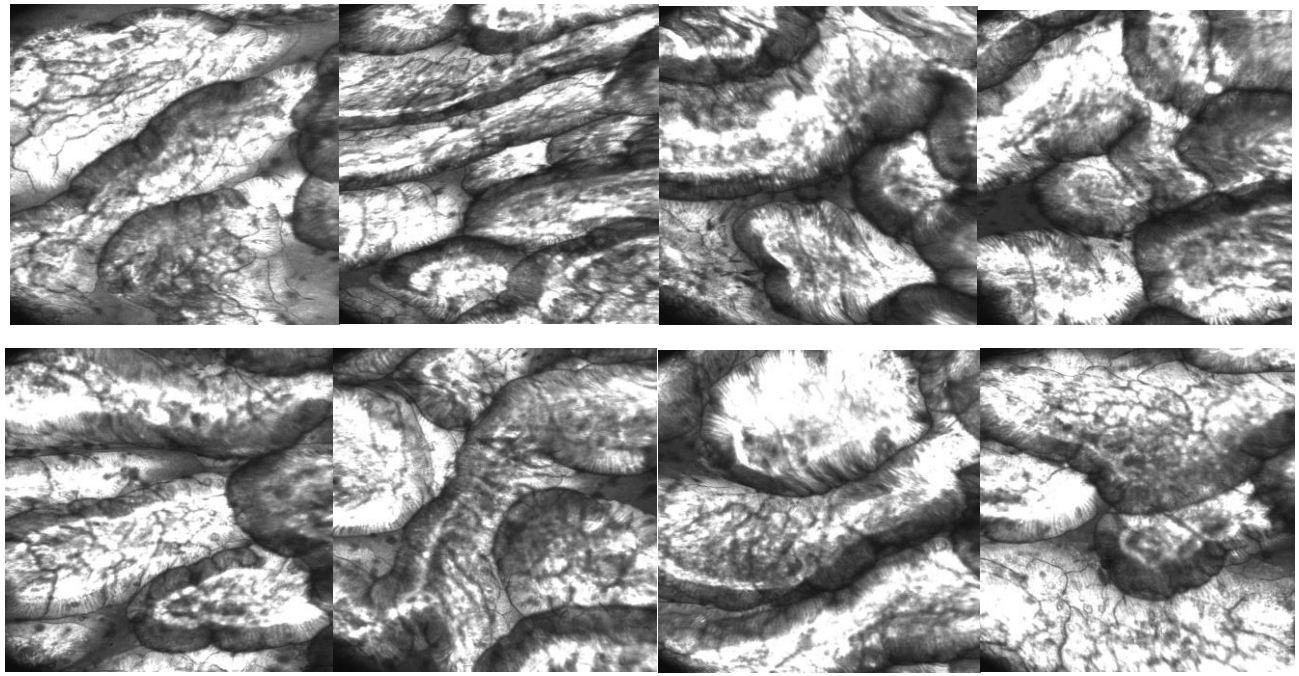
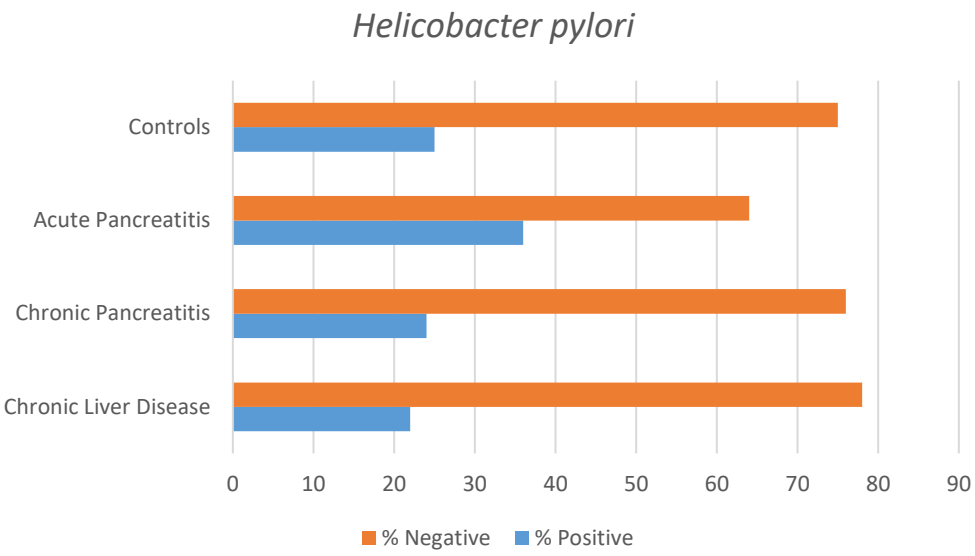


Figure 21. A set of images from a healthy control subject, post-selection, ready for scoring by separate blinded investigators. There was no evidence of fluorescein leakage in the images from this particular control subject.

6.4 *Helicobacter pylori* detection

All patients were tested for *Helicobacter pylori* by means of either rapid urease test on gastric antral biopsy or antibody testing of serum samples. All patients recruited during the pilot study had retrospective serum *H. pylori* IgG tested and those within the main study cohort had prospective urease testing of gastric tissue. 24% of the overall population in the study were positive for *Helicobacter pylori*, confirming current or past infection. 76% were negative. The urease test only detects active current infection. The serum antibody test is also positive in active infection, but can also be positive if there has been past infection, hence a positive results indicates either past or current infection. Individual cohorts were similar in their distribution (positive:negative): Controls: (23%:77%); AP (36%:64%); CP (24%:76%) and CLD (22%:78%). (Figure 22)

A



B

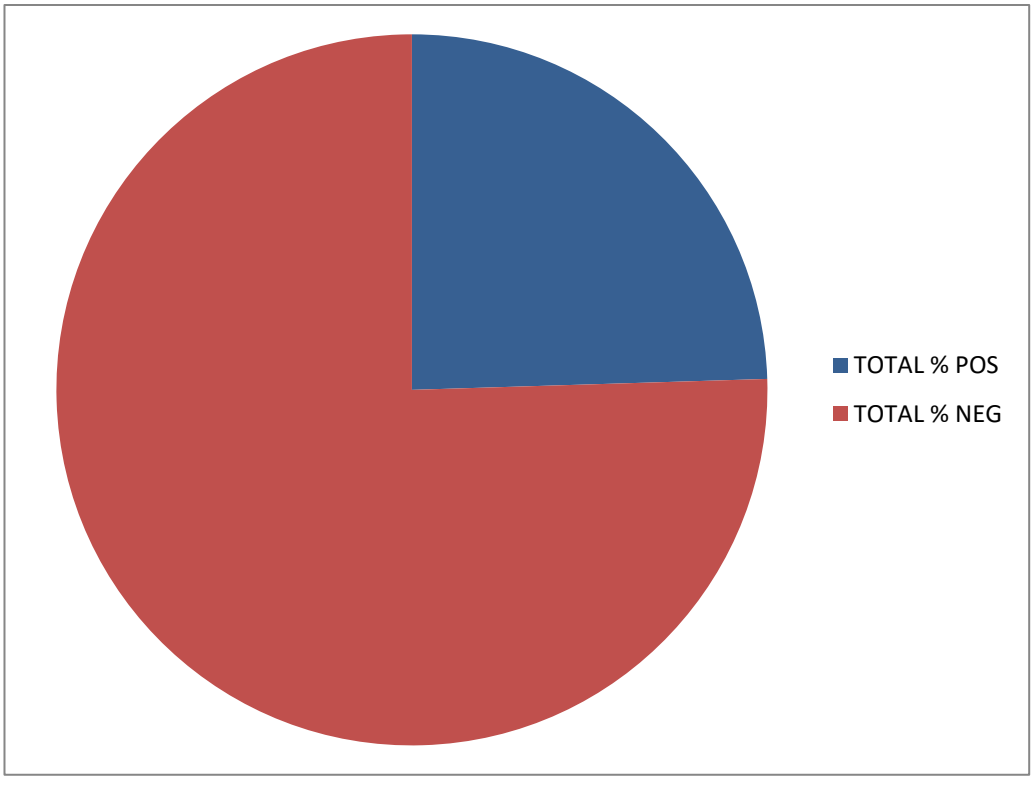


Figure 22. A. Percentage prevalence of *H. pylori* in the study population cohorts. B. Overall prevalence of *H. pylori* in the study population.

	Healthy Controls	Acute Pancreatitis	Chronic Pancreatitis	Chronic Liver Disease	Total
Number of patients recruited	48	54	47	33	**182**
Number of patients endoscoped	39	16	24	22	**101**
Number of patients with blood tests only	9	38	23	11	**81**
Cytokines analysed	48	54	47	33	**182**
Direct endotoxin analysed	24	13	25	21	**83**
Endotoxin IgG analysed	48	54	47	33	**182**
Endotoxin IgM analysed	48	54	47	33	**182**
***Helicobacter pylori* tested**	43	11	25	23	**102**
Number of patients with scorable images	31	16	20	17	**84**
Number of patients with L:M ratio tested	34	39	30	18	**121**
Number of patients with blood cultures taken	48	54	47	33	**182**
Number of patients with duodenal fluid cultured	33	16	20	20	**89**

Table 12. Tabulated summary of recruitment.

	Control Subjects	Acute Pancreatitis	Chronic Pancreatitis	Chronic Liver Disease
Total number of gastroscopies performed	39	16	24	22
Normal	24	13	17	4
Portal hypertensive gastropathy and / or varices	0	0	2	12
Gastric / Duodenal Ulcer	1	0	1	2
Gastritis / duodenitis	1	2	4	2
Oesophagitis	4	1	0	3
Hiatus Hernia	4	0	1	1
Pyloric stenosis	1	0	0	0
Benign fundic gland polyps	2	0	0	0
Barrett's oesophagus	1	0	0	0

Table 13. Table of gastroscopy findings across all cohorts.

6.5 Discussion

By means of the two combined studies a good number of patients were recruited allowing meaningful analyses. Multiple factors and variables were assessed generating a large depth of data and invaluable information from three pivotal study cohorts. Concordance of the new variable under scrutiny was measured and cohorts were divided into appropriate subgroups.

CHAPTER 7

RESULTS IV

CONFOCAL LASER ENDOMICROSCOPY AND LACTULOSE MANNITOL STUDIES DEMONSTRATE CHANGES IN INTESTINAL PERMEABILITY

7.1 Introduction

Further to the successful development of a protocol that could be adhered to for all cohorts studied, and completion of the pilot study, ethical approval was successfully obtained to study AP subjects. CLE was therefore used to assess fluorescein leakage as a measure of intestinal permeability in patients with CP, CLD and AP.

Various comparisons were feasible between disease groups and healthy controls, and subsequent sub-group analyses were also undertaken. These subgroups included patients with different severities of AP and conservatively versus surgically managed patients with CP.

CLE findings were also compared to the results of another intestinal permeability test in the same subjects, namely the lactulose:mannitol ratio. Lactulose:mannitol tests were sent to another laboratory at Alder Hey children's hospital for processing using high pressure liquid chromatography.

To consolidate our findings further in subjects with AP, we also measured the concentration of serum DAO (an enzyme expressed at the apical end of mature villus cells, which has been reported to demonstrate changes in activity with IP changes).

7.2 Fluorescein Leakage as a measure of intestinal permeability

7.2.1 CLE demonstrated increased fluorescein leakage in AP patients

Fluorescein is detected as the white element within each CLE image. It can be distinguished from villous architecture and is present within inter-villous capillaries when the intestinal barrier is intact (Figure 23A). Loss of barrier integrity results in fluorescein leaking out of the capillaries into the lumen (Figure 23B).

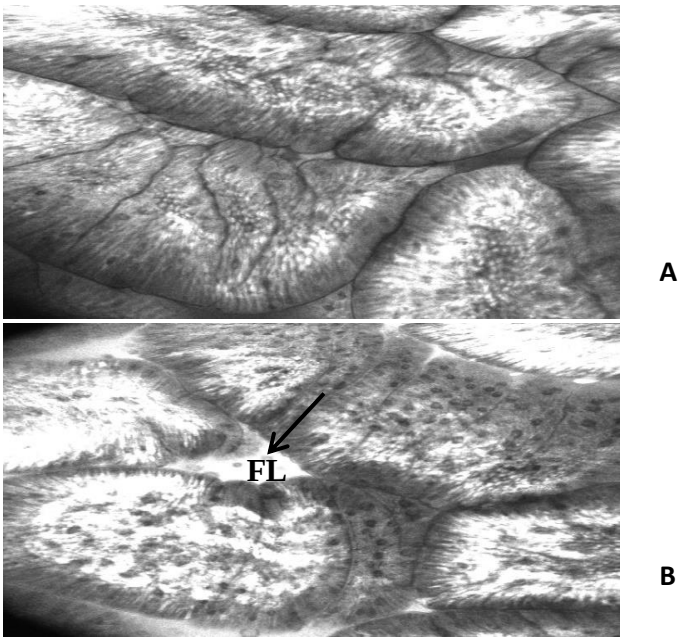


Figure 23.

(A) Duodenal villi in a control patient with no evidence of fluorescein leakage into the gastrointestinal lumen.

(B) Duodenal villi in a patient with Acute Pancreatitis showing fluorescein leakage (FL) into the gastrointestinal lumen consistent with loss of the permeability barrier.

Figure 24 demonstrates examples of images that were analysed from each of the study cohorts: chronic liver disease, chronic pancreatitis, severe acute pancreatitis, mild acute pancreatitis, moderate acute pancreatitis and healthy controls.

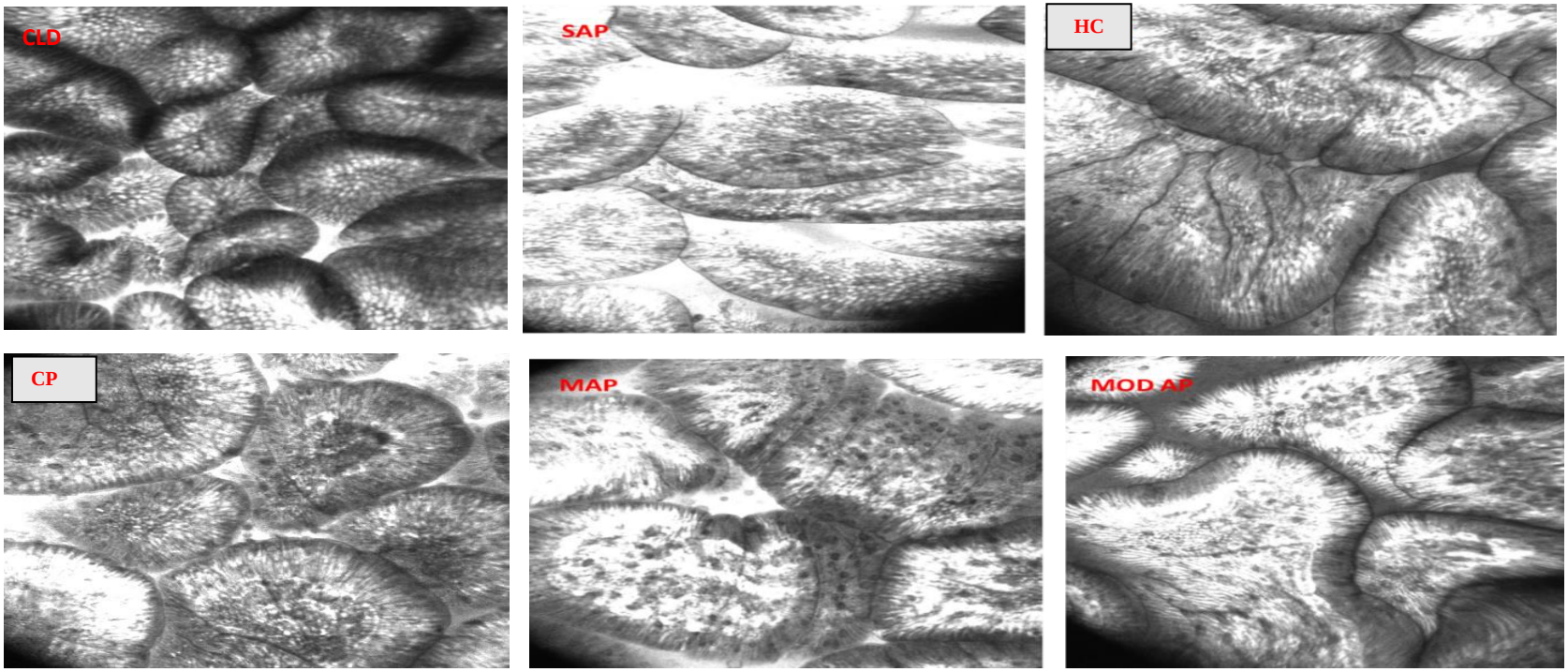


Figure 24. Representative images from each of the cohorts examined. Key: CLD – chronic liver disease; CP – chronic pancreatitis; SAP – severe acute pancreatitis; MAP – mild acute pancreatitis; HC – healthy control; MOD AP – moderate acute pancreatitis

Patients with mild, moderate and severe AP were grouped as 'AP' for initial analysis with further sub-group analysis being undertaken according to disease severity. Similarly, CP patients were initially analysed as a pooled cohort with further sub-group analysis being performed according to whether the disease was conservatively or surgically managed. By chi-square analysis, fluorescein leakage was found to be significantly higher in patients with AP compared with healthy controls (p=0.035) (Figure 25) and in patients with severe AP compared with healthy controls (p=0.015). This was the same in patients with CP compared with healthy controls (p=0.027) (Figure 25) and in patents with surgically managed CP compared with healthy controls (p=0.032) (Figure 26). Fluorescein leakage was also significantly higher in patients with mild AP compared to healthy controls (p=0.049) and in patients with severe AP compared with healthy controls (p=0.015). (Figure 27) A higher proportion of patients with CLD were also found to have fluorescein leakage than no leakage, but this was not statistically significant when compared with healthy controls.

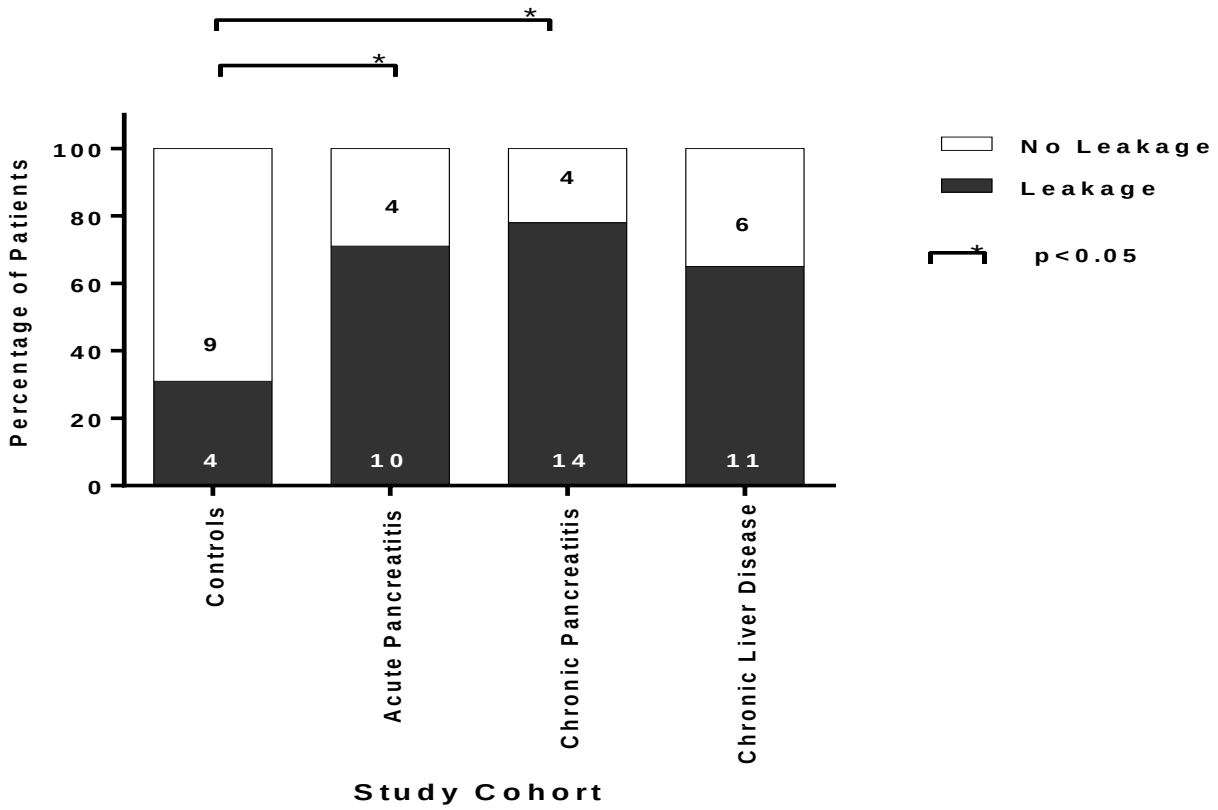


Figure 25. Fluorescein Leakage (FL) was significantly higher in patients with AP compared to healthy controls (*p=0.035) & in patients with CP compared with controls (*p=0.027) (chi-square *p<0.05).

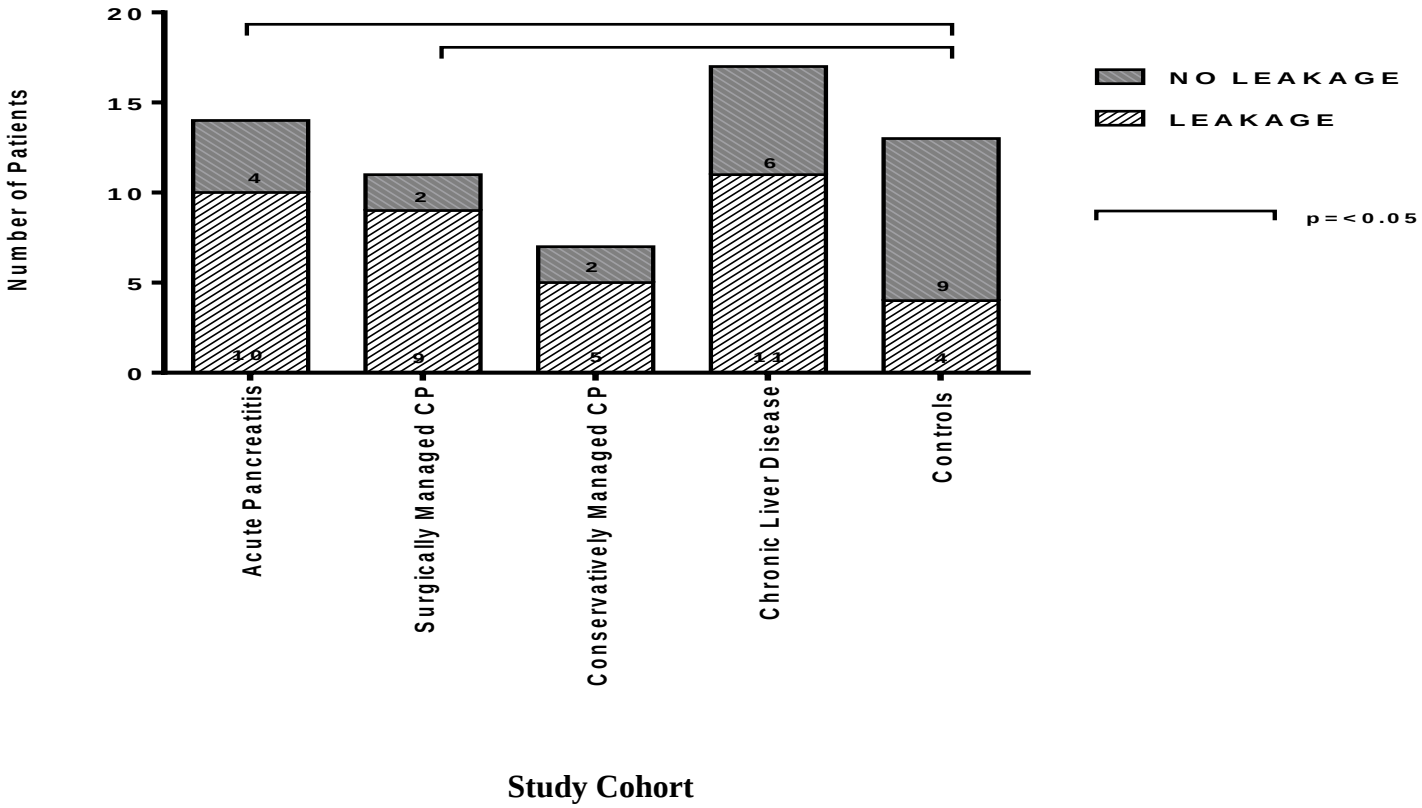


Figure 26. Chi-Square Analysis to compare the presence of fluorescein leakage on duodenal CLE images between cohort groups. Fluorescein leakage was significantly higher in patients with AP compared to healthy controls (*p=0.035) & in patients with surgically managed CP compared with healthy controls (*p=0.032).

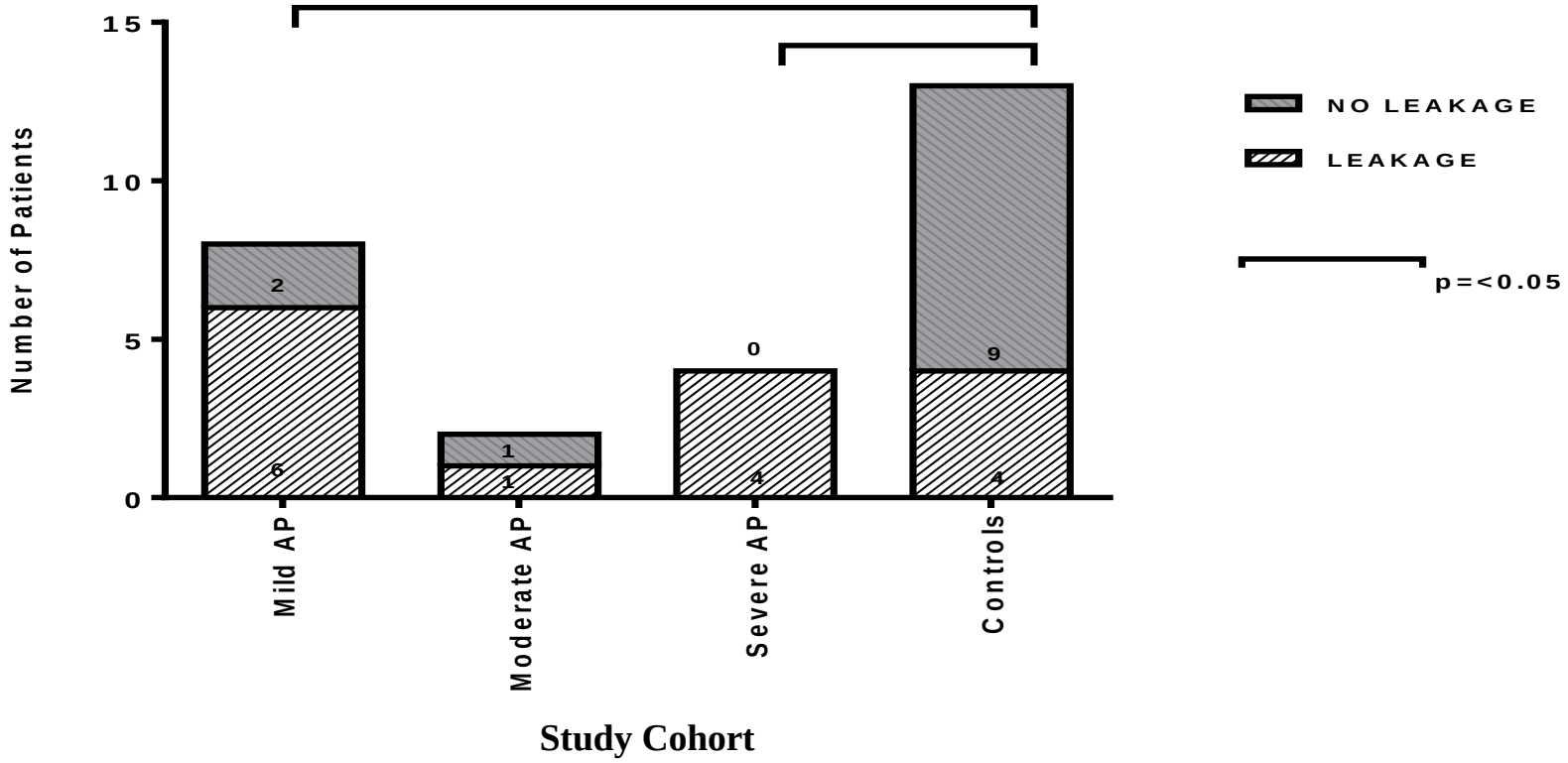


Figure 27. Chi-Square and Fisher's Exact Test Analyses to compare the presence of fluorescein leakage between patients with increasing severity of AP. Fluorescein leakage was significantly higher in patients with mild AP compared to healthy controls by chi-square test (*p=0.049) & in patients with severe AP compared with healthy controls by Fisher's Exact Test (*p=0.03).

7.2.2 CLE demonstrated increased fluorescein leakage in CP patients with a history of gastrointestinal surgery when compared with healthy controls

By chi-square analysis, fluorescein leakage was significantly higher in patients with CP who had a history of gastrointestinal (GI) surgery when compared with healthy controls (p=0.032). (Figure 28) There was no statistical significance between patients with CP who had not undergone surgery when compared with healthy controls; nor when compared with patients who did have a history of GI surgery.

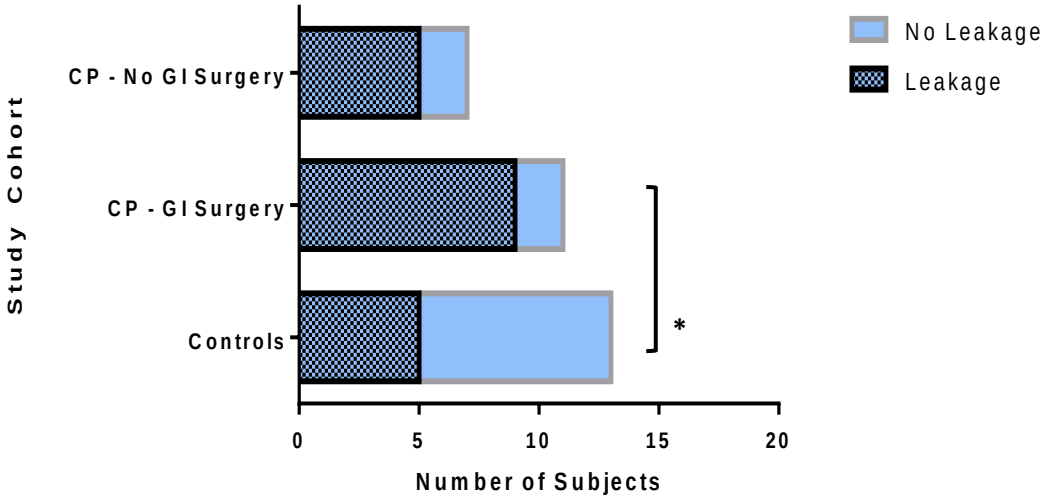


Figure 28. Fluorescein leakage was significantly higher in patients with CP who had a history of gastrointestinal (GI) surgery than healthy controls (*p=0.032).

7.2.3 CLE demonstrated increased fluorescein leakage in excluded controls when compared with healthy controls

To confirm that other factors have an impact on intestinal permeability, an analysis of subjects with dyspepsia who were not included in the 'healthy control' group due to co-morbidities (including a history of any previous surgery), endoscopic abnormalities or *H. pylori* positivity was undertaken. Figure 29 demonstrates that a combination of these factors in dyspeptic subjects resulted in significantly higher fluorescein leakage compared to healthy controls ($p=0.012$). On closer review of these subjects, it was noted that those with evidence of leakage frequently had a history of previous gastrointestinal surgery such as cholecystectomy or appendicectomy, sterilisation or hernia repair. Figures 30–32 demonstrate a comparison of fluorescein leakage between healthy controls and dyspeptic subjects who were not considered to be 'healthy controls' based on co-morbidities, positive gastroscopy findings, or positive *H. pylori* status, respectively.

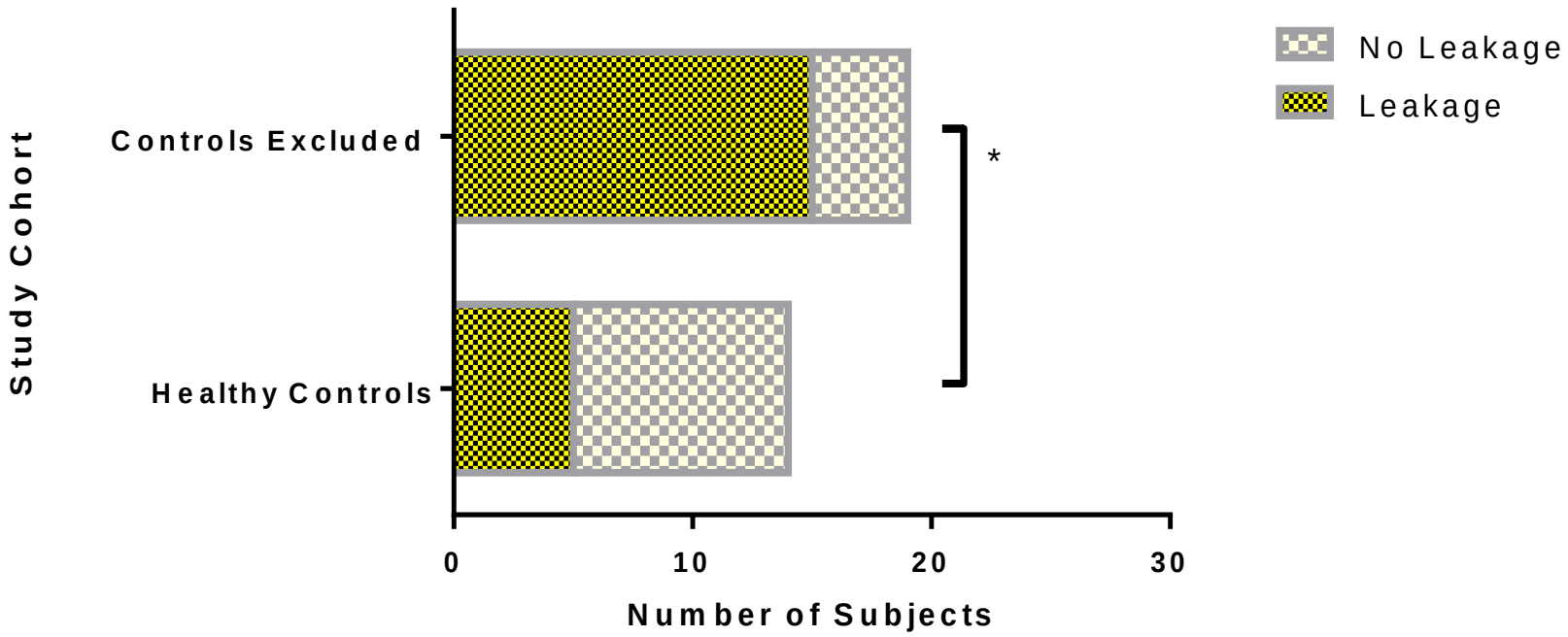


Figure 29. Fluorescein leakage was significantly higher in patients with non-ulcer dyspepsia who had co-morbidities, including a history of a long-standing medical problem requiring long-term medication; previous surgery or positive *H. pylori* status*;* and positive gastroscopy findings, compared with 'healthy controls' **(*p=0.012)**.

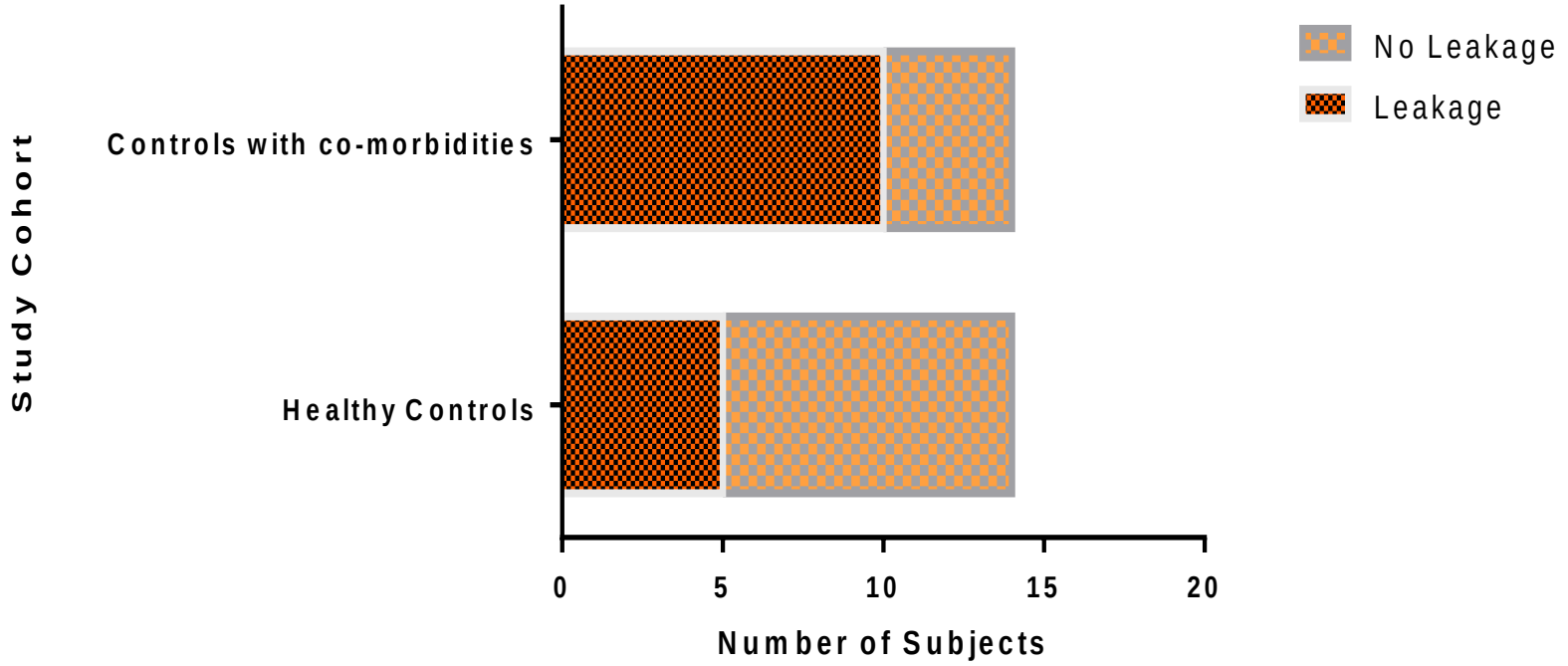


Figure 30. Fluorescein leakage was not found to be higher in subjects with non-ulcer dyspepsia with co-morbidities when compared with healthy controls. (p=0.058).

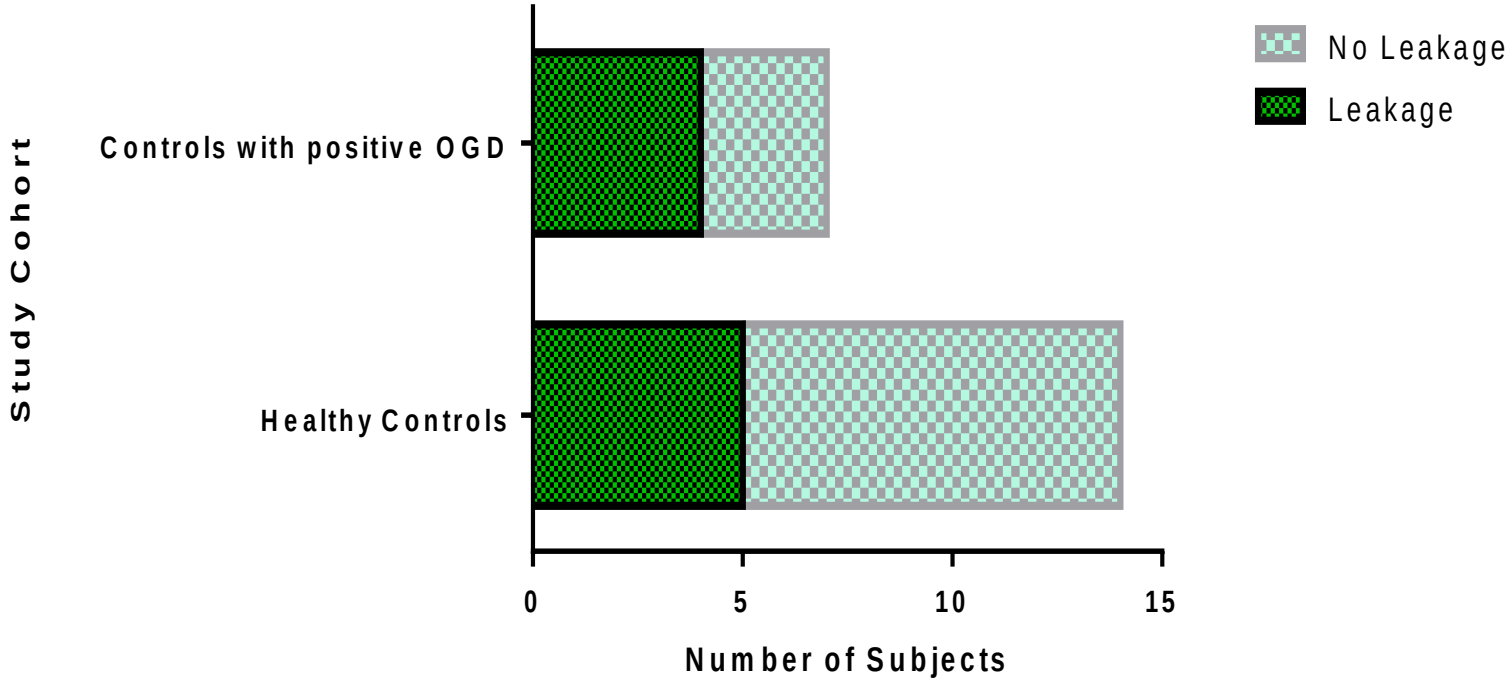


Figure 31. Fluorescein leakage was not higher in subjects with non-ulcer dyspepsia who had positive gastroscopy findings when compared with healthy controls. (p=0.350).

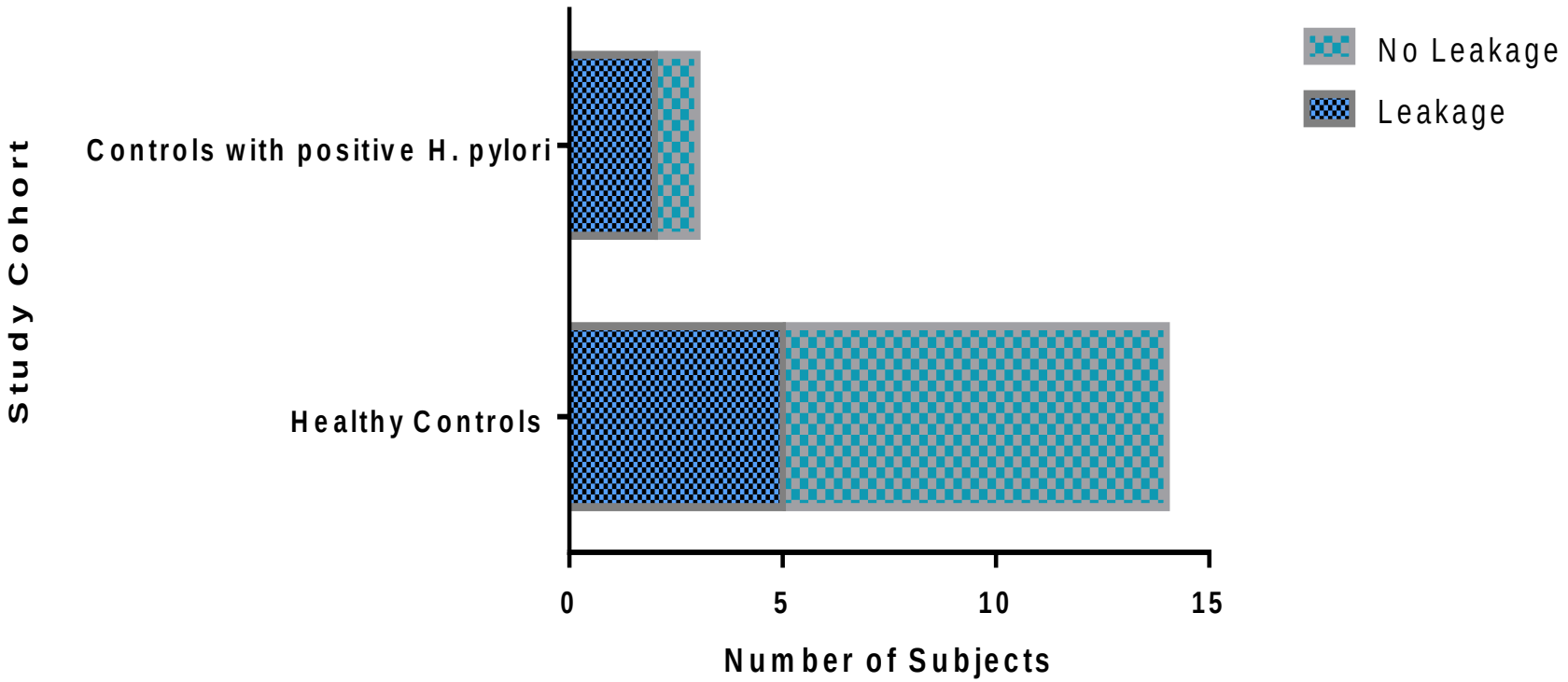


Figure 32. Fluorescein leakage was not higher in dyspeptic subjects who were *H. pylori* positive when compared with healthy controls. (p=0.323).

7.3 Average Scores of Fluorescein Leakage as a measure of intestinal permeability

7.3.1 When analysing the continuous variable of average scores of fluorescein leakage, there was no evidence to suggest that leakage presence statistically differs between groups

The parameters that were analysed and compared for the presence or absence of fluorescein leakage were also analysed according to average scores of fluorescein leakage. No significant differences were found. (Figures 33-34)

Fisher's Exact Test was carried out for various group comparisons. In an analysis of subjects with CLD compared with healthy controls p=1.00; CP compared with controls p=1.00; MAP compared with controls p= 1.00; Moderate AP compared with controls p=0.56 and SAP compared with controls p=1.00. Thus there was no evidence to suggest that leakage presence statistically differs between these groups.

In an analysis of CP patients who had a history of prior GI surgery compared to those without and healthy controls, comparison of the patients with a history of GI surgery against healthy controls reached statistical significance using this methodology (p=0.022). (Figure 35) The dyspeptic subjects that were excluded as healthy controls were also compared with healthy controls as described above. Average scores of fluorescein leakage in excluded control subjects who had positive gastroscopy findings and / or co-morbidities, including a history of previous surgery, showed higher levels of fluorescein leakage than healthy controls, and this also reached statistical significance (p=0.016). (Figure 36)

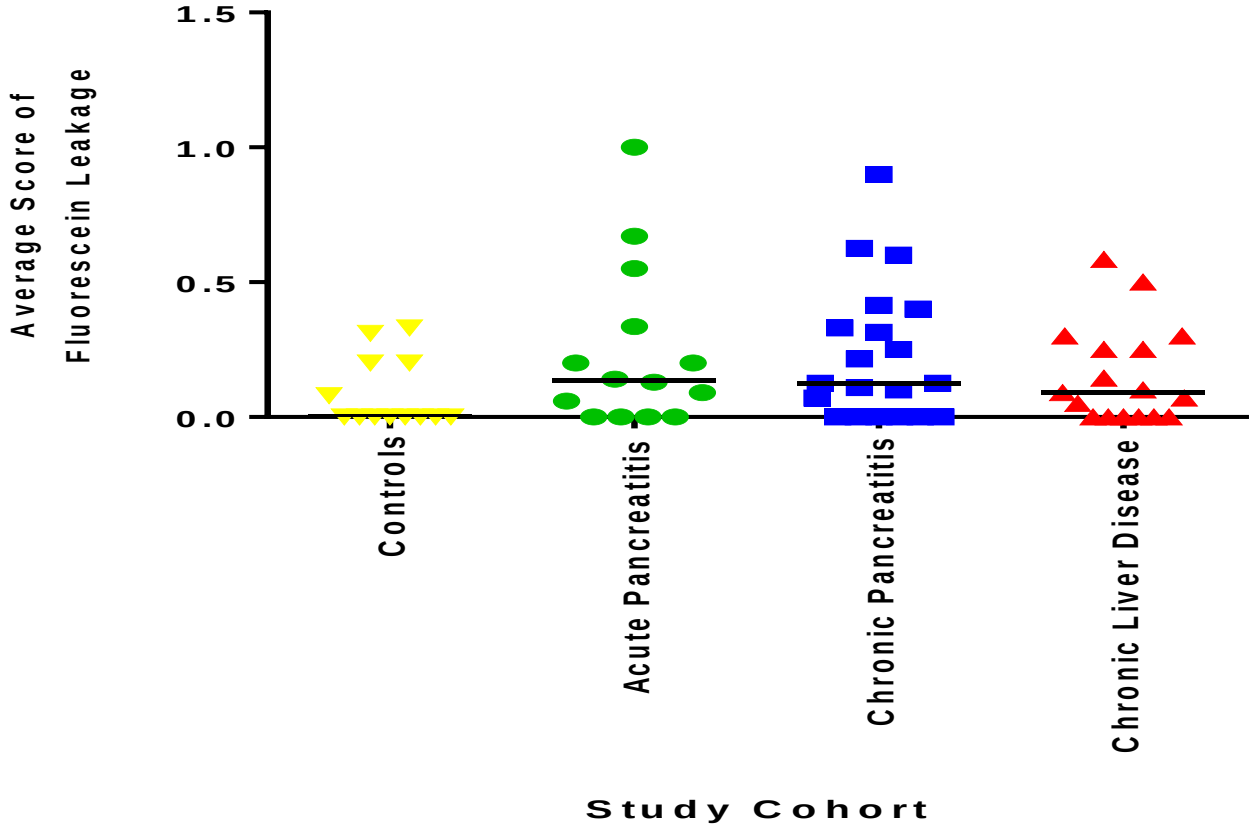


Figure 33. Average scores of fluorescein leakage were not found to be significantly higher in patients with AP, CP and CLD when compared to healthy controls. CLD compared with healthy controls p=1.00; CP compared with healthy controls p=1.00; AP compared with healthy controls p=0.106.

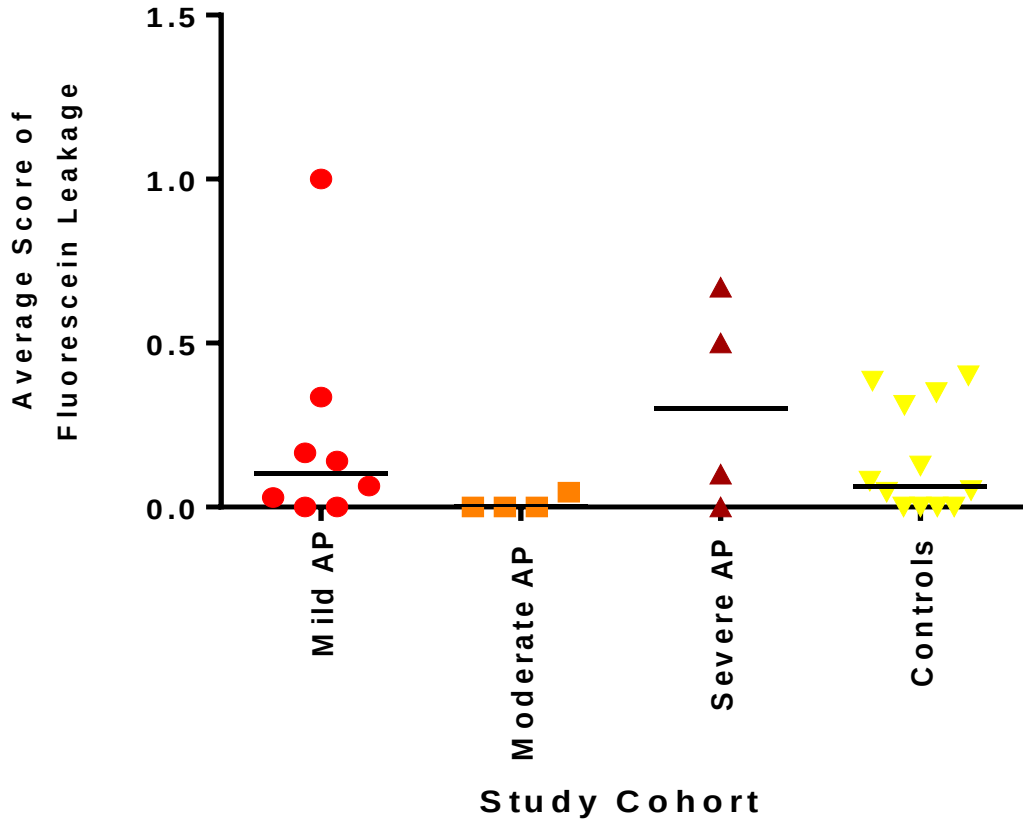


Figure 34. Average scores of fluorescein leakage were not found to be significantly higher than controls in patients with AP when sub-classified according to disease severity by Fisher's Exact Test. MAP compared with healthy controls p=1.00; moderate AP compared with healthy controls p=0.56; SAP compared with healthy controls p=1.00.

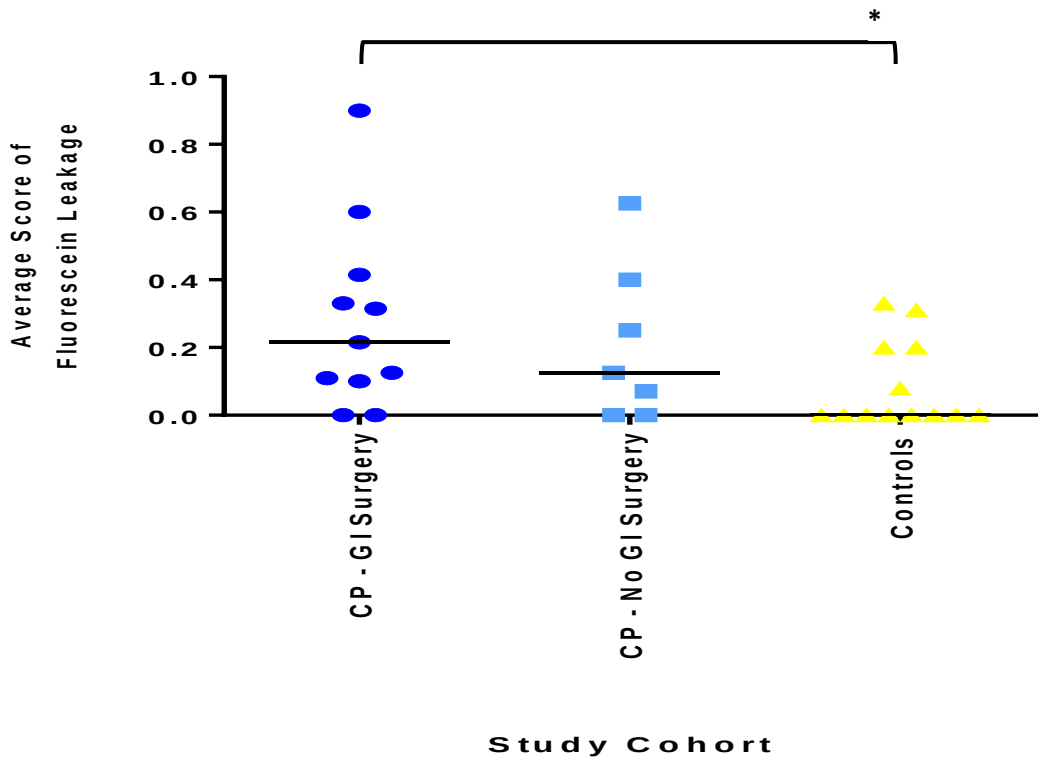


Figure 35. Average scores of fluorescein leakage were found to be significantly higher in subjects with CP who had undergone GI surgery when compared with controls. A comparison of the cohort of CP subjects with a history of GI surgery compared with healthy controls (p=0.022*) reached statistical significance. CP with GI surgery versus CP with no GI surgery p=0.608; CP with no GI surgery versus healthy controls p=0.1687.

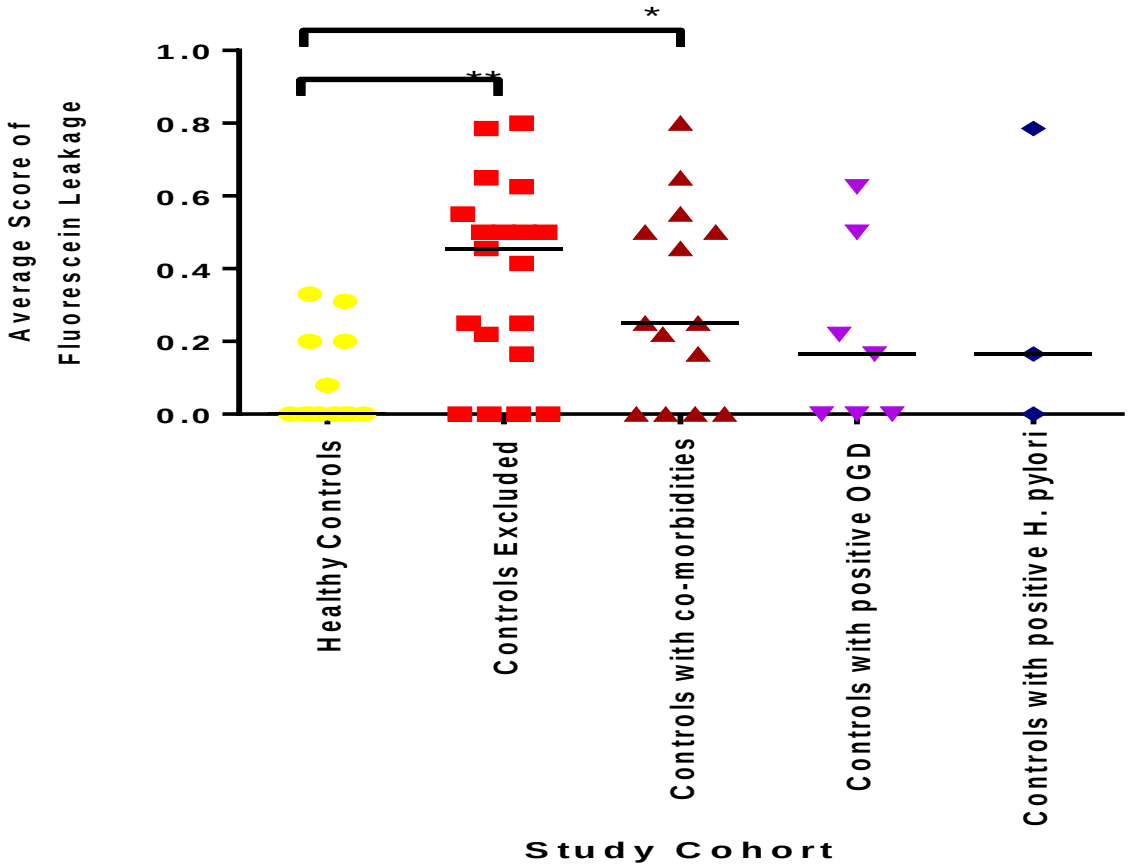


Figure 36. Average scores of healthy controls compared with controls who were excluded as a result of co-morbidities, (p=0.022*); positive *H. pylori* status (p=0.341)*;* OGD abnormalities (p=0.295); or a combination of these factors (p=0.016**).

7.4 Lactulose : Mannitol Ratio as a measure of intestinal permeability

7.4.1 Lactulose:mannitol ratio confirmed increases in IP in AP patients and surgically managed CP patients

The lactulose:mannitol (L/M) ratio was measured as a standard assay of IP in 96 patients (9 controls; 39 AP; 30 CP; 18 CLD). Control subject numbers were lower due to a delay in acquisition of the solution and a lack of uptake of recall appointments in this group. Serum absorption was measured 90 minutes after ingestion of the L:M solution with higher ratios indicating higher levels of intestinal permeability. Patients were grouped for analysis in the same way as described above for the confocal image analysis. HPLC analysis was performed by Andrew Hodgkinson at Alder Hey children's hospital, Liverpool. This is not a standard clinical test and therefore it is not readily available in all laboratories. The team had acquired the equipment and expertise for a previous research study investigating gut permeability in a paediatric cohort and were willing to assist our study both with equipment and skillset.

In the example chromatograph, (figure 37) the x-axis corresponds to elution time in minutes and the y-axis the current generated at the electrochemical cell. The time to elute from the chromatography column is displayed against each sugar, with the smaller sugars eluting earlier. The concentration of mannitol and lactulose in each sample is calculated from a 3 point standard curve for each sugar. The internal standard, melibiose is added to all standards and unknowns and taken through the extraction process which is then used in the calculation to correct for any losses in sample preparation or injection. The L:M ratio is simply the concentration of lactulose divided by the concentration of mannitol for each sample.

Mann-Whitney U tests were used to compare groups. The serum 90-minute L:M ratio was significantly increased in patients with AP compared to healthy controls ($p=0.031$). (Figure 38) An increase was also observed in patients who had surgically managed CP compared with healthy controls ($p=0.017$), and compared with CP patients who were conservatively managed ($p=0.013$). (Figure 39)

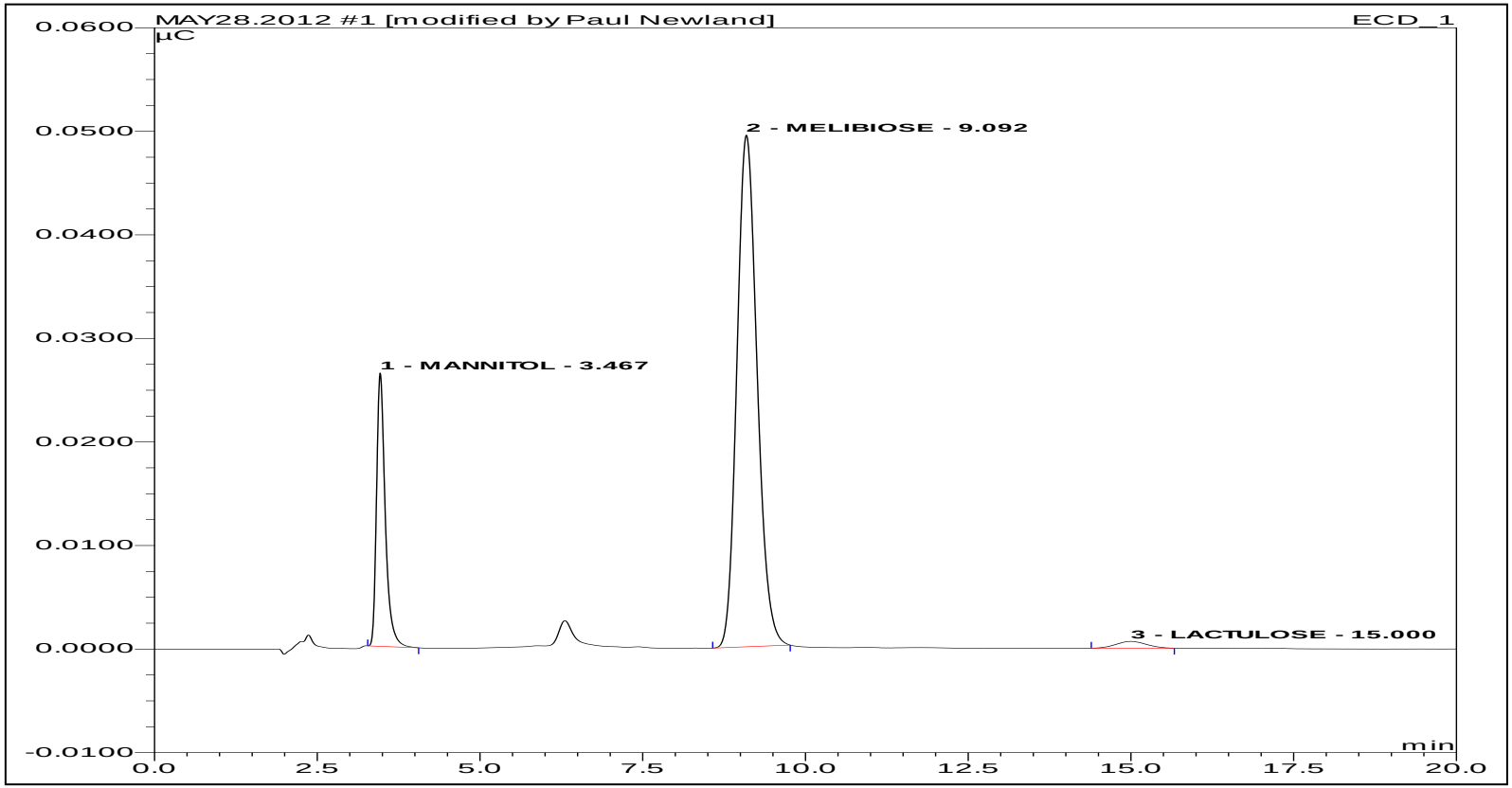


Figure 37. An example HPLC chart demonstrating concentrations of mannitol and lactulose and a standard sugar, melibiose. Generated and interpreted by Dr Andrew Hodgkinson, Alder Hey Hospital, Liverpool.

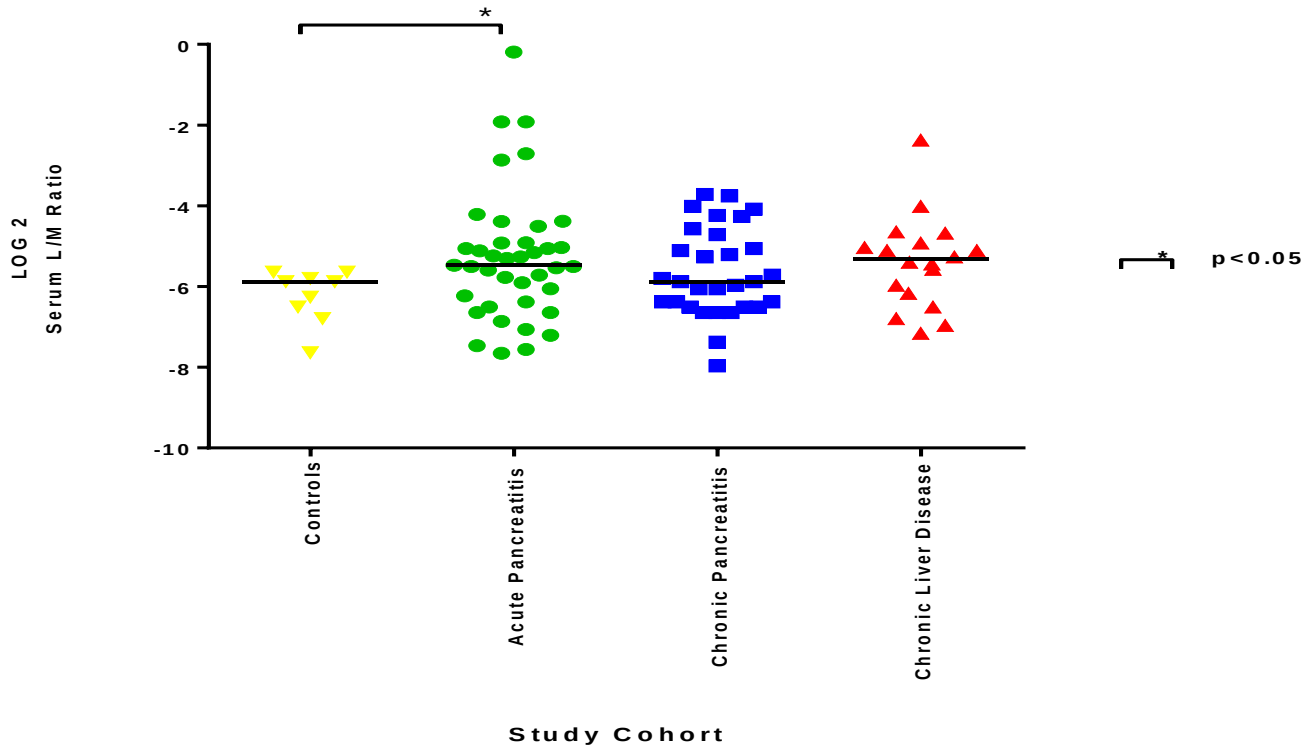


Figure 38. The lactulose:mannitol (L/M) ratio was recorded as a standard measure of intestinal permeability. Serum absorption was measured at 90 minutes with higher ratios indicating higher levels of permeability. Patients with AP were found to have significantly higher L/M ratios when compared to healthy controls (*p=0.031). (MW *p<0.05); (Kruskal Wallis – p=0.32).

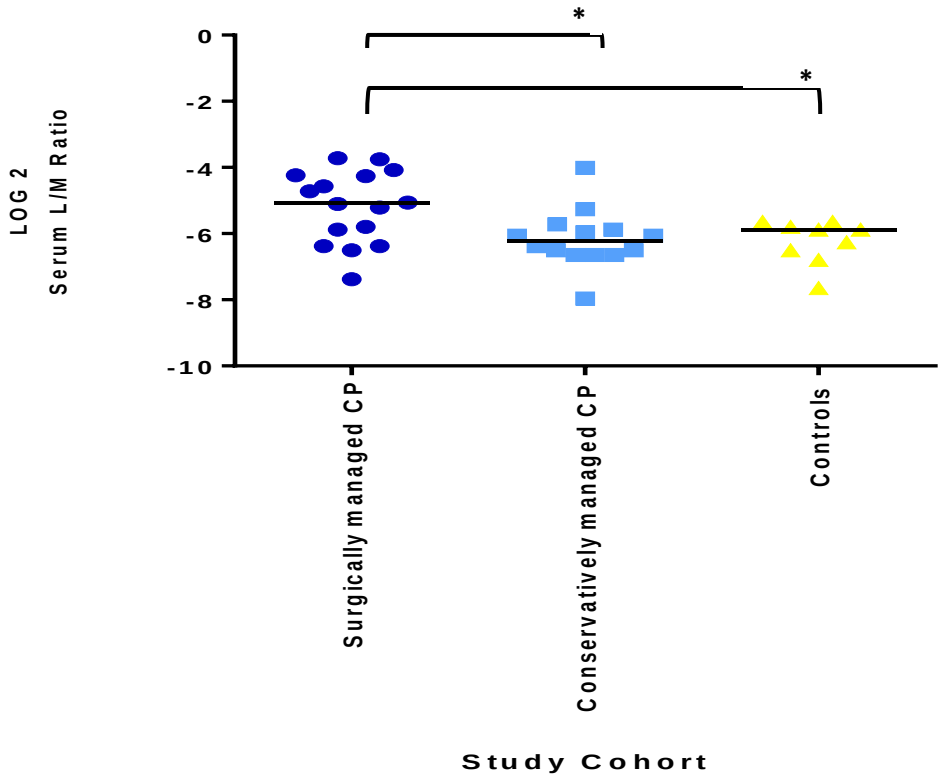


Figure 39. Mann-Whitney U Tests to compare intestinal permeability index between cohorts. Serum 90 minute lactulose/mannitol ratio was significantly increased in patients with AP compared to healthy controls (*p=0.031) & in patients with surgically managed CP compared with healthy controls (*p=0.017) and patients with conservatively managed CP *(*p=0.013); (Kruskal Wallis – p=0.03*).

7.4.2 Lactulose:mannitol ratio confirmed statistically significant increase in IP in patients with moderate AP, and in excluded controls when compared with healthy controls

The serum 90-minute lactulose:mannitol ratio was significantly higher in patients who had moderate AP compared to controls (p=0.033*). (Figure 40) An increase was also evident in patients who had moderate AP compared with those who had mild AP (p=0.028*), and compared with patients who had severe AP (p=0.046*). A comparison of healthy controls and dyspeptic subjects who were excluded from being controls also demonstrated significant differences. Serum 90-minute lactulose:mannitol ratio was significantly increased in dyspeptic patients with co-morbidities, including a history of previous surgery (p=0.011*); positive *H. pylori* status (p=0.0007***)*;* abnormal gastroscopy findings (p=0.017*); and a combined group of one or more of these factors (p=0.004**) compared with healthy controls.

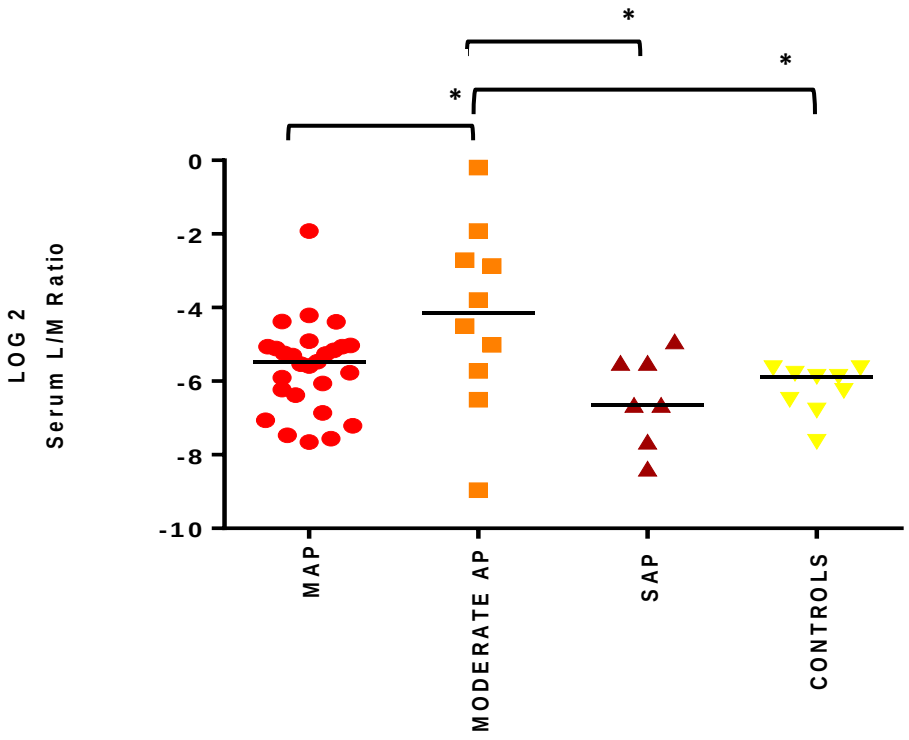


Figure 40. Serum 90-minute lactulose:mannitol ratio was significantly increased in patients with moderate AP compared to controls (p=0.033*); moderate AP compared with mild AP (p=0.028*), and moderate AP compared with severe AP (p=0.046*). Mild AP compared with controls p=0.173; MAP compared with SAP p=0.119; SAP compared with healthy controls p=0.633; (ANOVA – p=0.01*).

7.5 In patients with AP, Diamine oxidase concentration increased with increasing fluorescein leakage

Diamine oxidase (DAO) is specifically located at the apical end of mature villus cells. Its activity reflects the integrity and maturity of the small intestinal mucosa and it enters the blood as intestinal permeability increases. Serum DAO concentration was measured only in AP patients and those healthy controls who also underwent CLE, 14 and 13 subjects respectively. The serum concentration of DAO was analysed against the continuous variable of average score for fluorescein leakage, by means of Spearman's R correlation. This showed that serum DAO concentration increased with increasing fluorescein leakage, as demonstrated by CLE. Spearman's rank correlation was statistically significant between these two parameters ($p=0.035$). (Figure 41) When the discrete variable of fluorescein leakage and the lactulose:mannitol ratio were analysed for the same variables, statistical significance was not reached as demonstrated in figures 42 and 43 below.

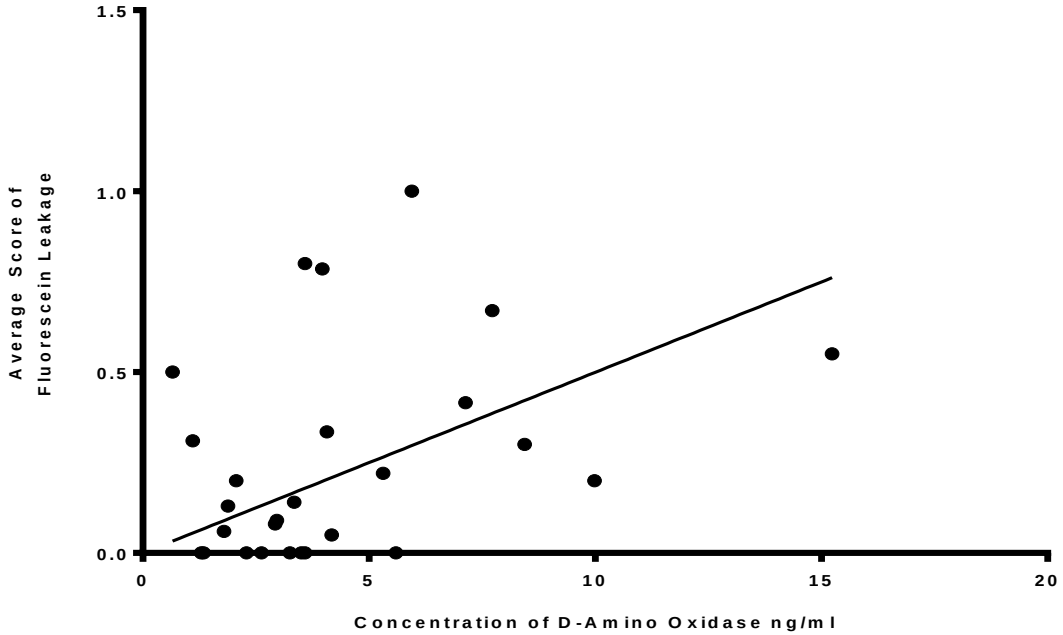


Figure 41: The concentration of DAO increased relative to the amount of fluorescein leakage, as demonstrated by CLE. Spearman's rank correlation was found to be statistically significant between these two parameters (r = 0.40, 95% CI – 0.02 to 0.68, p = 0.035)

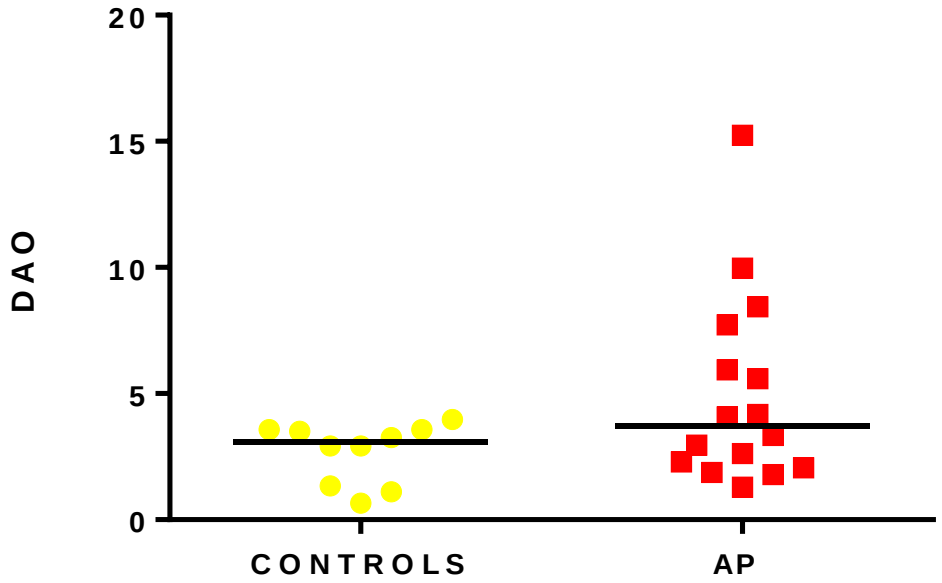


Figure 42: Patients with AP were not found to have higher serum DAO concentrations when compared with healthy controls. (p=0.150)

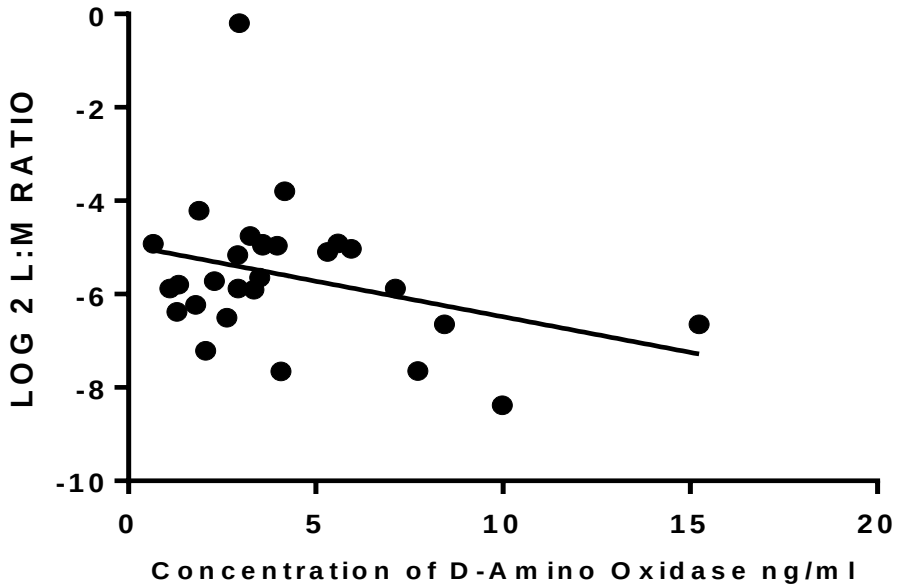


Figure 43. The concentration of DAO was not found to have increased significantly relative to the lactulose:mannitol ratio. Spearman's rank correlation p=0.363.

7.6 Duodenal Tissue

Due to the limitations of time on the study, duodenal tissue analysis could not be undertaken. Immuno-histochemical analysis was initially planned with staining for active caspase 3 activity and DNA fragmentation ELISA. Wax blocks were prepared by a pathology technician and initial discussions with experts undertaking immunohistochemistry were held, but this aspect was subsequently excluded from the study prior to experimental planning. For part of the main study cohort however, duodenal tissue was sent from patients with AP and controls to the laboratory in formalin for H&E analysis. (table 14) Tissue acquisition was impractical for those patients who were endoscoped on the intensive care unit. Thus, due to very limited numbers, meaningful analysis of this dataset is not feasible. Of the 16 patients with AP who underwent CLE, 12 had duodenal biopsy histology assessed, but only one of these was from the severe AP cohort, rendering it difficult to assess for changes based on severity. 10 of the 12 distal duodenal biopsy samples from patients with AP, and all 4 biopsies taken from control subjects, were reported as showing normal histology. Two patients with AP (1 mild, 1 moderate) were reported to have evidence of duodenal inflammation.

	Normal	**Inflammation**	**Total**
Healthy Controls	4	0	4
Mild Acute Pancreatitis	8	1	9
Moderate Acute Pancreatitis	1	1	2
Severe Acute Pancreatitis	1	0	1

Table 14. Summary table of duodenal histology results.

CHAPTER 8

RESULTS V

ASSESSMENT OF PARAMETERS OF INFECTION INCLUDING MICROBIAL FLORA AND ENDOTOXINAEMIA

8.1 Introduction

On the basis that intestinal permeability is associated with bacterial translocation and endotoxaemia, we undertook multiple further analyses to consolidate our findings further and investigate the potential of infection associated with the process.

We analysed cultures of duodenal aspirates from all patients undergoing CLE and assessment of endotoxinaemia using anti-endotoxin antibodies and a direct endotoxin assay. We have compared the findings from the various modalities used, to investigate the extent to which alterations in intestinal permeability correlated with changes in duodenal microbial growth and endotoxinaemia. We have focussed on subjects with acute pancreatitis but have also made comparisons between the other cohorts studied where relevant.

8.2 Intestinal Permeability and associated changes in duodenal fluid microbial growth

8.2.1 Fluorescein leakage and the presence of duodenal organisms in AP

Duodenal fluid was aspirated and cultured in all 62 patients who underwent CLE. Chi square analysis showed that patients with AP had significantly higher microbial growth in their duodenal fluid than healthy controls ($p=0.004$). (Figure 44B) The identification of fluorescein leakage by CLE into the gastrointestinal lumen was also significantly associated with the presence of cultured duodenal organisms in patients with AP ($p=0.003$). (Figure 44A) Patients with severe AP were also found to have significantly higher microbial growth in their duodenal fluid compared to healthy controls ($p=0.002$). (Figure 44B)

A sub-group analysis of patients with CP who had been managed surgically or conservatively did not show any statistically significant differences. (Figure 45)

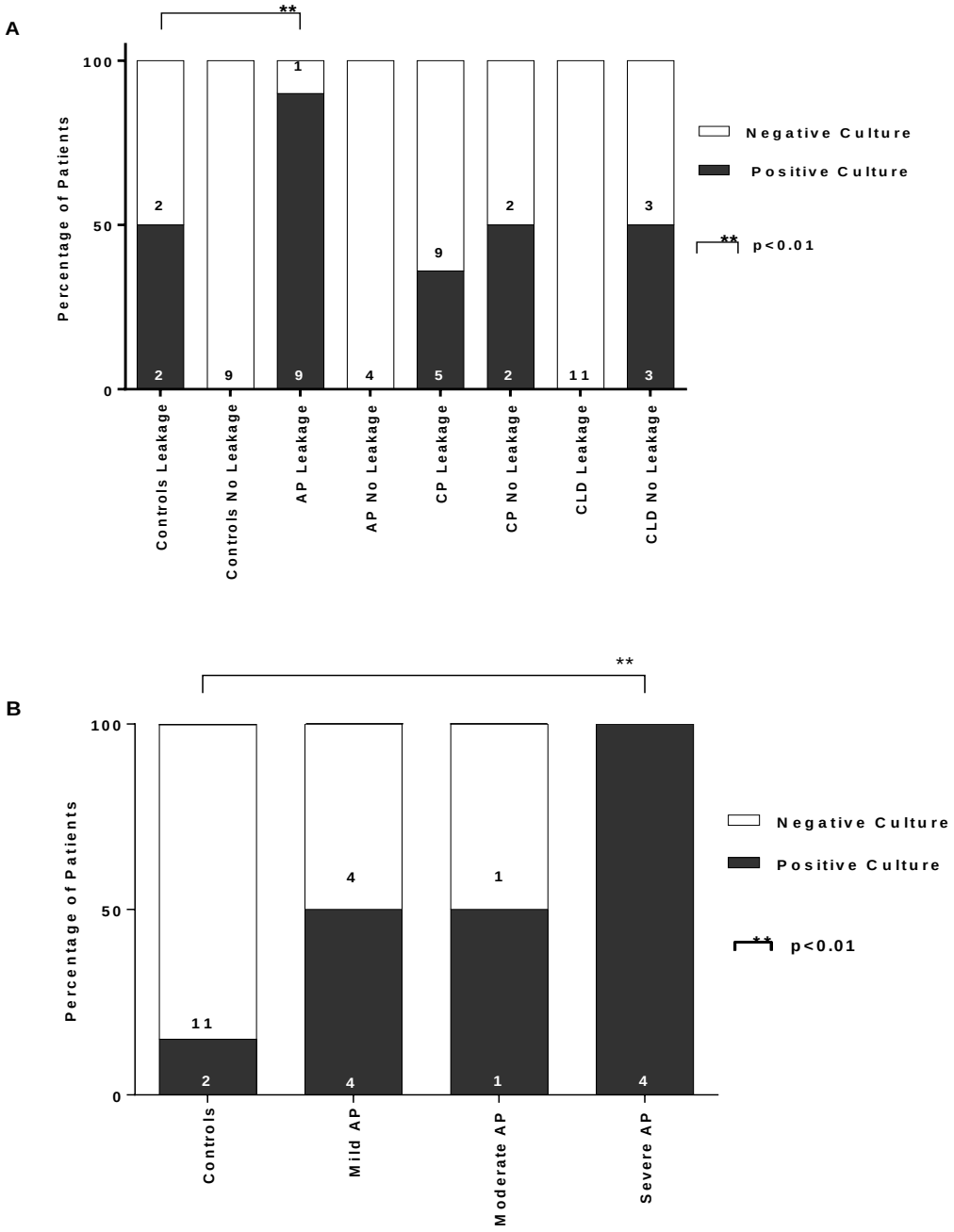


Figure 44. Chi Square analyses to assess association between fluorescein leakage and positive duodenal fluid cultures. (A) Patients with AP were found to have significantly higher microbial growth in the duodenal fluid than healthy controls (**p=0.004). The identification of fluorescein leakage by CLE into the gastrointestinal lumen was also significantly associated with the presence of positive duodenal cultures in patients with AP (p=0.003). (B) Patients with severe AP were found to have significantly higher microbial growth in the duodenal fluid than healthy controls (**p=0.002).

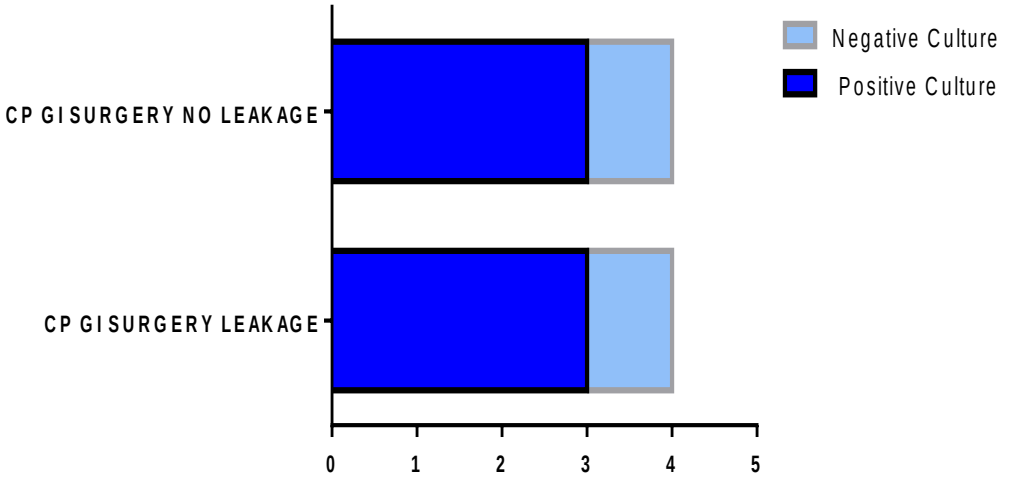


Figure 45. The identification of fluorescein leakage by confocal endomicroscopy into the gastrointestinal lumen was not significantly associated with the presence of positive duodenal fluid cultures in patients with conservatively (non-surgically) managed CP (p=0.488; p=1.00).

8.2.2 Distribution of identified duodenal organisms within the study cohorts

Several duodenal organisms were identified within subjects who had CP, CLD and particularly AP. These were grouped as positive duodenal fluid culture and included:

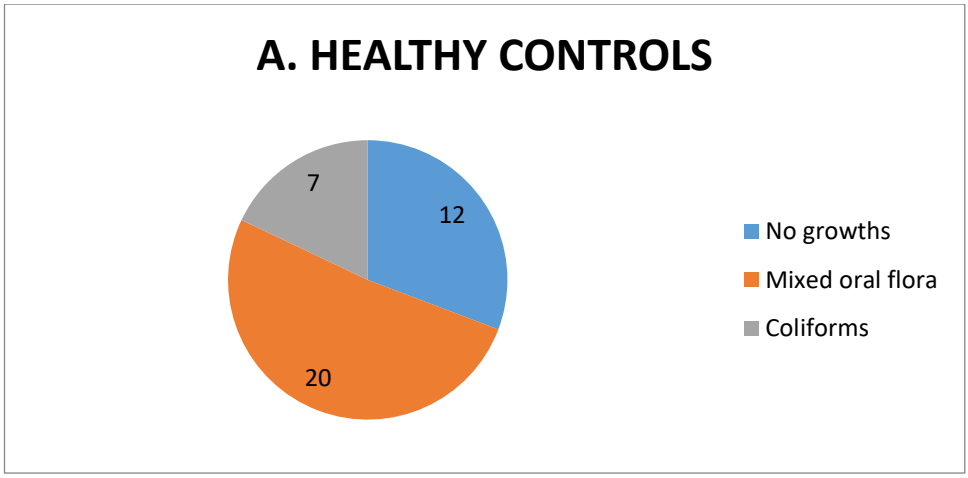
- Yeasts, Staphylococcus aureus, Coliforms, Pseudomonas, Enterococcus, Gut anaerobes

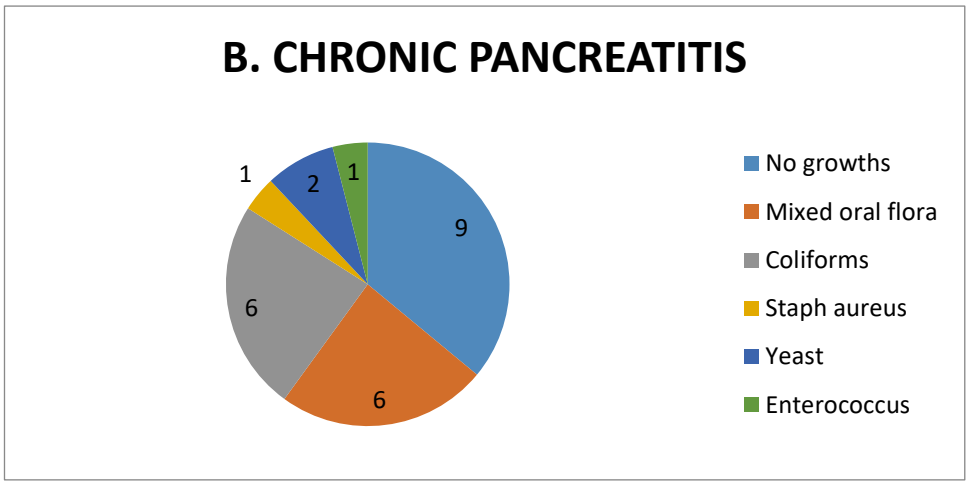
The majority of controls had negative duodenal fluid culture and were primarily reported as:

- No Growth or Mixed Oral Flora

Charts below demonstrate the distribution of organisms within the cohorts. (Figure 43)

Approximately 2/3 of subjects with CP, CLD and non-ulcer dyspepsia had negative duodenal fluid cultures. The small proportion of patients with positive duodenal fluid cultures in the CLD and CP cohorts showed the presence of Staphylococcus aureus, coliforms or yeast. The patients with AP had much higher proportions of positive cultures and diversity in microbial growth. Positive cultures in this cohort included the above organisms but also Pseudomonas and Enterococcus species. (Figure 44A and 44B)





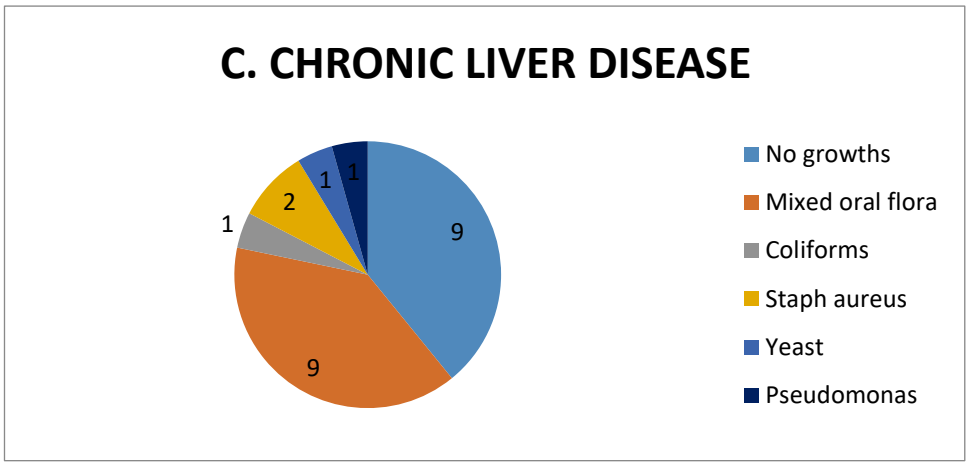
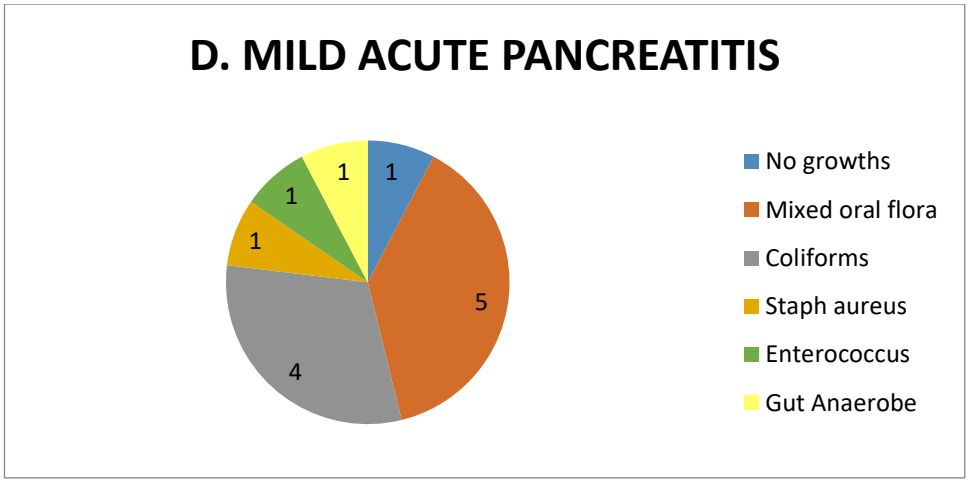
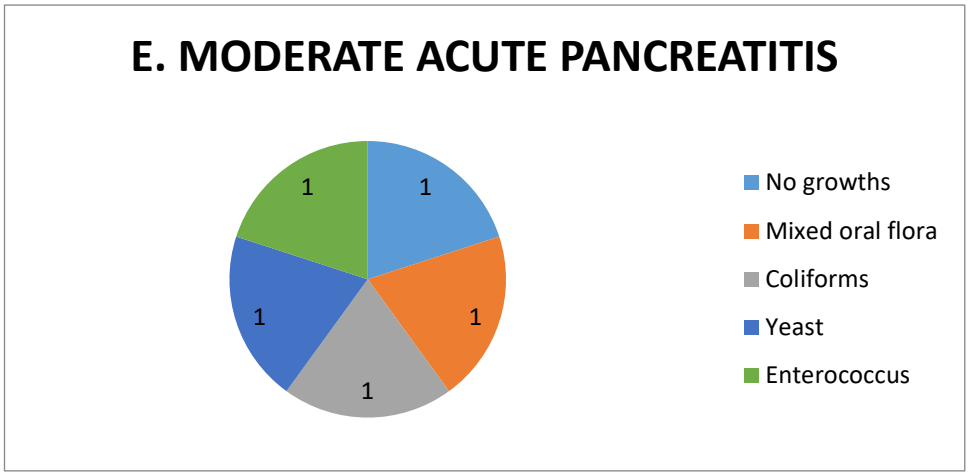


Figure 46. The pie charts demonstrate the duodenal culture findings from the patients from each of the cohorts: A – healthy controls; B – CP; C – CLD; The figures represent the number of patients with each of the gut flora mentioned. Approximately two thirds of subjects with CP, CLD and healthy controls had negative duodenal fluid cultures with either no growth or mixed oral flora only.





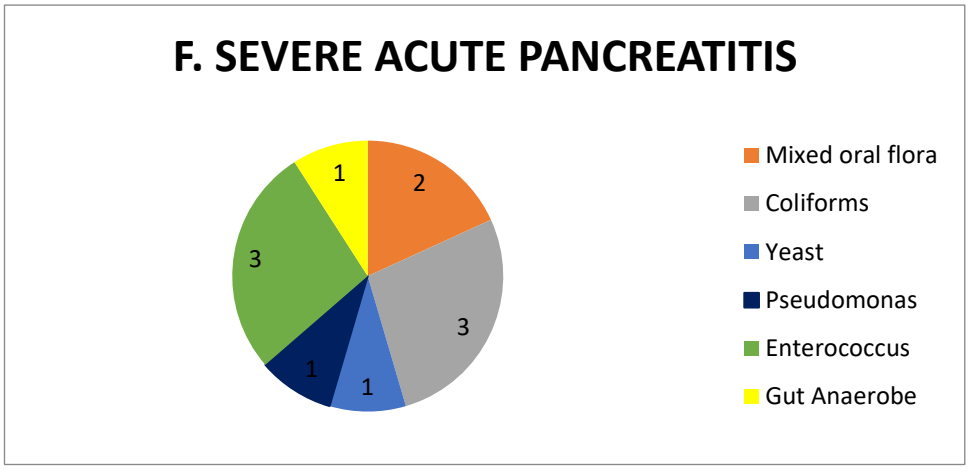
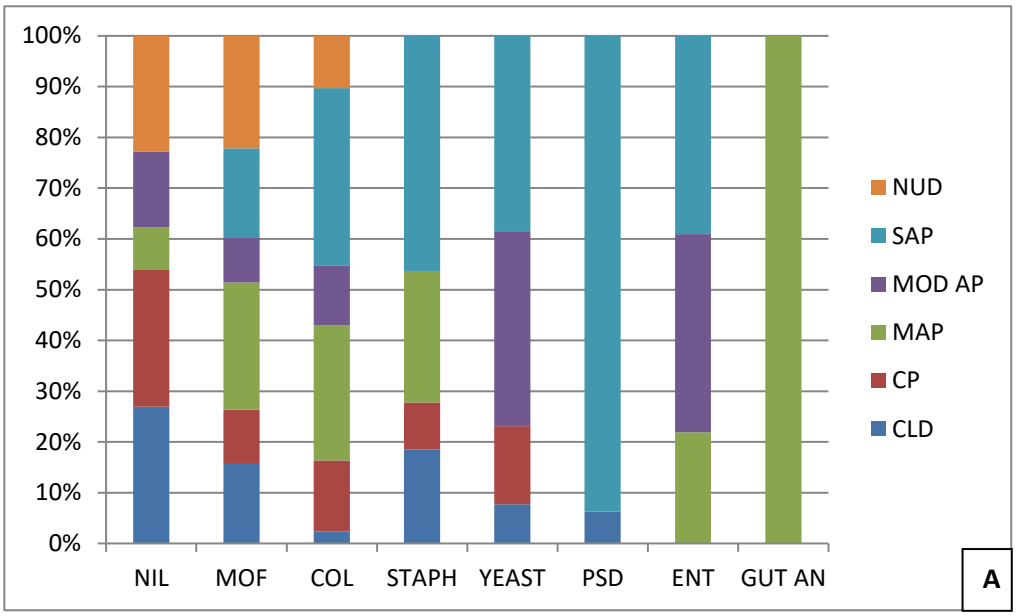
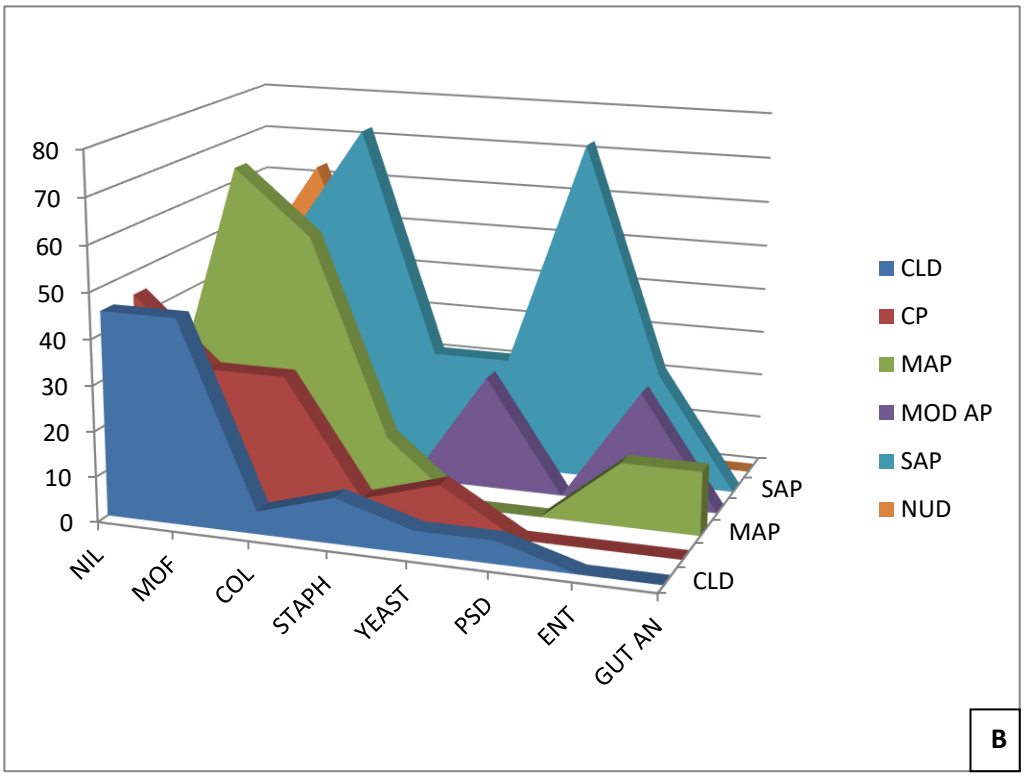


Figure 47. The pie charts demonstrate the duodenal culture findings from the patients from each of the cohorts: D – mild AP; E – moderate AP; F – severe AP. The figures represent the number of patients with each of the gut flora mentioned. **(D-F)** Patients with AP had a much higher proportion of positive cultures and diversity in growth.





Figures 48:

A and B. Bar chart and area graph to further demonstrate differences in duodenal fluid microbial growth within cohorts.

Key: MOF – mixed oral flora; COL – coliforms; STAPH – staphylococci; YEAST – yeast; PSD – pseudomonas; ENT – enterococci; GUT AN – Gut Anaerobes

8.3 Assessment of endotoxinaemia

8.3.1 Anti-endotoxin antibodies showed inverse correlation to severity of AP

We measured the Endocab ELISA IgG and IgM and performed Mann Whitney U analyses to compare levels between 54 patients with AP (35 mild AP; 11 moderate AP; 8 severe AP) and 13 healthy controls. IgG anti-endotoxin antibodies (EndoCab IgG) were significantly lower in patients with severe AP when compared to those with mild AP (p=0.046) and also in severe AP and moderate AP when compared to healthy controls (p=0.022; p=0.025, respectively). (Figure 49A) IgM anti-endotoxin antibodies (EndoCab IgM) were also significantly lower in patients with severe AP when compared to those with mild AP (p=0.036) and also in patients with severe AP, moderate AP and mild AP when compared to healthy controls (p=0.001; p=0.011; and p=0.041, respectively). (Figure 49B) Circulating endotoxins are bound by anti-endotoxin antibodies, hence the levels decrease inversely to the amount of endotoxin challenge, and the level of decline is a reflection of clinical severity.

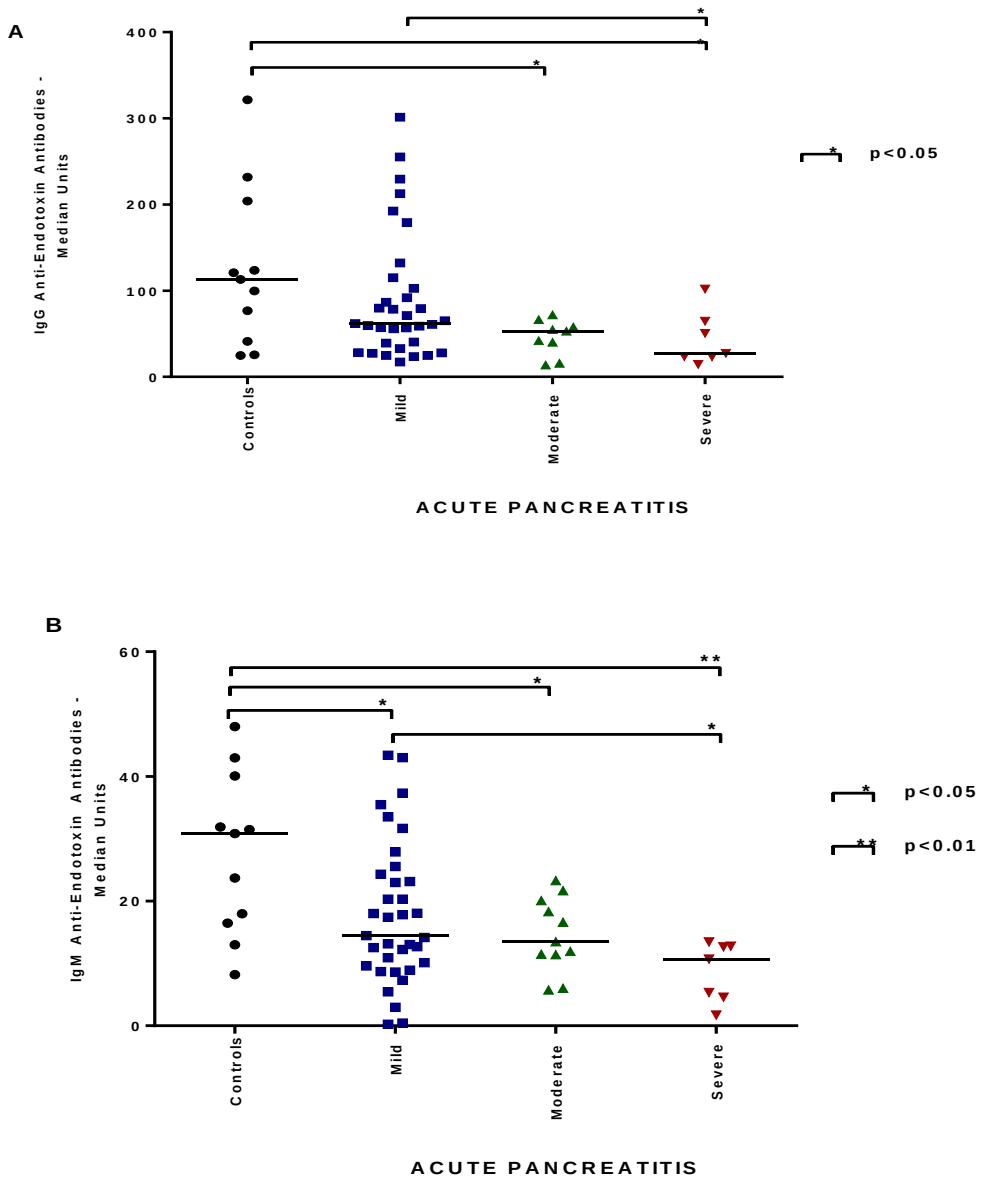


Figure 49. (A) IgG Anti-endotoxin antibodies (EndoCab IgG) were significantly lower in patients with severe AP when compared to mild AP (*p=0.046) and also in severe AP and moderate AP when compared to healthy controls (*p=0.022; *p=0.025, respectively). (B) IgM Anti-endotoxin antibodies (EndoCab IgM) were significantly lower in patients with severe AP when compared to mild AP (*p=0.036) and also in severe AP, moderate AP and mild AP when compared to healthy controls (**p=0.001; *p=0.011; & *p=0.041, respectively).

8.3.2 Direct Endotoxin Assay

The results from the Limulus amoebocyte assay were deemed unsuitable for further analysis. Of 90 randomly selected patients (24 controls; 20 AP; 25 CP and 24 CLD), only 4 discrete values were acquired: 2 from CP patients; 1 from a CLD patient and 1 from a healthy control subject. Due to interference, significant dilution was required meaning that the assay could only report values above 0.5 IU/ml with the first batch analysis or above 0.1 IU/ml with the second batch. 86 values were reported as *<0.5 or <0.1*, rendering further comparison difficult. Further testing using this assay for the full cohort was therefore not undertaken.

8.4 Blood Cultures

All patients in all cohorts were reported to have negative blood cultures from samples taken at the time of recruitment. It is however important to note that blood cultures in the patients with SAP on the intensive care unit were often performed at a late stage in their course of illness, after they had received antibiotic treatment for infective episodes.

CHAPTER 9

RESULTS VI

ANALYSIS OF IMMUNE RESPONSE BY

REVIEW OF CYTOKINE RESPONSE

9.1 Introduction

In the final stages of our study, we investigated immunological responses by means of analysing serum cytokine concentrations. We investigated variations as severity of acute pancreatitis increased, and also in the pilot study cohorts of chronic pancreatitis and chronic liver disease.

9.2 Cytokine Analysis

9.2.1 Measurement of Serum Cytokine concentrations to differentiate severity of AP patients

A comparison of the serum concentrations of various cytokines in patients with mild (n=8), moderate (n=11) and severe AP (n=35) and healthy controls is demonstrated in figure 50. IL-6 levels showed a statistically significant increase between each tier of AP disease severity (Kruskal Wallis: p=<0.0001). (Figure 51) A number of cytokines also demonstrated statistically significant increases in expression in mild AP, moderate AP and severe AP when compared with healthy controls, by Mann Whitney U tests. (Figure 52) These included IL-6, IL-12, GCSF, IL-1b, 1L-10, VEGF, IL-8, MIP-1a and FGF-Basic. A combination of (A) Immune stimulatory mediators, (B) Immunoregulatory mediators, (C) Vascular remodelling mediators, and (D) Chemotactic Mediators were identified as being up-regulated in AP.

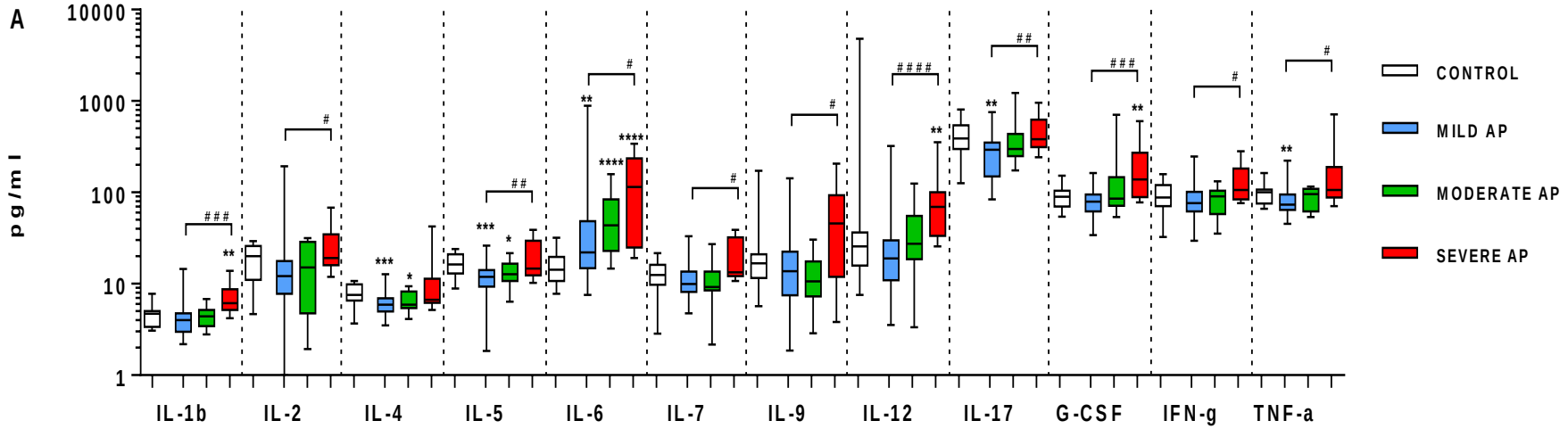
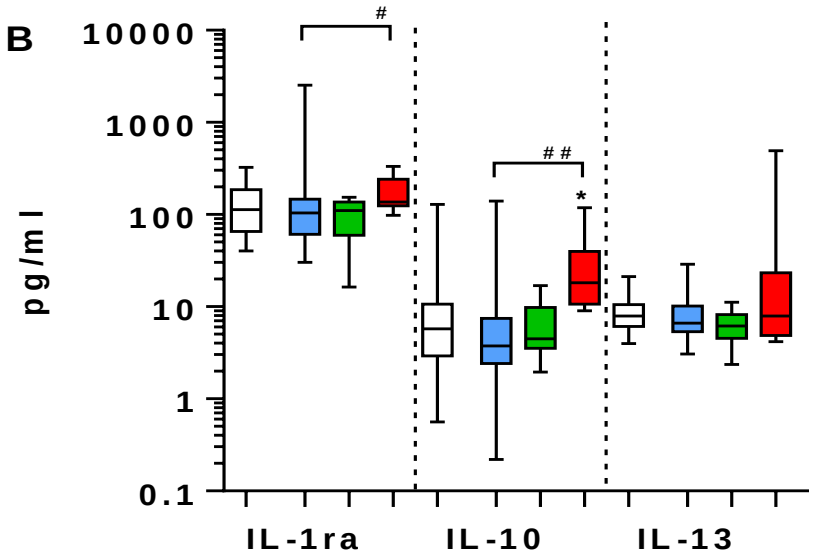


Figure 50 (A-D). Median and interquartile ranges for 24 cytokines in patients with varying severity of AP and healthy controls. Several cytokines demonstrated statistically significant increases in expression in MAP, moderate AP and SAP when compared with controls (* p=<0.05, **p<0.01, ***p<0.001, ****p,0.0001 - MW); and also in SAP when compared with MAP (# p=<0.05, ## p<0.01, ### p<0.001, #### p,0.0001 - MW). A combination of (A - above) 12 Immune stimulatory mediators, (B - below) 3 Immunoregulatory mediators, (C - below) 3 Vascular remodelling mediators, and (D - below) 6 Chemotactic Mediators were identified to be up-regulated in acute pancreatitis.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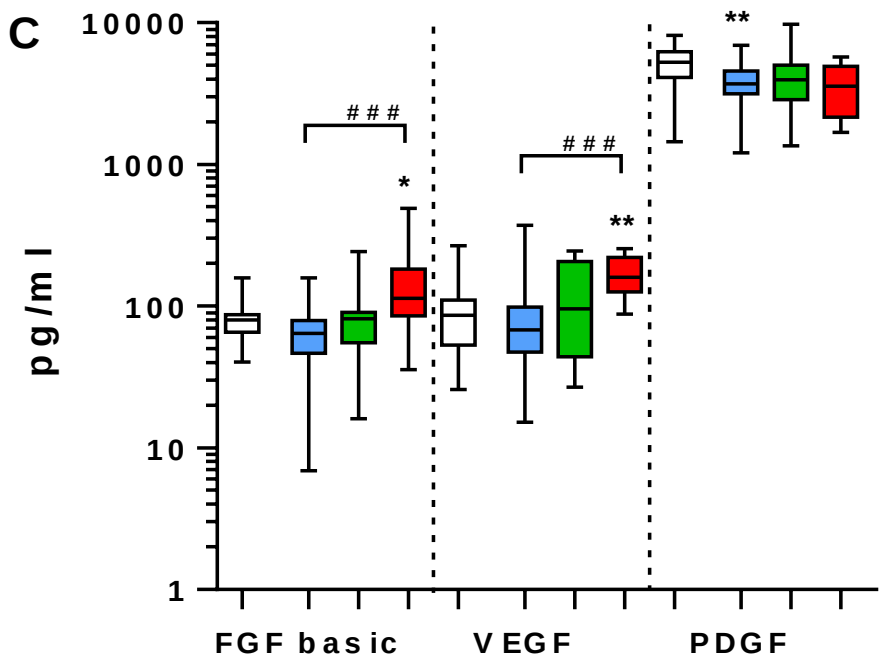
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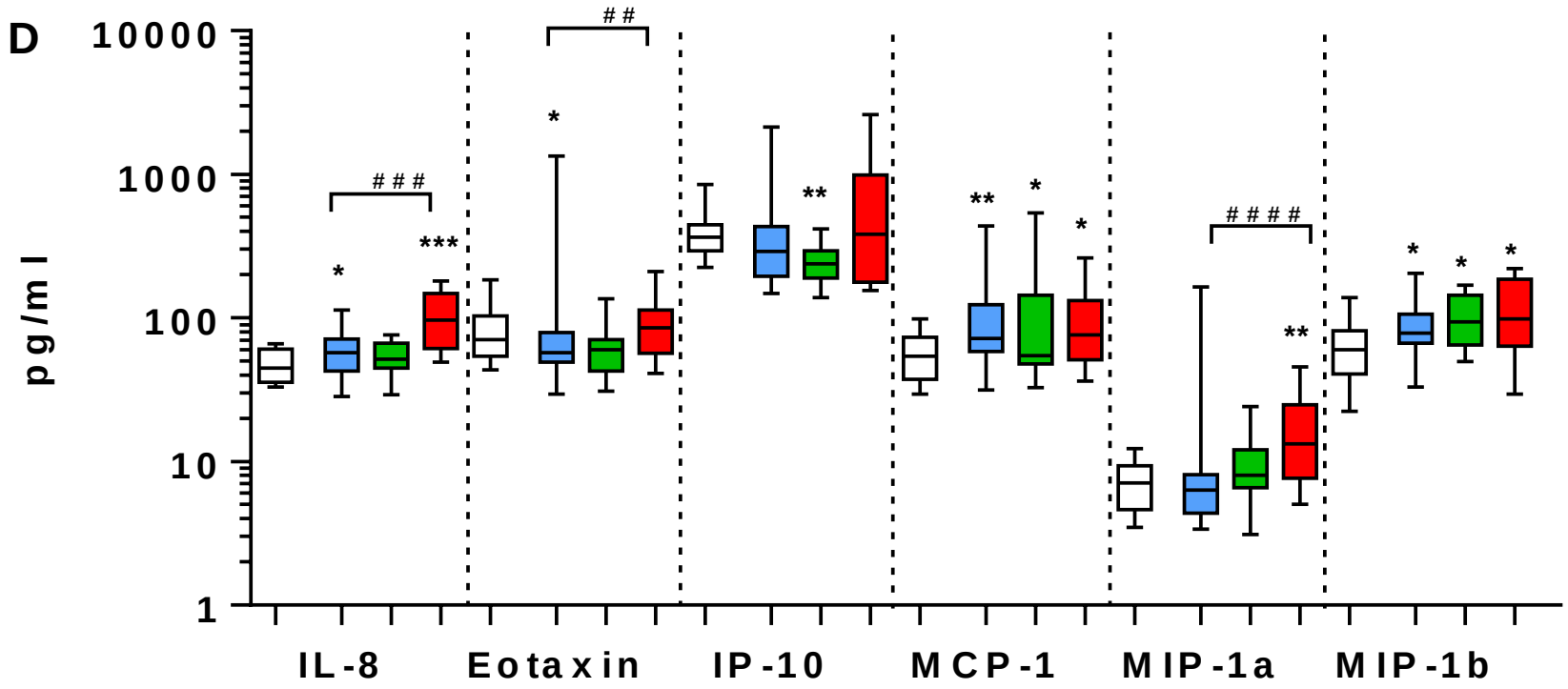
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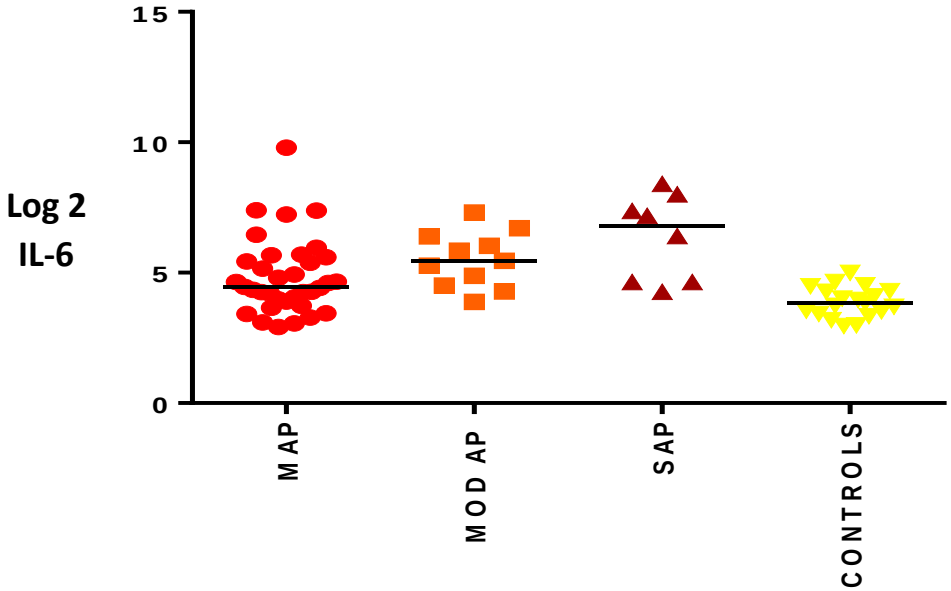


Figure 51. Log 2 IL-6 levels showed a statistically significant increase between each tier of acute pancreatitis correlating closely with disease severity and enabling differentiation between sub-groups (Kruskal Wallis: p=<0.0001).

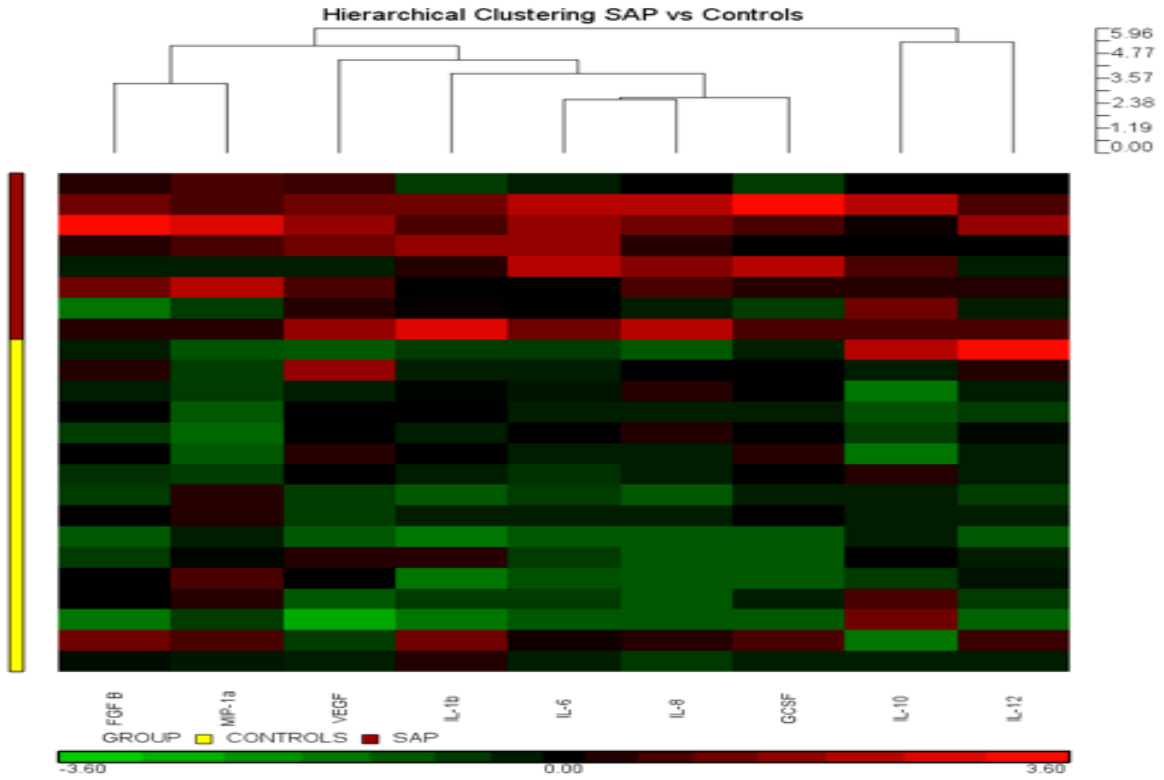
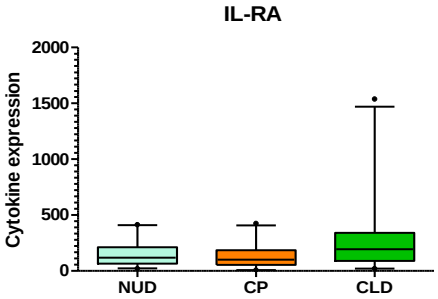


Fig. 52. Hierarchical Clustering Diagram showing 9 of 24 cytokines which demonstrated a significant increase in expression in patients with SAP when compared with healthy controls and patients with MAP – *figure produced by Brian Lane*.

9.2.2 Measurement of Serum Cytokine concentrations in CP and CLD cohorts

The same cytokines were also analysed for the patients with CLD and CP. Only one of the cytokines, IP-10, demonstrated a statistically significant increased serum concentration in CLD, by Mann Whitney test as shown in figure 54. The results from the other cytokines examined are also shown in figure 53. Although up-regulation of cytokine expression was seen in patients with CP and CLD, in many cases it was also seen in the healthy controls also and hence the differences were not found to be statistically significant.

The chemokine that did show a statistically significant rise in expression in CLD, IP-10, was further analysed to look at sub-groups of patients with liver disease as shown in figure 55. No statistically significant differences were seen between patients with CLD of varying severity and aetiology in this cohort.


IL-RA
Cytokine expression
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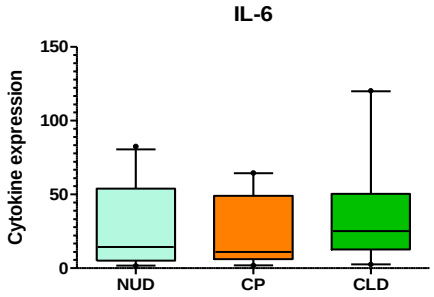
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Cytokine expression
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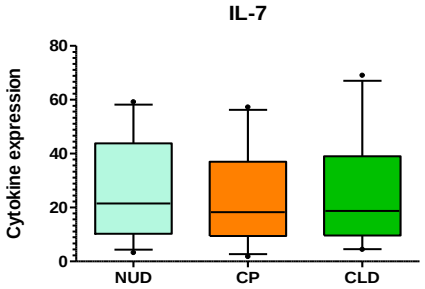
CLD


IL-7
Cytokine expression
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40
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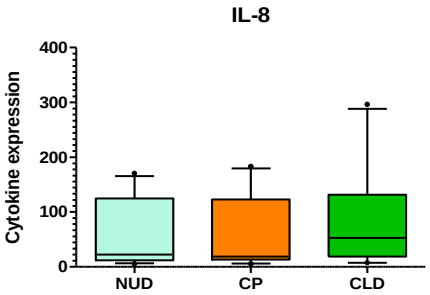
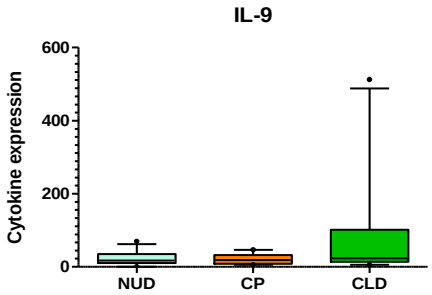
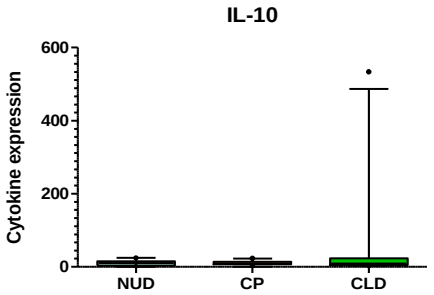
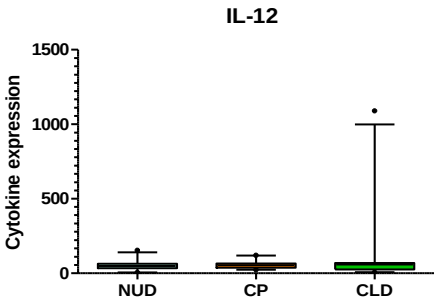
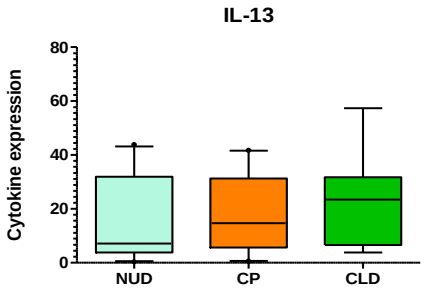
NUD
CP
CLD

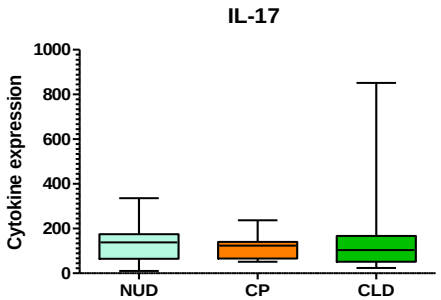

IL-8
Cytokine expression
400
300
200
100
0
NUD
CP
CLD


IL-9
Cytokine expression
600
400
200
0
NUD
CP
CLD


IL-10
Cytokine expression
600
400
200
0
NUD
CP
CLD


IL-12
Cytokine expression
1500
1000
500
0
NUD
CP
CLD


IL-13
Cytokine expression
80
60
40
20
0
NUD
CP
CLD


IL-17
Cytokine expression
1000
800
600
400
200
0
NUD
CP
CLD

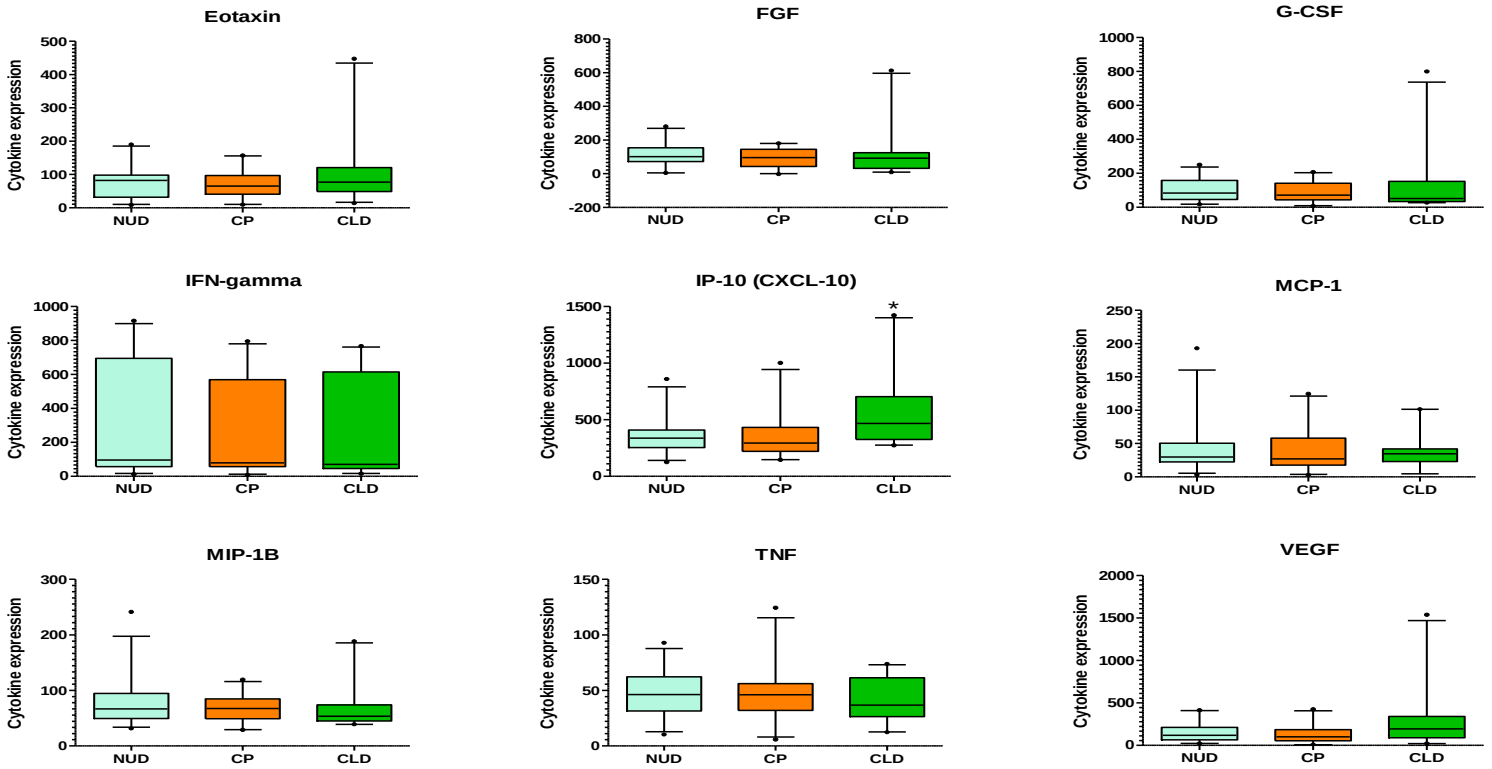


Figure 53. Box and whisker plots of cytokine expression as analysed using the LUMINEX assay, for patients with CP, CLD and non-ulcer dyspepsia (NUD). Only IP-10 in CLD showed a statistically significant up-regulation in any cohorts compared with NUD.

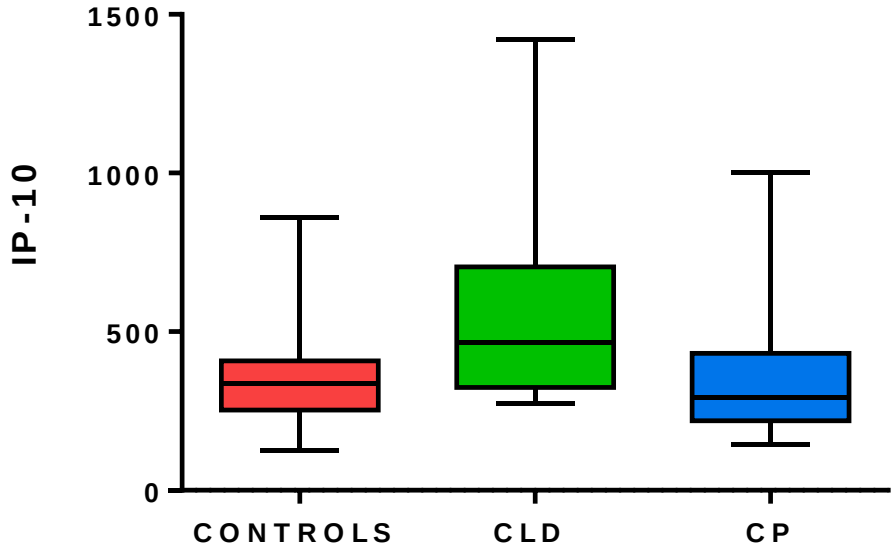


Figure 54. Mann Whitney analysis confirmed statistically significant up-regulation of IP-10 in patients with CLD when compared with the non-ulcer dyspepsia cohort (p=0.007).

Key: y axis – IP10 in pg/ml

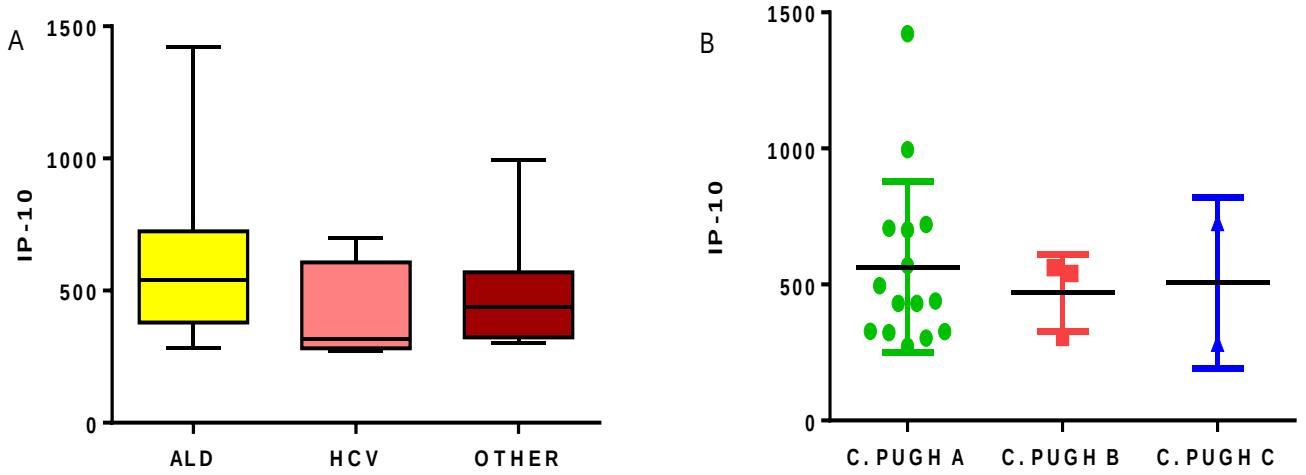


Figure 55. A sub-analysis of IP-10 expression in sub-groups of patients with liver disease was undertaken to see if expression was affected by aetiology (A) or severity (B). No statistically significant differences were found.

Key: C. Pugh = Child's Pugh Score of liver cirrhosis; y axis – IP10 in pg/ml

CHAPTER 10

DISCUSSION

10.1 Overview

Using fluorescein leakage by CLE, we demonstrated an increase in intestinal permeability in patients with acute pancreatitis, severe acute pancreatitis, chronic pancreatitis and surgically managed patients with CP, when compared with healthy controls. We were also able to demonstrate that other co-morbidities and positive gastroscopy findings and *H. pylori* status also impacted on intestinal permeability and showed statistically significant increases in fluorescein leakage, when compared with healthy controls. The discrete variable of positive or negative fluorescein leakage was found to be of greater utility during this study, than the continuous variable of 'average score'. Trends for 'average score' were similar to those described above but analyses did not reach statistical significance when this parameter was analysed. The concentration of the enzyme DAO also increased relative to the amount of fluorescein leakage in patients with acute pancreatitis. The trend was also higher in patients with AP compared with healthy controls, but this did not reach statistical significance.

The lactulose:mannitol ratio confirmed an increase in intestinal permeability in patients with acute pancreatitis and in patients with chronic pancreatitis who had been managed surgically. Also, similar to CLE, L:M ratios were also increased in those dyspeptic patients who had other co-morbidities, positive gastroscopy findings or positive *H. pylori* status. In this study, lactulose:mannitol ratios did not show statistically significant increases in patients with severe acute pancreatitis when compared with healthy controls or patients with mild and moderate AP. L:M ratios in moderate AP, were however found to be higher than in patients with mild and severe AP and healthy controls. It is important to note however, that the number of patients with severe AP was very small. These patients were also recruited at a later stage in their disease process than those with mild and moderate disease, who were mostly recruited at the time of admission. It is therefore possible that L:M ratio is maximum at the onset of disease, whereas epithelial changes may persist for longer, potentially explaining the findings of this study.

In our study of infection and microbiology, patients with acute pancreatitis, and particularly those with severe acute pancreatitis, had significantly higher microbial growth in their duodenal fluid compared with healthy controls. Organisms that were demonstrated in 'positive' duodenal cultures included Pseudomonas, Enterococcus, gut anaerobes, yeasts, Staphylococcus aureus and coliforms, whereas 'negative' duodenal cultures were reported as showing either no growth or mixed oral flora. Patients with AP had much higher proportions of positive cultures and a greater diversity of cultured organisms, including the presence of Pseudomonas and Enterococcus.

In summary, the measurement of fluorescein leakage using CLE was found to be a useful means of detecting increased intestinal permeability in patients with acute pancreatitis, chronic pancreatitis and chronic liver disease. Findings were confirmed by the lactulose:mannitol ratio and further corroborated by the results of duodenal fluid cultures. These findings support the concept that bacterial translocation is associated with increases in intestinal permeability, though our methodology does not allow us to comment on the mechanism by which this occurs. Fluorescein leakage has been identified by *in vivo* histology, but the site of leakage or 'gap' in epithelium has not been identified as may have been the case if the Watson grading system had been used.

Positive duodenal cultures were more commonly observed in the severe acute pancreatitis cohort, along with increased fluorescein leakage by means of CLE. Both these findings support the hypothesis that intestinal permeability increases as the severity of acute pancreatitis increases, along with bacterial translocation. A key aim in the management of patients who have acute pancreatitis, is the identification of the level of severity of disease at the time of presentation.

Further to the intestinal permeability changes demonstrated by CLE, we have also investigated a number of parameters that have been reported to alter with increasing severity of disease to further support our findings. For example anti-endotoxin antibodies showed a significant inverse correlation with the severity of acute pancreatitis. In terms of immunological response, we have demonstrated that the serum concentrations of a number of cytokines were also found

to be statistically significantly increased in patients with AP, with IL-6 found to enable differentiation of disease severity.

In summary, the key findings of our study are in keeping with the literature reports with relation to intestinal permeability, infection and the gut microbiome and immunology. Most significantly, we have demonstrated a rise in gut flora, IL-6, and a relative correlation with endotoxinaemia as intestinal permeability rises also with increasing severity of acute pancreatitis.

10.2 The Gut Microbiome in Acute Pancreatitis

The typical healthy person is inhabited with trillions of microbes whilst no two healthy people will share the same microbiome. Multiple factors influence the microbiome from the time in utero through birth, infancy and life, including diet, environment, the aging process, medications and stress. [284] Most of the human adult microbiota lives in the gut with the colonisation of over 1000 different species. A core gut microbiome is shared by healthy adults and plays a role in the maintenance of health status, digestion, immune system development, defense against infections, synthesis of vitamins, fat storage, angiogenesis regulation, and behaviour development. Thus, it is postulated that alterations of the human gut microbiome can play a role in disease development. Interestingly, microbial richness, intended as bacterial diversity, is usually considered an indicator of a healthy status whilst reduced bacterial diversity has been related to obesity and immune-related and inflammatory diseases. [285, 286] There is an overlapping developmental course of the gut microbiota and intestinal barrier. The intestinal barrier acts as a shield which can be modified by the gut microbiota or its metabolites.

Actual evidence of the link between microbiota imbalance and pancreatic diseases remains limited. Dysbiosis appears to be present in acute and chronic pancreatitis. As compared to healthy controls, the blood and neutrophil-associated microbiomes in the patients were significantly altered, with an expansion in Bacteroidetes and Firmicutes as well as a decrease in Actinobacteria. [287] Another study investigated alterations of the intestinal bacteria and

its associations with the inflammation in acute pancreatitis. Dramatic alterations in the predominant faecal microbiota were observed in most of both MAP and SAP patients. In addition, the rates of the multiorgan failures and infectious complications in the patients with SAP with altered intestinal microbiota were significantly higher than in those whose intestinal microbiota remained unaltered. Enterococcus increased and Bifidobacterium decreased in the patients with SAP compared to the patients with MAP. Serum IL-6 were positively correlated with Enterobacteriaceae and Enterococcus and negatively correlated with Bifidobacterium, whereas plasma endotoxin positively correlated with Enterococcus. The intestinal bacteria most frequently altered in both the patients with MAP and those with SAP significantly correlated with inflammation, which indicated that the intestinal microbiota may be involved in the progression of AP. [288] The increase in Enterococcus reported in this study, along with the findings related to IL-6 and endotoxin are in keeping with our own findings strengthening further the outcomes from our evaluation.

More recently, Li et al conducted a prospective observational case series exploring the microbial landscape in peripheral blood and neutrophils in SAP patients. They were able to identify diverse bacterial microbiomes within peripheral blood and neutrophils and discovered that the microbiome is altered in SAP patients. They also suggest that the perturbation of blood microbiota, which likely represents a disease-provoking state, might be involved in the progression of sepsis. They report that the blood microbiome is mainly composed of gut-associated organisms in SAP patients. It is therefore speculated that a bacterial consortium from the gut, rather than single or several organisms, might migrate into systemic circulation during sepsis. [289]

The symbiosis between the microbe and its host has been described to be disturbed in a multitude of diseases, especially in critically ill patients. Trauma, sepsis, systemic inflammatory response syndrome, and other conditions lead to shifts in the composition of the intestinal microbiome, which are correlated with clinical outcome. Studies have shown that critical illness though not related directly to bacterial infections, demonstrate alterations in the microbiome and the subsequent dysbiosis impacts on the pathogenesis of critical illness. The

microbiome can be manipulated therapeutically, as has been shown by the success of faecal microbiota transplantation in the treatment of refractory Clostridium difficile infection. [290, 291]

The intestinal microbiota is described as symbiotic in nature and involved in various processes, including the breakdown and absorption of nutrients, the production of vitamins and hormones, and the prevention of colonization by pathogens. The gut barrier plays a key role in the avoidance of inflammatory responses to the microbiota and is regulated by a finely tuned network of immune mechanisms for microbial recognition and tolerance. [292]

Several studies report the use of commensal bacteria and probiotics to promote intestinal barrier integrity in vivo [293] although some studies have been negative or inconclusive. Many studies report enhancement of the intestinal barrier in vitro or protection from barrier disruption by probiotics. For example, the probiotic E.coli Nissle 1917 has been shown to prevent barrier disruption caused by infection with an enteropathogenic E. coli strain. [294] Metabolites secreted by Bifidobacterium infantis Y1, one of the components of the probiotic product VSL#3, leads to an increase in expression of ZO-1 and occludin while reducing expression of claudin-2 leading to enhanced effects on transepithelial resistance and altered ion secretion. [295] Probiotics have been shown to have beneficial effects in patients with IBD where E.coli Nissle 1917 is used to support maintenance of remission in patients with UC and VSL#3 reduces and protects against pouchitis in patients with UC. [296] [297] Recent reports have also demonstrated the value of probiotics for treatment in IBS and in fatty liver disease. Some of the probiotic trials in IBS demonstrated that the effects are correlated with an improvement of the intestinal permeability. An example is short-term active lactic acid bacteria treatment for diarrhoea-predominant IBS improved mucosal barrier function. [298]

Since the intestinal permeability barrier has been shown to be compromised in critically unwell patients, we could assume that probiotic use would be of benefit in those patients who are generally treated on the intensive care setting. The classical treatment of loss of barrier function in the ICU patient is usage of antibiotics directed against gram-negative bacteria and improvement of intestinal perfusion by catecholamines and volume. Some studies suggested

a beneficial effect of selected probiotics and synbiotics on sepsis complications in patients with major abdominal surgery, but trials are few and patient numbers small to be able to extract a meaningful outcome of probiotic use in these patients. [299] Early evidence from animal studies and a small placebo-controlled trial suggested that probiotic prophylaxis stabilised intestinal barrier dysfunction, minimised bacterial translocation and reduced infectious complications. [181] [182] A major randomised placebo-controlled trial of probiotic prophylaxis in severe acute pancreatitis however contradicted these findings and demonstrated a significant increase in mortality associated with probiotic administration (due to jejunal necrosis), and showed no reduction in infection rates. [35].

Enteral feeding on the other hand is thought to be effective in these patients as described earlier in this thesis.

We have shown that CLE can be used to demonstrate increased intestinal permeability as AP severity increases, and that duodenal bacterial growth is also increased. CLE has enabled non-invasive assessment of the cellular and molecular properties of tissues in real time, resulting in the concept of optical biopsy. [300] It is rapidly evolving and continues to be developed for new applications having been used in early studies in inflammatory bowel disease and Barrett's oesophagus. [1, 301] More recent reports demonstrate its usefulness in the identification of pancreatobiliary pathology and GI malignancies. [302] [303, 304] Recent literature has shown that the intestinal permeability barrier is being identified as a significant aspect of many disease processes and hence a potential therapeutic target, not just in acute pancreatitis. With this in mind, the method of direct visualisation by confocal laser endomicroscopy and the identification of leakage or even mucosal barrier defects has been investigated and methodology improved. [305] [306] An interesting study conducted in Lusaka, Zambia evaluates the use of CLE to evaluate intestinal permeability to investigate patients with Environmental Enteropathy. [306] They comment that CLE is the only technique that can visualise epithelial cell shedding and potentially sites of barrier loss in patients during endoscopy in real time, enabling an understanding of the severity of epithelial disruption. The authors propose that it is plausible that initial minor defects in the barrier lead to the ingress

of bacteria increasing tissue concentrations of TNF and increasing pathology in a positive feedback loop. They report that they have shown a significant epithelial barrier defect in Environmental Enteropathy *in vivo*, which provides a plausible pathway for the bacterial translocation observed in many populations.

10.3 A brief overview of immunological aspects in Acute Pancreatitis

Cytokine concentrations have been demonstrated to be an accurate and objective method for the assessment of the severity of acute pancreatitis since they are involved in both the local and the systemic inflammatory responses in acute pancreatitis. The inflammatory process results in excessive leukocyte activation and increased migration of neutrophils to the inflamed area, with a consequent release of pro-inflammatory mediators including interleukins which influence progression to necrosis and SIRS (IL-1b, IL-6, IL-8, IL-10, IL-18 and TNF-α). [202] Multiple studies detailed previously in this thesis have identified IL-6 to be a key mediator in the inflammatory response in AP. It is an excellent marker of the severity of acute pancreatitis with peak concentrations. TNF- α is also among one of the inflammatory cytokines that is produced once necrosis develops. Current research is working towards TNF as a potential target for therapy in AP since blocking the production and the effects of this cytokines is a strategy that makes sense in its treatment. A trial of infliximab therapy in AP patients is currently close to initiation and will provide some very interesting results. One case report however reports a case of AP induced infliximab, a monoclonal anti-TNF-α antibody. [208] Both pro-inflammatory and anti-inflammatory cytokines are released during the disease process. Pro-inflammatory mediators in acute pancreatitis include TNF-α, IL-1, IL-2, IL-6, and IL-18, and IL-8, platelet-activating factor, and reactive-oxygen and reactive-nitrogen species. With the release of these pro-inflammatory mediators, anti-inflammatory cytokines are concomitantly produced, leading to a compensatory response syndrome. Cytokines are involved in all aspects of the cascade leading to systemic inflammatory response syndrome and multiple organ dysfunction syndrome and indeed also in the release of mediators that are likely to exacerbate alterations in intestinal permeability. A recent study reported cytokine

responses in AP on day 3 from onset at a tertiary centre. Genotyping was done to correlate IL-6, TNF-α and MCP-1 gene polymorphisms with cytokine levels. Serum IL-6 added significantly to the predictive value of SIRS for severe AP. [307] In a similar study, significant differences were found between patients with severe AP and those with mild or moderately severe AP in IFN-γ, IL6, TNF-α, GM-CSF, IL4, IL1b, and IL13 concentrations. Interferon-γ, IL6, and TNF-α were associated with severe AP, whereas GM-CSF, IL4, IL1b, and IL13 were associated with mild or moderately severe AP. The IL13/IFNγ ratio was significantly higher in patients with mild AP. The authors concluded that a TH1 profile was associated with severe AP and a TH2 profile with mild or moderately severe AP. [213] Infection with microbes results in upregulated expression of immunoregulatory genes in epithelial cells with increased production of pro-inflammatory cytokines or inadequate synthesis of anti-inflammatory cytokines. [5] [27]

10.4 Development of a pilot study facilitated a complex protocol to be optimised prior to the recruitment of acutely unwell patients with acute pancreatitis

This thesis aimed to identify the role of confocal endomicroscopy as a tool to measure intestinal permeability alterations in patients with acute pancreatitis, and to assess whether this method could differentiate between levels of disease severity. The severity of acute pancreatitis is a key factor in determining prognosis and the severe forms of the disease are associated with a mortality of 20-30%. [90] Identification of those patients who have severe disease is central to optimisation of treatment, but remains a controversial clinical assessment. The development of novel clinical tools to assess disease severity early during the course of illness is therefore of high clinical importance.

As previously discussed, the nature of this translational study was complex. The different components required significant patient involvement and cooperation. Patients with acute pancreatitis are often acutely unwell in-patients albeit with a broad spectrum of clinical severity. It was thus important to establish a practical and robust protocol prior to embarking

upon the recruitment of this patient cohort. With this in mind, we conducted a pilot study involving groups of patients who are also reported to have intestinal permeability disturbances, but who have more chronic stable courses of disease. We therefore decided to investigate patients with stable, compensated chronic liver disease or cirrhosis and patients with chronic pancreatitis, as well as control subjects.

The study was conducted at the Royal Liverpool University Hospital. This centre had large patient cohorts in all study groups as well as the clinical, endoscopic and scientific equipment and expertise required.

Training in the skill of confocal endomicroscopy was initially acquired from Professor Kiesslich in Mainz. Meetings were also held with Professor Watson in order to learn how to assess the 'Watson score' [1] While this formed the initial structure of our imaging process, the method was subsequently amended and simplified in order to minimise inter-observer variability. Watson's group had previously reported the identification of plumes of fluorescein leakage from identifiable sites in the epithelium and developed the concept of 'epithelial gaps', which allow the entry of bacteria into the lumen by a combination of transcellular and paracellular pathways. [4, 21, 22] Our modified scoring system simply assessed for the presence of fluorescein leakage rather than assessing the individual sites from which this leakage occurred.

Following completion of the pilot study we developed a revised, optimised protocol for use in the acutely unwell AP cohort. The pilot study also served its second purpose of generating important results from patients who had chronic pancreatitis and chronic liver disease.

10.5 Protocol implemented in patients with acute pancreatitis

Following completion of the pilot study and development of a final protocol, ethical approval was granted to recruit patients who had acute pancreatitis. Approximately 120 patients per annum are admitted to the Royal Liverpool University Hospital with acute pancreatitis. All such patients were identified daily by means of an alert through the clinical biochemistry laboratory for patients who had elevated serum amylase activity. Such patients were

approached for recruitment to the NIHR PBRU biobank and to this current study. The number of patients in the mild AP cohort was significantly greater than in the moderate and severe AP cohorts. Moreover, the severe AP patients were mostly recruited at a later stage in their disease course than those with mild and moderate disease, who were mostly recruited at the time of admission.

10.6 Intestinal permeability alterations were observed in patients with AP, CP, CLD and patients with a history of gastrointestinal surgery

The intestinal permeability barrier becomes defective in AP 48-72 hours after disease onset and further increases gradually during the course of disease. [101, 144, 180, 200] Increased severity of pancreatitis appears to correlate with an increase in intestinal permeability and in turn, this results in increased bacterial and endotoxin translocation. Evidence for the association between gut injury and subsequent development of local infection, infected necrosis, multi-organ failure, mortality and outcome continues to increase. [107] Early gut mucosal dysfunction appears to contribute to the pathophysiology of severe AP by permitting the entry of endotoxin and bacteria from the intestinal lumen through the intestinal epithelium into the systemic circulation as IP increases. [104] [199]

In CP, reports of changes in intestinal permeability are limited, but there is extensive evidence to suggest that increased IP affects the pathogenesis of chronic liver disease, cirrhosis and its complications. In this condition, dysfunction of the intestinal epithelial barrier may again enhance the risk of translocation of bacteria and bacterial products into the systemic circulation. [244] A number of studies (as described in chapter 1) have also reported alterations in intestinal permeability in patients who have undergone gastrointestinal and even cardiac surgery.

Using the detection of fluorescein leakage by CLE, we have demonstrated an increase in intestinal permeability in patients who had acute pancreatitis, severe acute pancreatitis, chronic pancreatitis, conservatively or surgically managed, when compared with healthy controls. We were also able to demonstrate that other co-morbidities, positive gastroscopy

findings and positive *H. pylori* status also affected intestinal permeability and showed statistically significant increases in fluorescein leakage in such patients relative to healthy control subjects.

The serum concentration of the enzyme DAO increased relative to the amount of fluorescein leakage in patients with acute pancreatitis. There was also a trend towards higher serum DAO concentrations in patients with AP compared with healthy controls, but this difference did not reach statistical significance.

Lactulose:mannitol ratios also confirmed an increase in intestinal permeability in patients with acute pancreatitis, as well as in patients with chronic pancreatitis which had been managed surgically. The lactulose:mannitol ratio was also increased in control patients who had other co-morbidities, positive gastroscopy findings or positive *H. pylori* status. It is probable that the lactulose:mannitol ratio peaks at the onset of disease and may subsequently decrease, whereas epithelial changes are likely to persist for longer, hence fluorescein leakage may still be seen in patients at a later stage in their disease course, as reflected in the results from fluorescin leakage.

10.7 Confocal Laser Endomicroscopy was used to investigate intestinal permeability alongside a commonly reported measure of the same variable

Our study confirmed that patients with AP have increased IP compared to healthy controls. We however used serum lactulose:mannitol ratios as described by Fleming, instead of the more frequently reported urinary test. [144] [145] [104] [146] [19]. These biochemical findings were supported by the new technique under investigation in our study, CLE. By means of CLE, we demonstrated that fluorescein leakage, the marker of IP for this method, was also significantly higher in AP patients compared to healthy controls and that this was particularly apparent in patients who had severe AP. Reports of CLE as a measure of IP, though limited, support our findings. [4, 274, 275] We took great care to use a robust method to assess fluorescein leakage to reduce the possibility of selection bias. The selection and scoring processes were conducted separately and involved blinded co-investigators who were

unaware of patients' clinical details and comorbidities. A unique and unidentifiable identification number was allocated to each subject and only this was provided at the stage of image selection and scoring. Cohen's kappa coefficient, which was used to assess the level of concordance between scorers, showed significant agreement.

Increased IP was also observed in surgically managed CP patients by both lactulose:mannitol testing and CLE. However, conservatively managed CP patients were not significantly different from healthy controls using either technique. In fact, there was a significant difference in lactulose:mannitol ratios between the surgically and conservatively managed CP cohorts, suggesting that the gut barrier may be compromised in CP patients who have undergone surgical intervention.

In all the above cases, fluorescein leakage as a discrete variable gave similar results to intestinal permeability assessed by lactulose:mannitol ratios. However, when fluorescein leakage was assessed by means of average scores and as a continuous variable, although the patterns were similar, statistical analysis did not demonstrate significant differences.

The cohort of patients with CLD also demonstrated increases in lactulose:mannitol ratios and fluorescein leakage, but these changes were not statistically significant compared to healthy controls. A future study involving larger numbers of patients would therefore be required to assess this disease state more effectively.

As a further sub-analysis, we analysed the patients who had been recruited as potential controls but who were eventually excluded from the study for one or more reasons. We identified that there were increases in intestinal permeability in those patients who had been excluded from the study compared to healthy controls. This was confirmed by all three modalities measured: lactulose mannitol ratio; fluorescein leakage by CLE and average scores of fluorescein leakage by CLE. Exclusion of patients from the 'healthy control' group was based on abnormal gastroscopy findings including the presence of oesophagitis or gastritis; positive *H. pylori* status and/or a history of co-morbidities including a history of previous surgery. On closer review of past medical histories, it was interesting to note that a number of subjects who had

evidence of increased intestinal permeability had a history of previous surgery such as cholecystectomy or duodenal ulcer surgery.

We also measured serum diamine oxidase (DAO) concentrations in control subjects and in those patients with AP who underwent CLE, and demonstrated increased serum DAO concentrations in those patients who showed fluorescein leakage. DAO is an enzyme that catalyses the oxidation of diamines, and in humans and rodents it is specifically located at the apical end of mature intestinal villi. Several studies have demonstrated an inverse correlation between DAO and small intestinal permeability, as its activity reflects the integrity and maturity of the small intestinal mucosa. [308] We propose that its increased concentration in this study may be related to the increased intestinal permeability that was observed in the AP patient cohort, though we have not demonstrated that this was a result of cell shedding.

10.8 Bacterial translocation increases with increased intestinal permeability

AP patients and also subjects who were identified as showing fluorescein leakage by CLE demonstrated significantly higher frequencies of positive bacterial/fungal cultures in duodenal fluid relative to healthy controls. Organisms that were demonstrated in 'positive' duodenal cultures included Pseudomonas, Enterococcus, gut anaerobes, yeasts, Staphylococcus aureus and coliforms, whereas 'negative' duodenal cultures were reported as showing either no growth or mixed oral flora. Positive duodenal cultures were most prevalent in patients who had severe AP, consistent with previous reports that have suggested a correlation between severity of disease, endotoxaemia and bacterial translocation. We found that patients with severe AP had the highest levels of endotoxaemia, along with the most positive duodenal bacterial cultures. The observation of positive duodenal cultures in those patients who showed fluorescein leakage at CLE supports a hypothesis of bacterial translocation, suggesting that alteration of the gastrointestinal barrier occurs in AP, though the mechanism remains unclear. The identification of fluorescein leakage by CLE was also associated with the presence of positive bacterial cultures from the duodenal fluid in patients with CLD. The small proportion of patients with CLD and CP who had positive duodenal fluid cultures showed the presence

of Staphylococcus aureus, coliforms or yeast. Patients with AP had much higher proportions of positive cultures and a greater diversity of cultured organisms. Positive cultures in this cohort included the above but also Pseudomonas and Enterococcus.

In all our subjects, blood cultures were negative at the time of recruitment, suggesting an absence of bacteraemia. However, it is important to note that in the patients who had mild AP, we would not expect positive blood cultures, and in the moderate and severe cases, recruitment often occurred later in the disease course and after antibiotic treatment had been initiated.

Despite the absence of bacteraemia, endotoxaemia was evident, in particular in those patients who had severe AP. Endotoxaemia without bacteraemia is well-recognised and in severe AP it may result from local sepsis, or pancreatic and intraabdominal necrosis leading to the absorption of gut derived endotoxin. [194] Endotoxaemia is common in patients who have sepsis (79%) and liver cirrhosis (46%), and correlates with mortality in patients who have severe AP. [309] Circulating endotoxins are bound by anti-endotoxin antibodies. Hence the levels of these antibodies decrease inversely to the amount of endotoxin challenge and the level of decline is a reflection of clinical severity.

10.9 The effect of surgery, and other factors, on intestinal permeability, in particular in CP

It was apparent from the sub-analyses that were performed that multiple factors contribute to intestinal permeability changes. In patients with CP, it is not clear whether the increased IP that is observed occurs due to the combined effect of surgery and CP or due to the effect of surgery alone. IP changes have not previously been extensively investigated and reported in CP; similarly, there are very few published reports of IP changes in post-operative patients. One study which investigated bacterial translocation and IP in patients following abdominal surgery, concluded that increased IP was closely related to bacterial translocation, but this observation was made only a few hours after abdominal surgery. [252] In our cohort, surgical intervention occurred months or years prior to recruitment, rendering the relative contributions of CP and previous surgery to increased IP unclear.

The sub-analysis involving patients who were excluded from the healthy control cohort further supports the concept that surgery and other factors that affect the gastrointestinal tract affect intestinal permeability. A combined group of dyspeptic subjects who had co-morbidities including previous surgery; positive *H. pylori* status and/or abnormal findings on gastroscopy showed a statistically significant increase in IP compared to 'healthy controls'.

10.10 Differentiating severity in acute pancreatitis

A comparison of serum cytokine concentrations between patients who had different severities of AP demonstrated statistically significant increases in the concentrations of multiple cytokines in patients with severe AP when compared with healthy controls. In the subset of pro-inflammatory cytokines, we found significantly higher serum concentrations of IL-6 in particular, but also IL-8, and IL-10 in patients who had severe AP. [144] Serum IL-6 concentrations showed a statistically significant increase between each tier of severity of AP, suggesting that further investigation of this cytokine is warranted to explore its suitability as a biomarker of severity in patients with AP.

It has been postulated that endotoxaemia is a key component of a positive feedback loop that is associated with cytokine release and increased IP, impaired host immunity and enhanced bacterial translocation. It is also possible that the inflammatory response and cytokine release are key to the subsequent release of endotoxin and that these factors initiate an inflammatory cascade which results in intestinal epithelial cell shedding, epithelial gaps and bacterial translocation. [7]

The titres of IgG and IgM anti-endotoxin antibodies were significantly lower in patients who had severe AP when compared to patients with mild AP and controls. This is consistent with increased endotoxaemia in patients who had increased severity of disease. These results are similar to those previously reported. [7, 99, 101, 104, 140, 144, 180]

The histopathological analysis of duodenal tissue was incomplete and so we cannot reach any meaningful conclusions from these data. Nonetheless, there was evidence of duodenal

inflammation in two patients who had AP. It would be interesting to investigate this further to assess whether duodenal inflammation increases with increasing severity of AP.

10.11 A significant increase in the serum concentration of IP-10 was seen in the CLD cohort

The chemokine IP-10, also called IFN-γ induced protein or CXCL-10 is secreted in response to IFN-γ. The only report of note of its mention in liver disease is in hepatitis C viral infection, where it has been shown to play a role in the assessment of treatment response. [310] Although we analysed whether this chemokine was associated with a specific aetiology or severity of chronic liver disease, no associations were identified.

10.12 Limitations of the study

The main limitation of this study was the difficulty that we had in recruiting patients with severe AP immediately after the onset of their disease. Many of these patients were tertiary referrals and were therefore recruited sometime after their initial presentation. Other patients who had more severe forms of the disease were unable to consent to participating in the study due to a lack of consciousness or capacity. This particularly limited the numbers of patients with severe acute pancreatitis, which was the cohort of most interest.

The study of patients with CLD and CP was primarily undertaken with the aim of establishing a protocol by studying stable patient cohorts. More valuable results could have been generated by recruiting larger numbers of patients with compensated and decompensated disease in the former group, and patients with more recent surgery in the latter group. The time taken to establish a protocol and then re-submit an ethics proposal for the AP cohort also reduced the time available to actually recruit this group of patients.

The scoring system that we formulated and utilised reduced inter-observer variability, but limited our ability to identify the point of release of fluorescein and hence the ability to identify epithelial gaps. Further work to establish an improved scoring system that assesses this parameter but still maintains good inter-observer agreement level, would be very useful.

Similarly, we have established that there was a significant change in duodenal microbial growth in patients with AP, but we have been unable to demonstrate how this has occurred. Analysis of the microbiome rather than culture would also have provided more informative results and remain a potential consideration for investigation.

An analysis of cytokine responses at different stages in the disease process along with IP analysis in a similar way would render meaningful data but was not feasible during the course of this study. It was also not possible to randomise patients treated with enteral feeding against those without and to assess reciprocal changes in intestinal permeability.

10.13 Future Directions

This study has provided an excellent platform for further studies using CLE as a modality to assess intestinal permeability using a direct *in vivo* technique.

Further studies involving larger cohorts of patients would be of great benefit to assess the clinical implications of assessing IP by CLE in patients with AP, but also in CP and particularly in CLD of varying severity. In patients who had chronic pancreatitis, we have found an interesting connection between increased IP and a history of previous surgery. It would therefore be interesting to directly study subjects with CP prior to and following surgical intervention to be able to objectively assess intestinal permeability changes post-operatively more closely.

With respect to patients with acute pancreatitis, it would be useful to increase the number of patients studied in the moderate and severe AP cohorts, particularly as the numbers in these groups were limited due to the reasons outlined earlier, and to assess IP at different timepoints.

Much further research is needed to improve the treatment of acute pancreatitis which is sometimes a life-threatening condition. Improved understanding of the mechanisms responsible for alterations in intestinal permeability and bacterial and endotoxin translocation would permit further research into potential therapeutic targets. Labelled fluorescein with closer investigation of paracellular and transcellular pathways similar to investigations in mouse models, could be a potential means of studying these variables.

This study has therefore highlighted that intestinal permeability changes and alterations in duodenal bacterial growth are key features of acute pancreatitis. It would also be very useful to enhance the work on the gut microbiome by assessing stool for intestinal pathogens and blood also as comparative factors to the duodenal cultures for a broader analysis of the microbiome.

The actual mechanisms involved in gut barrier dysfunction, intestinal permeability changes and bacterial translocation have not been investigated in this study and are crucial to understand to be able to develop therapies and targeted treatments.

10.14 Conclusions

We have shown that patients with AP had evidence of increased IP by two methods, namely fluorescein leakage at CLE and lactulose:mannitol tests. They were also found to have increased concentrations of serum DAO. Endotoxaemia, positive duodenal bacterial cultures, and increased serum concentrations of several cytokines were associated with increasing severity of AP.

The increase in positive duodenal bacterial cultures in patients who also demonstrated fluorescein leakage at CLE supports a hypothesis of bacterial translocation, suggesting that the gastrointestinal barrier is altered in AP. We were unable to confirm the mechanism for bacterial translocation, hence further work is required to establish whether this occurs through epithelial gaps. Evidence for increased IP was also observed in surgically managed CP patients, suggesting that the gut barrier may also be compromised in some CP patients.

CLE is therefore a novel technique that may have a role in assessing gut barrier function in patients with acute and chronic pancreatitis and also subjects who have chronic liver disease. Further studies should probably concentrate on investigating the mechanisms involved to potentially allow the development of targeted therapies in the future. Further work is also needed to develop clinically relevant scoring tools to assess intestinal permeability, as our work suggests that alteration in the gut barrier plays a major role in exacerbating the clinical manifestations of acute pancreatitis.

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APPENDICES

Appendix A: Patient Invitation Letter - Pilot Study

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

UNIVERSITY OF LIVERPOOL

PATIENT INVITATION LETTER

Patient details

Prescot Street
Liverpool
L7 8XP

Tel no: 0151 706 2000
Ext: 4487 / 4877
Mobile: 07931 246347

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH CHRONIC PANCREATITIS AND CHRONIC LIVER DISEASE

DEAR SIR / MADAM,

You have been invited to attend **the gastroenterology department on**_____ for an upper gastrointestinal endoscopy or endoscopic ultrasound as part of a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. To allow for this, I have arranged to meet you before the procedure so that I can discuss details with you fully and give you an information leaflet that details the study; the reasons for the study and what involvement would be required from you.

Please report to the main desk in the foyer at the main entrance of the ground floor of the Royal Liverpool University Hospital with this letter on _____ at _____.

I shall come to see you on the day of your procedure to check that you have had a chance to read the information and to discuss everything with you again in person, in more detail. If there is anything that is not clear, or if you would like more information, please ask at this time. If you have any questions beforehand, please do not hesitate to contact myself on the telephone number at the top of this letter. We appreciate your time and thank you for reading the attached information. This is a voluntary project and, if when you have heard about the study, you would prefer not to take part; your decision will be accepted without question and will not affect the standard of care you receive.

Many Thanks,
Dr S Bharucha, Clinical Research Fellow

Appendix B: Patient Information Leaflet - Pilot Study

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH CHRONIC PANCREATITIS AND CHRONIC LIVER 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. If there is anything that is not clear, or if you would like more information, please ask.

We appreciate your time and thank you for reading this information leaflet.

This is a voluntary project and if, when you have heard about the study, you would prefer not to take part your decision will be accepted without question and will not affect the standard of care you receive.

What is the purpose of the study?

Pancreatic diseases develop in >50,000 people per year in the UK, causing major illness and even death**.** Chronic pancreatitis (CP) is a disease that occurs over a period of time and results in permanent structural changes in the pancreas. This can result in hormonal problems such as diabetes and other significant problems with the digestive system that cause symptoms of pain, weight loss and an unusual form of diarrhoea called steatorrhoea. How chronic pancreatitis occurs has not been completely understood. It is a painful, wasting disease that has a greatly increased risk of pancreatic cancer. The risk of pancreatic cancer developing in people with chronic pancreatitis is over ten times that of normal. The diagnosis of chronic pancreatitis is challenging and prevention and effective treatment are difficult to achieve.

Liver cirrhosis is a consequence of chronic liver disease characterized by replacement of liver tissue by fibrosis, scar tissue and regenerative nodules (lumps that occur as a result of a process in which damaged tissue is regenerated), leading to loss of liver function. Cirrhosis is most commonly caused by alcoholism, hepatitis B and C, and fatty liver disease, but has many other possible causes. Some cases are idiopathic, i.e., of unknown cause. There are an estimated 30,000 people living with cirrhosis in the UK and at least 7000 new cases being diagnosed each year. The disease is recognised to have significant complications which are life-threatening and mortality rates are high.

New ways of identifying the earliest stages of inflammation are needed, as are new ways of identifying how well the body is responding to this and healing. Confocal endoscopy offers a means to identify inflammation at the earliest stage, and to predict what will happen more correctly. This work will be a thorough test of new methods of diagnosis, to find new ways to improve the care of patients with pancreatic and liver disease. The use of confocal endomicroscopy will be assessed to identify inflammation and its effects at an earlier stage, to prevent it or to treat, and to monitor the effects of treatment.

The ultimate goal of this study is to study the use of confocal microscopy to assess and identify inflammation and its effects in patients with chronic pancreatitis and chronic liver disease, to prevent it or to treat, and to monitor the effects of treatment.

We aim to recruit about 100 patients with chronic pancreatitis or liver cirrhosis or neither (control subjects) and investigate them with confocal endomicroscopy (a camera with an end microscope inserted into the stomach and duodenum or bowel). Patients will be identified and recruited for the study from the Gastroenterology unit at the Royal Liverpool University Hospital.

Why have I been chosen?

You have been chosen because you have been diagnosed with a disease of the digestive tract and we aim to study factors involved in the severity of these disease processes.

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. You are still free to withdraw from the study at any time without giving a reason. This will not affect the standard of care you receive in any way.

What will happen to me if I take part?

All patients who decide to take part in this study will receive the usual clinical care. If you decide to take part, the following will also take place:

a) A blood sample (approximately 20 mls) will be taken to try and identify genes and markers for diagnosis and assessment of severity.

b) A camera test with a confocal endomicroscope (a camera with an end microscope) will be carried out and tissue samples and fluid will be removed from the small or large bowel. Multiple images will be taken with the camera and the confocal endomicroscope. Suitable, safe chemicals will be developed to obtain fluorescence in relevant parts of the body. The test will be carried out in the Gastro Unit at the Royal Liverpool University Hospital.

- In most cases a gastroscopy will be carried out with the confocal endomicroscope. You will be asked to fast before attending for the test. You will be given two lots of sedative agents and throat spray to make sure you are comfortable throughout the test. A thin tube will be passed through the back of the throat, into the gullet, the stomach and the first part of the small bowel. The test will take about twenty minutes to complete.
- In some patients, we may conduct a colonoscopy which is an examination of the large bowel and a tube is passed through the back passage until the small bowel is reached. The same sedation would be given. This test may take approximately thirty to forty minutes to complete.

c) The tissue samples that are taken at endoscopy will be utilised for processing in our research. The samples used for research purposes will be those that are surplus to that which is required for clinical purposes.

d) The images that are taken at endoscopy will be analysed for our research.

e) We will also ask you for a few questions about your general health and that of your family in relation to digestive diseases, in the form of a questionnaire.

f) A review of you case-notes will be carried out by the research doctor.

g) On another occasion, we will ask you to drink a sugary liquid which will be a solution of sugar and a diuretic. This will be made up into approximately 100ml of liquid. After ninety minutes, we will take a very small amount of blood from you before you are discharged. This test will look at the absorption of sugar in your body.

IMPORTANT NOTE related to point *g)*:

Not all patients will be asked to participate in part ***g)*** detailed above. The first 5-10 patients in the study (at least) will not be asked to take part in this section of the study. Thereafter, patients will be given the option to take part in ***g)*** or to refuse, and to only participate in the rest of the study.

What are the possible disadvantages and risks of taking part?

There may be some minor but short-lasting discomfort from having a blood test. The camera test comes with a 1 in 1000 risk of causing a small hole in your digestive tract (perforation) or

of bleeding (haemorrhage). The procedure will be carried out by a specialist with extensive experience to minimise these risks. Taking part in the study will not affect your current treatment.

What are the possible benefits of taking part?

The study will not be of direct benefit to you however, it may benefit patients in the future who develop chronic pancreatitis or cirrhosis of the liver.

What will happen to my tissue samples, blood tests and images?

Blood will be divided into serum and genetic samples which will be stored at the University of Liverpool. DNA will also be extracted from the genetic sample at the University. If something significant is found when your DNA is analysed, we will inform you of the results. If necessary we will advise you and your family on how to go about having further genetics tests and on how to seek further opinions from a clinical geneticist, who is a specialist in this field.

A large number of microscopic images will be retrieved and analysed. We shall identify patterns which are deemed to be normal and make a comparison with more abnormal patterns. Particularly meaningful images will be developed in photoimages for further analysis.
The urine sample will be assessed for the concentration of the sugar absorbed previously and compared to the concentration of the diuretic within the same solution.
Your tissue samples will be frozen in liquid nitrogen or fixed in formalin for subsequent sectioning, staining and analysis.
Some of the results obtained from these blood and tissue tests will be linked with information that we obtain about your current and past medical history from the questionnaire and from your casenotes.

It is important to note that all blood and tissue samples going outside the hospital, and any notes relating to it, will be identified only by a code number. All clinical details will be kept securely, either at the Royal Liverpool University Hospital or the University of Liverpool. Once the study has been completed, we will anonymise your clinical details and blood and tissue samples, and therefore it will not be possible to trace the samples back to you. After anonymisation, it will also not be possible for you to withdraw the samples. Once anonymised, the DNA samples may be used for other research, but as this cannot be traced back to you, it will have no direct bearing on your clinical care. Approval will be sought from the ethics committee for any future studies.

Your blood and tissue samples will be considered to be a gift to the University of Liverpool, which will act as custodian of all the samples obtained as part of this project. In some cases, a small amount of your sample will be provided to other researchers either in the UK or other parts of the world. However, it is important to remember that this will only be identified by a code.

In the short-term, it is unlikely that the samples will be of any commercial value to the University or the hospital. However, it is possible that they may be some commercial value in the future, although it is important to note that any commercial value is likely to be due to findings in a group of patients rather than from samples from a single patient. You will not be paid for taking part in the study, nor will you get financial benefit from future discoveries.

Will my taking part in this study be kept confidential?

As stated above, your sample will be anonymised, and therefore the genetic information obtained from it will be kept strictly confidential and not be disclosed to anyone. All information collected about you during the course of the research will also be kept strictly confidential. Any information about you, which leaves the research centres taking part, will have your name and address removed so that you cannot be recognised from it. We will inform your GP that you have participated in the study.

What will happen to the results of the research study?

Results from the project will be published in leading international medical journals.

Who is organising and funding the research?

This study has been designed by The University of Liverpool and the Royal Liverpool and Broadgreen University Hospital NHS Trust. It is funded by the National Institute of Health Research (NIHR) and the Pancreatic Biomedical Research Unit. The principal investigators are Professor Robert Sutton and Professor Mark Pritchard.

Who has reviewed the study?

The research has been reviewed by the North West Research Ethics Committee and has been granted approval.

Contact for further information

If you need further information or have any concerns or questions, do not hesitate to contact Dr Shameena Bharucha, the Research Fellow running the study, on 0151 706 4487/4877.

Thank you for reading this information leaflet.

Appendix C: Consent Form - Pilot Study

UNIVERSITY OF LIVERPOOL

CONSENT FORM

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH CHRONIC PANCREATITIS AND CHRONIC LIVER DISEASE

Name of principal investigators: Professor Robert Sutton, Professor Mark Pritchard

Name of Researcher: **Please initial boxes**

1. I confirm that I have read and understood ***version 4*** of the information sheet dated ***27/06/11,*** for the above study and have had the opportunity to discuss the research and ask questions ☐
2. I understand that my participation is voluntary and that I may withdraw at any time without needing to give a reason, and without my medical care or legal rights being affected ☐
3. I understand that the results will not be added to my medical records but that my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust where it is relevant to my taking part in this research. I give permission to these individuals to have access to this information ☐
4. I agree to have an endoscopic procedure with additional samples of intestinal tissue and images to be taken with the confocal endomicroscope ☐
5. I agree to have blood samples taken for tests on factors that may be involved in the process of digestive disease ☐
6. I ***have/have not*** been asked to undertake a further test called the lactose:mannitol test which involves drinking a liquid given to me, and collecting a small blood sample after ninety minutes. If asked, I ***have/have not*** agreed to undertake this test ☐
7. I understand that my samples will be anonymised and will be stored, and it will not be possible to trace the samples back to me ☐
8. I understand that my samples may be used in the future for more genetic tests as there are more scientific advances ☐
9. I agree to take part in the study ☐

……	……..	……
Name of patient	Date	Signature
……	……..	……
Name of researcher	Date	Signature

Appendix D: GP Letter - Pilot Study

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

UNIVERSITY OF LIVERPOOL

Prescot Street
Liverpool
L7 8XP

Tel no: 0151 706 4487

GP LETTER

GP name and address	*Patient details*

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH CHRONIC PANCREATITIS AND CHRONIC LIVER DISEASE

Dear Doctor,

Your patient has kindly consented to participate in the above study which is one of the projects taking place in the NIHR-funded Pancreatic Biomedical Research Centre at Royal Liverpool and Broadgreen Hospitals NHS Trust in collaboration with the University of Liverpool. The study has been approved by Liverpool (Adult) Research Ethics Committee. The aims of this project will be to determine the role and prognostic significance of intestinal epithelial gaps in patients with gastrointestinal diseases including chronic pancreatitis, chronic liver diseases and non-ulcer dyspepsia, and to define clinical applications of *in vivo* confocal endoscopic microscopy in these digestive diseases.

The patient has consented to having an additional endoscopic examination in the form of confocal endomicroscopy with additional gastrointestinal biopsies and multiple image capture of endoscopic findings. Consent has also been given for just over 20ml blood test to be taken. Some patients will also undertake a lactose:mannitol test to investigate intestinal permeability. The first patients in the study will not undergo this test and thereafter, patients will be given the option to participate, or not, with this particular additional test.

The clinical findings at endoscopy will be sent to you in the usual way. These blood and biopsy samples, and images, will be analysed in an anonymised fashion and used to identify relationships between epithelial gaps, biomarkers and disease processes. The patient will not be required to participate any further in the study. If you require any further information please contact Professor Robert Sutton, Executive Director of the Liverpool NIHR Pancreatic Biomedical Research Unit, Professor Mark Pritchard, Professor of Gastroenterology and Honorary Consultant Gastroenterologist, or Dr Shameena Bharucha, Clinical Research Fellow / Gastroenterology SpR at Royal Liverpool University Hospital.

Yours sincerely,

Professor Robert Sutton, Professor Mark Pritchard, Dr Shameena Bharucha

Appendix E: Participant Information Form - Pilot Study

PARTICIPANT INFORMATION SHEET FOR CEM STUDY

IDENTIFICATION NUMBER	
PATIENT NAME	
DATE OF BIRTH	
AGE	
HOSPITAL RQ6 NUMBER	
REASON FOR ENDOSCOPY	
PAST MEDICAL HISTORY	
MEDICATIONS	
SMOKER	**Y / N QUANTITY:**
ALCOHOL IN UNITS / WEEK	
ENDOSCOPY FINDINGS	

Appendix F: Patient Invitation Letter - Main Study

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

PATIENT INVITATION LETTER

Prescot Street
Liverpool
L7 8XP

Tel no: 0151 706 2000
Ext: 4487 / 4877
Mob: 07931 246347

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH ACUTE PANCREATITIS

DEAR SIR / MADAM,

I would like to invite you 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. I have attached an information leaflet with this letter that details the study; the reasons for the study and what involvement would be required from you. Please take time to read the information carefully. If you agree to take part, an appointment will be made for you to attend the gastroenterology department for an upper gastrointestinal endoscopy. If you are on a higher level care bed, we shall transfer the equipment to you and conduct the procedure on the Intensive Care or High Dependency Care Unit.

I shall come to see you on the day of your procedure to check that you have had a chance to read the information and to discuss everything with you again in person, in more detail. If there is anything that is not clear, or if you would like more information, please ask at this time. If you have any questions beforehand, please do not hesitate to contact myself on the telephone number at the top of this letter. If you are too sick to contact a member of the research team yourself, or in case of an emergency, or for any other reason, the nursing team or a member of your family can contact the team on internal extension 4487, or the above telephone number, on your behalf.

We appreciate your time and thank you for reading the attached information. This is a voluntary project and, if when you have heard about the study, you would prefer not to take part, your decision will be accepted without question and will not affect the standard of care you receive.

Many Thanks,

Dr S Bharucha, Clinical Research Fellow

Appendix G: Patient Information Leaflet - Main Study

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH ACUTE PANCREATITIS

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. If there is anything that is not clear, or if you would like more information, please ask.

We appreciate your time and thank you for reading this information leaflet.

This is a voluntary project and if, when you have heard about the study, you would prefer not to take part your decision will be accepted without question and will not affect the standard of care you receive.

What is the purpose of the study?

Pancreatic diseases develop in over 50,000 people per year in the UK and cause major illness and death. Acute pancreatitis is a common disease with many causes. It is severe in one of four people affected, and one out of every four people with severe disease dies. Infection in the blood makes this illness worse but it is often difficult to tell when infection is present, making the disease difficult to treat and even more difficult to prevent.

The gastrointestinal tract is one of the largest points of contact the human body has to the outside environment. One of its jobs is to stop harmful intruders from getting into the bloodstream. This is called the gut barrier. A lot of work has been done to show that the gut barrier is damaged in pancreatitis.

Confocal microscopy allows us to look at a slice of tissue too small to be seen with the naked eye. We can see cells, tiny building blocks of living things, to help us with the diagnosis and treatment of disease. Endoscopes are long, black tubes passed into body cavities like the gullet or stomach to look for disease. Confocal probes have been put into endoscopes to look at the bowel lining at microscopic level, to find disease more accurately. The technique is called confocal endomicroscopy. Recent work using this technique has identified gaps in the lining of the bowel that increase in certain diseases, which let harmful substances into the bloodstream.

New ways of identifying the earliest stages of major illness are needed, as are new ways of identifying how well the body is responding to this and healing. Confocal endoscopy offers a means to identify inflammation at the earliest stage, and to predict what will happen more correctly. This work will be a thorough test of new methods of diagnosis, to look for disease and then look to see how providing earlier treatment can improve the condition of the patient and the course of illness.

We aim to recruit up to 50 patients with acute pancreatitis and investigate them with confocal endomicroscopy (a camera with an end microscope inserted into the stomach and duodenum or bowel). Patients will be identified and recruited for the study from the Surgical Wards at the Royal Liverpool University Hospital.

Why have I been chosen?

You have been chosen because you have been diagnosed with Acute Pancreatitis and we aim to study factors involved in the severity of this disease.

Do I have to take part?

No. 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. You are still free to withdraw from the study at any time without giving a reason. This will not affect the standard of care you receive in any way.

What will happen to me if I take part?

All patients who decide to take part in this study will receive the usual clinical care. If you decide to take part, the following will also take place:

h) A blood sample (approximately 20 mls/4 teaspoons) will be taken to try and identify genes and markers for diagnosis and assessment of severity.

a) A camera test with a confocal endomicroscope (a camera with an end microscope) will be carried out. The test involves passing a slim endoscope through the mouth and down into oesophagus and stomach and the first part of your small bowel. Please see the attached *gastroscopy information leaflet* for more detailed information about the actual procedure. Please note that the procedure will be slightly longer than the time detailed on this sheet. The total procedure time is between 15-30 minutes. To ensure that you are as comfortable throughout this period, you will be given both sedation and throat spray by the endoscopist.

b) During this procedure, tissue samples and fluid will be removed from the small or large bowel. Multiple images will be taken with the camera and the confocal endomicroscope. Suitable, safe chemicals will be developed to obtain fluorescence in relevant parts of the body.

c) The tissue samples that are taken at endoscopy will be utilised for processing in our research. The samples used for research purposes will be those that are surplus to that which is required for clinical purposes.
d) The images that are taken at endoscopy will be analysed for our research.
e) We will also ask you for a few questions about your general health and that of your family in relation to digestive diseases, in the form of a questionnaire.
f) A review of you medical notes will be carried out by the research doctor.
g) Soon after your endoscopy, and possibly during your observation and recovery period, we will ask you to drink a sugary liquid which will be a solution of sugar and a laxative. This will be made up into approximately 100ml (20 teaspoons) of liquid. After ninety minutes, we will take a very small amount of blood from you also. This test will look at the absorption of sugar in your body.
h) We would look to repeat the procedure, blood test and sugar test again once the illness has settled down, to assess disease progression. If you have a long hospital stay, we shall do this during this time, but if you improve quickly and are discharged, we would invite you back as an out-patient to see how things have improved, and to conduct the repeat endoscopic procedure. We are likely to carry out the procedure twice, but up to a maximum of three times, if you were willing to have the repeat procedure(s). We would discuss this with you prior to every planned procedure and allow you time to make an informed decision each time, separately.

What are the possible disadvantages and risks of taking part?

There may be some minor but short-lasting discomfort from having a blood test.

The camera test comes with a 1 in 1000 risk of causing a small hole in your digestive tract (perforation). This is rare and if it happened would require an admission into hospital for treatment. There is a very small risk of bleeding if samples of tissue are taken (usually minor). There is a small chance of a reaction to the medicines used for sedation or occasionally even from throat spray. You may have a slightly sore throat following the procedure, which will usually wear off within 24 hours. You must let the nurse know if you have any loose teeth, caps or crowns, as there is a risk they could become dislodged. The risks from this procedure, including the risk of death, are minimal and are not increased in the context of acute pancreatitis. The procedure will be carried out by a specialist with extensive experience to minimise these risks. *Please also read the more detailed gastroscopy information leaflet that is enclosed with this leaflet).*

The dye given to you may cause slight discoloration of your skin, eyes and urine for twenty four hours, but it will then pass out of your system in your urine. The sugar test involves a laxative, which may cause you to open your bowels. Taking part in the study will not affect your current treatment.

What are the possible benefits of taking part?

The study will not be of direct benefit to you however, it may benefit patients in the future who develop Acute Pancreatitis.

What will happen to my tissue samples, blood tests and images?

Blood will be divided into serum and genetic samples which will be stored at the University of Liverpool. DNA will also be extracted from the genetic sample at the University. If something significant is found when your DNA is analysed, we will inform you of the results. If necessary we will advise you and your family on how to go about having further genetics tests and on how to seek further opinions from a clinical geneticist, who is a specialist in this field.

A large number of microscopic images will be retrieved and analysed. We shall identify patterns which are deemed to be normal and make a comparison with more abnormal patterns. Particularly meaningful images will be developed in photoimages for further analysis.

Some blood will be assessed for the concentration of the sugar absorbed previously and compared to the concentration of the diuretic within the same solution.

Some of the results obtained from these blood and tissue tests will be linked with information that we obtain about your current and past medical history from the questionnaire and from your medical notes.

It is important to note that all blood and tissue samples going outside the hospital, and any notes relating to it, will be identified only by a code number. All clinical details will be kept securely, either at the Royal Liverpool University Hospital or the University of Liverpool. Once the study has been completed, we will anonymise your clinical details and blood and tissue samples, and therefore it will not be possible to trace the samples back to you. After anonymisation, it will also not be possible for you to withdraw the samples. Once anonymised, the DNA samples may be used for other research, but as this cannot be traced back to you, it will have no direct bearing on your clinical care. Approval will be sought from the ethics committee for any future studies.

Your blood and tissue samples will be considered to be a gift to the University of Liverpool, which will act as custodian of all the samples obtained as part of this project. In some cases, a small amount of your sample will be provided to other researchers either in the UK or other parts of the world. However, it is important to remember that this will only be identified by a code.

In the short-term, it is unlikely that the samples will be of any commercial value to the University or the hospital. However, it is possible that they may be some commercial value in the future, although it is important to note that any commercial value is likely to be due to findings in a group of patients rather than from samples from a single patient. You will not be paid for taking part in the study, nor will you get financial benefit from future discoveries.

Will my taking part in this study be kept confidential?

As stated above, your sample will be anonymised, and therefore the genetic information obtained from it will be kept strictly confidential and not be disclosed to anyone. All information collected about you during the course of the research will also be kept strictly confidential. Any information about you, which leaves the research centres taking part, will have your name and address removed so that you cannot be recognised from it. We will inform your GP that you have participated in the study.

What will happen to the results of the research study?

Results from the project will be published in leading international medical journals and disseminated through oral and poster presentations at national and international conferences.

Who is organising and funding the research?

This study has been designed by The University of Liverpool and the Royal Liverpool and Broadgreen University Hospital NHS Trust. It is funded by the National Institute of Health Research (NIHR) and the Pancreatic Biomedical Research Unit. The principal investigators are Professor Robert Sutton and Professor Mark Pritchard. The study is being undertaken as part of a PhD project by a gastroenterology registrar, Dr Shameena Bharucha.

Who has reviewed the study?

The research has been reviewed by the North West / Preston Research Ethics Committee and has been granted approval.

Contact for further information

If you need further information or have any concerns or questions, do not hesitate to contact Dr Shameena Bharucha, the Research Fellow running the study, on 0151 706 4487/4877 or 07931 246347.

If you wish to complain about any aspect of the study or wish to discuss any concerns or questions with someone that is not linked to the investigations, please feel free to speak to a member of the clinical team on your ward. Alternatively, PALS (Patient Advice and Liaison Services) is a service that provides on the spot help for patients, relatives and carers. PALS is independent of all the hospital wards and departments where patients are seen and treated. The PALS team can be contacted on 0151 706 4903 or freephone Kerfone #159 from the bedside in-patient phone. You can find more information and contact options on the PALS website: www.rlbuht.nhs.uk/pals.asp.

Thank you for reading this information leaflet.

Appendix H: Consent Form - Main Study

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

UNIVERSITY OF LIVERPOOL

CONSENT FORM

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH ACUTE PANCREATITIS

Name of Principal Investigators: Professor Robert Sutton, Professor Mark Pritchard

Name of Researcher:

		Please initial boxes
1.	I confirm that I have read and understood ***version 2*** of the information sheet dated ***06/06/12,*** for the above study and have had the opportunity to discuss the research and ask questions	
2.	I understand that my participation is voluntary and that I may withdraw at any time without needing to give a reason, and without my medical care or legal rights being affected	
3.	I understand that the results will not be added to my medical records but that my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust where it is relevant to my taking part in this research. I give permission to these individuals to have access to this information	
4.	I agree to allow the research team to consult my medical notes	
5.	I agree to have an endoscopic procedure with additional samples of intestinal tissue and images to be taken with the confocal endomicroscope	
6.	I agree to have blood samples taken for tests on factors that may be involved in the process of digestive disease	
7.	I agree to undertake a further test called the lactulose:mannitol test which involves drinking a liquid given to me, and collecting a small blood sample after ninety minutes	
8.	I agree to have the endoscopic procedure, blood sampling, and lactulose:mannitol test repeated upto a maximum of two further times	
9.	I understand that my samples will be anonymised and will be stored, and it will not be possible to trace the samples back to me	
10.	I understand that my samples may be used in the future for more genetic tests as there are more scientific advances	
11.	I agree to take part in the study	
12.	I agree for my GP to be informed of my participation in the study and for them to be informed of any results generated from the tests related to the study	

……	……..	……
Name of patient	Date	Signature
……	……..	……
Name of researcher	Date	Signature

Appendix I: Participation Information Form - Main Study

PARTICIPANT INFORMATION SHEET FOR CEM STUDY

IDENTIFICATION NUMBER	
PATIENT NAME and GENDER	
DATE OF BIRTH and AGE	
HOSPITAL RQ6 NUMBER	
TIME and DATE OF ONSET OF SYMPTOMS OF AP	
SYMPTOMS OF AP	
PAST MEDICAL HISTORY INC CO-EXISTING GI DISEASE	
MEDICATIONS	
US / CT / MR FINDINGS	
SMOKER	**Y / N QUANTITY:**
ALCOHOL IN UNITS / WEEK	**Y / N QUANTITY:**
HELICOBACTER PYLORI	
AMYLASE	
ENDOSCOPY FINDINGS	
HISTOLOGY FINDINGS	

ORIGINAL 1992 ATLANTA CLASSIFICATION: **MILD = Parenchymal inflammation with no local comp. or sys. Involvement** **SEVERE = Above with local complication:** • **Necrosis on Enhanced CT ≥3cm or 1/3 of pancreas** • **Pseudocysts or acute fluid collection** • **Abscess** **or systemic complications:** • **Transient or persistent organ failure**	
OVERALL GRADING BY 1992 ATLANTA CLASSIFICATION	
REVISED 2012 ATLANTA CLASSIFICATION: **MILD = No local or sys comp** **MODERATE** • **Local or sys comp without persistent organ failure** • **Transient organ failure < 48hrs** **SEVERE** • **Persistent single or multiple organ failure > 48hrs**	
OVERALL GRADING BY 2012 ATLANTA CLASSIFICATION	
SOFA SCORE: **MILD = No local or sys comp** **MODERATE** • **Necrosis OR transient organ failure** **SEVERE** • **Persistent organ failure OR Infected pancreatic necrosis** **CRITICAL** • **Persistent organ failure AND infected pancreatic necrosis**	
OVERALL GRADING BY SOFA SCORE	

Appendix J – Case Record Form - Main Study

WORKSHEET FOR THE COLLECTION OF SAMPLES FOR THE CEM STUDY

SAMPLE COLLECTION CODE: **KIT GENERIC LABEL START**

Consent Taken By:	Date:	PATIENT REF NO:	AP
		CEM_____	

DATA SHEET COMPLETED	COLLECTED OGD FORM	IMAGES DOWNLOADED	IMAGES SAVED TO DISC AND HARD DRIVE

Date of Endoscopy: _____

Image Stacks

D2 STACK	IMAGE NUMBERS	SELECTED IMAGES
SITE 1		
SITE 2		
SITE 3		
SITE 4		
SITE 5		

Collection of Tissue

3 Bx for Path		Time into Formalin	
3 Bx to snap freeze in 3 Blue Cryovials		Time into Liquid Nitrogen	

Collection of Fluid

Fluid for Micro in Cryovial		Time Taken		Volume into Pyrogen Free Cryovial	
Fluid for Neat Storage in Falcon		Time Taken		Volume into Falcon	

Collection of Blood: **Date:**

Sample Type	Date and Time Collected	Collected By	Inverted x 10
2 ml EDTA			
3.5 ml Serum			
4.5ml Na Cit			
Blood Cultures 5ml x 2			

Intestinal Permeability Test: **Date:**

Date of Test		Collected / Administered By		
1. 2ml Serum at start			Time Taken	
Sugar Solution Drink completed			Time Given	
2. 2ml serum at 90mins			Time Taken	

BLOOD SAMPLE PROCESSING

EDTA TUBE

Time Stored at -80^{0}C	Label	Freezer	Confocal Box	Position of tube

SERUM

Stood for 30 mins after collection	Time centrifuged at 1500g for 10 mins	No of White Cryovials	Labels

Time Stored at -80^{0}C	Freezer	Tray	Box	Position of tubes

NA CITRATE TUBE

Time centrifuged at 1600g for 15 mins AT 20^{0}C		No of Red Cryovials with Plasma		No of Eppendorfs with cell pellet	
Time Red Top Cryovials Stored at -80^{0}C	Labels	Freezer	Tray	Box	Position of tubes
Time Eppendorfs Stored at -80^{0}C	Labels	Freezer	Tray	Box	Position of tubes

BLOOD CULTURE BOTTLES

Contacted 4718	Collected By Micro	Time

SERUM FOR IP TEST – **Date:**_____

Sample	Stood for 30 mins after collection	Time centrifuged at 1500g for 10 mins		No of Cryovials	Labels
1 at 0 mins					
2 at 90 mins					
Sample	**Time Stored at -80^{0}C**	**Freezer**	**Tray**	**Box**	**Position of tube**
1 at 0 mins					
2 at 90 mins					

PROCESSING / STORAGE OF TISSUE and FLUID

Tissue for Paraffin Wax Embedding:

No. of samples (Max=3)	
Date and Time taken to Pathology	
Slides received from Pathology	

Tissue for Storage in Isopentane:

No. of samples (Max=3)					
Labels of Cryovials					
Location in Freezer	**Time Stored at -150^{0}C**	**Freezer**	**Tray**	**Box**	**Position of tube**

PROCESSING / STORAGE OF FLUID

Fluid for Microbiology

Contacted 4718	**Collected By Micro**	**Time**

Neat Fluid

Time Stored at -80^{0}C	**Label**	**Freezer**	**Confocal Box**	**Position of tube**

Appendix K: Microbiology Request Form

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

FAO: PAUL ROBERTS:4718

SHAMEENA BHARUCHA / PBRU CONFOCAL STUDY : MICROBIOLOGY FORM		
PATIENT NUMBER:	CEM	
DATE:		
SPECIMEN OF:		
Name of Medical Officer:	Dr Shameena Bharucha	
Signature of Medical Officer:		
Contact Details:	07931 246347 / 0151 706 4487 shameena_b@yahoo.co.uk	

Appendix L: Pathology Request Form

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

FAO: LEAH CLEMENT

SHAMEENA BHARUCHA / PBRU CONFOCAL STUDY : PATHOLOGY FORM		
PATIENT NUMBER:	CEM	
DATE:		
SPECIMEN OF:		
Name of Medical Officer:	Dr Shameena Bharucha	
Signature of Medical Officer:		
Contact Details:	07931 246347 / 0151 706 4487 shameena_b@yahoo.co.uk	
For Laboratory Use		
No. Of Cassettes:		
No. Of Ribbons:		
Date of Collection:		
Signatures:	**MO:**	**Lab:**

Appendix M: Lactulose Mannitol Test PI Leaflet

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

PATIENT INFORMATION: MANNITOL & LACTULOSE SMALL

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH CHRONIC PANCREATITIS AND CHRONIC LIVER DISEASE

Dear _____,

Many thanks for agreeing to attend for the sugar absorption test, which is the second part of the study that you previously took part in.

Please find enclosed a leaflet detailing the test and your appointment.

I look forward to seeing you again. If you need further information or have any concerns or questions, do not hesitate to contact me on 0151 706 4487 / 0151 706 4877 or 07931 246347.

Many Thanks,

Dr Shameena Bharucha

Pancreas Biomedical Research Unit

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

PATIENT INFORMATION: MANNITOL & LACTULOSE SMALL INTESTINAL PERMEABILITY TEST

CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH CHRONIC PANCREATITIS AND CHRONIC LIVER DISEASE

Brief Outline

Sugar absorption tests can be used to assess leakiness of the bowel, in a variety of diseases. The sugars are given in water and blood levels of two sugars are determined before and after the solutions are given.

Patient Preparation and Procedure

- Please do not eat or drink anything after midnight before your appointment.
- You are allowed to drink small amounts of water.
- Please avoid taking aspirin/diclofenac/ibuprofen/nurofen or other drugs from this group the night before the test.
- Do not drink any alcohol the day before the test.
- **Please report to the main desk in the foyer at the main entrance of the ground floor of the Royal Liverpool University Hospital with this letter on _____ at _____.**
- A small blood test will be taken on your arrival and then you will be asked to drink a sugar solution containing **lactulose*,** mannitol and water.
- You will be asked to stay in the facility for ninety minutes. It may be beneficial to bring reading material to occupy yourself during this time.
- A second small blood test will be taken at this point and then you will be discharged.

*** Please note that lactulose is a laxative and may cause you to open your bowels and pass loose motions. It is however a negligible dose and therefore is more likely to have no effect.**

Contact for further information

If you need further information or have any concerns or questions, do not hesitate to contact me on 0151 706 4487 / 0151 706 4877 or 07931 246347.

Thank you for reading this information leaflet

Appendix N: Lactulose Mannitol Prescription

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

SCOPE STUDY PRESCRIPTION

Trust reference number: 4067

To be completed by Investigator or Designée

Patient Name: _____

Patient Hospital Number: _____

Pt hospital label:
(Affix here)

Please Supply: 100ml of Lactulose Mannitol Oral Solution

Date and time of patient visit: _____

Doctor's name (Please print): _____
Date: ___ / ___ / ___

Signed: _____ **Ext. No/Bleep No.** _____

To be completed by Pharmacy

Dispensed by (Print Name and Sign):
_____ **Date:** __/__/__

Checked by (Print Name and Sign): _____
Date: __/__/__

Appendix O: Regional Ethics Committee Approval

Health Research Authority

National Research Ethics Service
NRES Committee North West –Preston
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ
Telephone: 0161 625 7434

07 June 2012
Professor Robert Sutton
Executive Director Liverpool NIHR Pancreas Biomedical Research Unit
NIHR Pancreas Biomedical Research Unit; Royal Liverpool University Hospital
Royal Liverpool University Hospital
Daulby Street
Liverpool
L69 3GA

Dear Professor Sutton

Study title:	**STUDY TO INVESTIGATE THE ROLE OF CONFOCAL IMAGING IN THE INVESTIGATION AND MANAGEMENT OF PATIENTS WITH ACUTE PANCREATITIS**
REC reference:	**12/NW/0420**
Protocol number:	**UoL000874**

Thank you for your email of 06 June 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair and Dr Monks.

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites
NHS sites
The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC RandD office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("RandD approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the RandD office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		08 May 2012
Evidence of insurance or indemnity		31 July 2011
Investigator CV		Robert Sutton
Investigator CV		Shameena Bharucha
Letter from Sponsor	Liverpool University	08 May 2012
Letter from Sponsor	NHS Co-Sponsor Letter	02 May 2012
Letter from Statistician	Trevor Cox	10 May 2012
Letter of invitation to participant	1	21 March 2012
Letter of invitation to participant	2	06 June 2012
Other: RandD Application Form		21 March 2012
Other: Research Registration Form Liverpool University		
Other: NHS Indemnity Cover		
Other: In-patient gastroscopy information leaflet from Royal Liverpool University Hospital		
Participant Consent Form	1	21 March 2012
Participant Consent Form	2	06 June 2012
Participant Information Sheet	1	21 March 2012
Participant Information Sheet	2	06 June 2012
Protocol	1	21 March 2012
REC application	3.4	10 May 2012
Referees or other scientific critique report		08 May 2012
Response to Request for Further Information		06 June 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

Notifying substantial amendments
Adding new sites and investigators
Notification of serious breaches of the protocol
Progress and safety reports
Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.
Feedback
You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After

Please quote this number on all correspondence: 12/NW/0420

With the Committee's best wishes for the success of this project
Yours sincerely

Dr Patricia Wilkinson
Chair

Email: Anna.bannister@northwest.nhs.uk
Enclosures:

"After ethical review – guidance for researchers"

Copy to:

Mrs Lindsay Carter
Professor Robert Sutton, NIHR Pancreas Biomedical Research Unit; Royal Liverpool University Hospital