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Mitochondrial damage-associated molecular patterns (DAMPs) in Inflammatory Bowel Disease

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CONTENTS

DECLARATION

I declare that the work contained within this thesis is my own, produced with support from my supervisors.

Any data generated with the assistance of others has been acknowledged appropriately within the body of the manuscript.

This work has not been submitted for any other professional qualification or degree.

Ray K. Boyapati

27 February 2018

PUBLISHED PAPERS

Papers published in peer-reviewed journals relating to this thesis

- **Boyapati RK** et al (2018). Mitochondrial DNA is a pro-inflammatory damage-associated molecular pattern (DAMP) released during active IBD, *Inflammatory Bowel Disease,* doi: 10.1093/ibd/izy095

- **Boyapati, R.**, Satsangi, J. and Ho, GT (2015) 'Pathogenesis of Crohn's disease', *F1000Prime Reports*, 7(44). doi: 10.12703/P7-44.

- Ho, GT., **Boyapati, R.** and Satsangi, J. (2015) 'Ulcerative colitis', *Medicine*, 43(5):276–281. doi: 10.1016/j.mpmed.2015.02.004.

- **Boyapati, R.K.** et al. (2016) Biomarkers in Search of Precision Medicine in IBD, Am J Gastroenterol, 111(12):1682-1690. doi: 10.1038/ajg.2016.441.

- **Boyapati, R.** et al. (2016) 'Gut mucosal DAMPs in IBD: from mechanisms to therapeutic implications', *Mucosal Immunology*, 9(3):567–82. doi: 10.1038/mi.2016.14.

- **Boyapati, R. K.** et al. (2017) 'Advances in the understanding of mitochondrial DNA as a pathogenic factor in inflammatory diseases', *F1000Research*, 6(169). doi: 10.12688/f1000research.10397.1.

Abstracts

Oral Presentations

Digestive Diseases Week 2017

(Session: “IBD: Cytokines, Signaling and Receptors”)

- **Boyapati RK**, Dorward DA, Tambrowska A, Rossi AG, Satsangi J, Ho GT. Mitochondrial DNA is a damage-associated molecular pattern (DAMP) released during active IBD promoting TLR9-mediated inflammation

Posters

European Crohn’s Colitis Organisation conference 2017

- **Boyapati RK**, Dorward DA, Tambrowska A, Rossi AG, Satsangi J, Ho GT. Mitochondrial DNA is a damage-associated molecular pattern (DAMP) released during active IBD promoting TLR9-mediated inflammation

Other IBD Publications 2014-2018

- Torres, J.*, **Boyapati, R.K.*** & Kennedy, N.A., Louis, E., Colombel, J.F. & Satsangi, J. (2015). 'Systematic Review of Effects of Withdrawal of Immunomodulators or Biologic Agents From Patients With Inflammatory Bowel Disease'. *Gastroenterology*, 149(7)1716-30. doi: 10.1053/j.gastro.2015.08.055 *co-first author

- **Boyapati, R.K.**, Ho, GT. and Satsangi J. (2017). 'Can Thiopurines Prevent Formation of Antibodies Against Tumor Necrosis Factor Antagonists After Failure of These Therapies?', *Clinical Gastroenterology & Hepatology*, 15(1):76-78. doi: 10.1016/j.cgh.2016.09.152

- Kalla R., Kennedy N.A., Ventham N.T., **Boyapati R.K.,** Adams A.T., Nimmo E.R., Visconti M.R., Drummond H., Ho GT., Pattenden R.J., Wilson D.C, Satsangi J. (2016). 'Serum Calprotectin: A Novel Diagnostic and Prognostic Marker in Inflammatory Bowel Diseases'. *American Journal of Gastroenterology,* 111:1796–1805. doi: 10.1038/ajg.2016.342

- Ho, G. T., Liu, B., **Boyapati, R. K.**, Kennedy, N. A., Dorward, D. A, Noble, C.L, Shimizu, T, Carter, R.N, Chew, E.T.S, Morton, N.M, Rossi, R.B, Iredale, J.P, Satsangi, J. (2018) 'MDR1 deficiency impairs mitochondrial homeostasis and promotes intestinal inflammation', *Mucosal Immunology*, 11(1):120-130. doi: 10.1038/mi.2017.31

DEDICATION

Aye, Edinburgh yer proper bonnie but that icy wind could cut right through ye

For Rachel, Charlie and Rory.

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ABSTRACT

Background

The inflammatory bowel diseases (IBD) ulcerative colitis (UC) and Crohn's disease (CD) are chronic relapsing inflammatory disorders which have a rising incidence and cause significant morbidity. There are currently several treatment options with many more in the drug pipeline, but there are a lack of accurate biomarkers for decisions on treatment choice, assessment of disease activity and prognostication. There is a growing interest and desire for personalised or 'precision' medicine in IBD where novel biomarkers may help individualise IBD care in terms of diagnosis, choice of therapy, monitoring of response and detection of relapse. One class of functionally active biomarkers which have yet to be thoroughly investigated in IBD is damage-associated molecular patterns (DAMPs) including mitochondrial DNA (mtDNA). It has been recently shown that gut mitochondrial dysfunction can result in loss of epithelial barrier function and the development of colitis. Mitochondrial DAMPs have recently been described as elevated in several inflammatory diseases.

Hypothesis

The primary hypothesis of this thesis is that circulating levels of mtDNA is elevated in IBD. Secondary hypotheses are: (a) levels of other mitochondrial DAMPs are elevated in IBD, (b) circulating mtDNA can be used as a novel biomarker in IBD and (c) mtDNA is released locally at sites of inflammation in IBD.

Methods

Plasma and serum were collected prospectively from recruited IBD patients and non-IBD controls. Faeces and colonic tissue were collected from a subset of these patients. mtDNA in serum, plasma and faeces was measured using qPCR (amplifying COXIII/ND2 genes). Mass spectrometry was used to detect mitochondrial formylated peptides in the plasma of a subset of patients. IBD tissue was assessed for (a) mitochondrial damage using transmission electron microscopy (TEM) and (b) TLR9 expression, the target for mtDNA.

Results

97 patients with IBD (67 UC and 30 CD), and 40 non-IBD controls were recruited. Plasma mtDNA levels were increased in UC and CD (both $p<0.0001$) compared to non-IBD controls; with significant correlations with blood (CRP, albumin, white cell count), clinical and endoscopic markers of severity; and disease activity. In active UC, we detected significantly higher circulating mitochondrial formylated peptides and faecal mtDNA levels (vs. non-IBD controls [$p<0.01$ and <0.0001 respectively]) with demonstrable TEM evidence of intestinal mucosal mitochondrial damage. In active IBD, TLR9+ lamina propria inflammatory cells were significantly higher in UC/CD compared to controls (both $p<0.05$).

Conclusions

Taken together, the findings suggest mtDNA is released during active inflammation in inflammatory bowel disease and is a potential novel mechanistic biomarker.

LAY SUMMARY

The mitochondria (‘batteries’ for the living cells) provide energy and regulate many key biological processes that maintain cellular health and function. The mitochondria within the lining of the large bowel are particularly exposed to a number of detrimental factors, which can damage the mitochondria. In health, damaged mitochondria (‘faulty batteries’) are effectively re-cycled or packaged away for safe disposal.

In the inflammatory bowel diseases (IBD) Crohn’s disease and ulcerative colitis, it has been shown that these protective processes do not work properly or are overwhelmed. The unhealthy colon therefore leaks damaged mitochondria and their products (including their genetic ‘code’: DNA) into the internal environment of the cells and importantly into the blood circulation. Of interest, the mitochondria, which reside in all cells, are evolutionarily derived from helpful bacteria maintaining a mutually beneficial relationship with the host cells. The mitochondria share many similar properties with bacteria – including their ability to activate the inflammatory and immune system. It has already been shown that in many human inflammatory diseases, levels of mitochondrial DNA and other products have been found to be elevated in the blood circulation.

In this thesis, a well-characterised patient population was recruited and compared to healthy people. Mitochondrial product levels were found to be

significantly higher in patients with IBD compared to healthy people, and higher levels were found within the IBD cohort for those with active inflammation. This information may be useful as a ‘biomarker’ to assess for disease activity, predict how patients will do, or how they may respond to therapy.

High levels of mitochondrial products were also found in the faeces, and cells expressing the receptor that is activated by these products were also found to be higher in the lining of the gut. These findings suggest that the inflamed gut may be the primary source of these mitochondrial products.

Importantly, this data adds to the growing evidence suggesting blockade of the inflammatory effects arising from damaged mitochondria within the lining of the gut may benefit some patients with IBD. This requires further investigation.

In summary, this work has identified a highly novel, specific and measurable factor in inflammatory bowel disease. This sets the platform for further research into this area to investigate how mitochondrial DNA may be used as a biomarker, and its precise contribution to inflammatory bowel disease.

ABBREVIATIONS

AIM2 - absent in melanoma 2

ALK – anaplastic lymphoma kinase

AMI – acute myocardial infarction

ANOVA – analysis of variance

APC – antigen presenting cell

ASUC – acute severe ulcerative colitis

ATP adenosine triphosphate

AUC – area under the curve

AUROC – area under the receiver operating characteristic curve

BSA - bovine serum albumin

cGAS – cyclic GMP-AMP synthetase

CD – Crohn's disease

CDAI – Crohn's Disease Activity Index

CID - collision induced dissociation

CRP – C-reactive protein

CAD – coronary artery disease

COX – cytochrome c oxidase

COXIII – cytochrome C oxidase subunit III

CSF – cerebrospinal fluid

CV – coefficient of variation

Cyt b – cytochrome b

DAMP – damage-associated molecular pattern

DEPC – diethyl pyrocarbonate

D-loop – displacement loop

DMEM - Dulbecco's Modified Eagles Medium

DNA – deoxyribonucleic acid

DNase - deoxyribonuclease

DSS – dextran sulfate sodium

ECM – extracellular matrix

ED – Emergency Department

EDTA – ethylenediaminetetraacetic acid

EGFR – epidermal growth factor receptor

EIM – extra intestinal manifestation

ELISA – enzyme-linked immunosorbent assay

ER stress – endoplasmic reticulum stress

ESR – erythrocyte sedimentation rate

FCS – foetal calf serum

FPR1 – formyl peptide receptor 1

FPR2 – formyl peptide receptor 2

GWAS – genome wide association studies

HAART – highly active anti-retroviral therapy

HBI – Harvey Bradshaw Index

HBSS – Hank's Balanced Salt Solution

HC – healthy controls

HCD - Higher Collision Dissociation

HER2 - human epidermal growth factor receptor 2

HIV – human immunodeficiency virus

HLA-DR – human leukocyte antigen – antigen D related

HMGB1 – high-mobility group box 1 protein

IBD – inflammatory bowel disease

IBS – irritable bowel syndrome

ICU – intensive care unit

IEC – intestinal epithelial cell

IFN – interferon

IL – interleukin

IQR – interquartile range

KO – knockout

KRAS – v-Ki-ras2 Kirsten rat sarcoma

LC-MS – liquid chromatography mass spectrometry

LRTI – lower respiratory tract infection

MAPK – mitogen-activated protein kinase

MMP – matrix metalloproteinase

MODS – multiple organ dysfunction syndrome

MS – mass spectrometry

mtDNA – mitochondrial DNA

mtROS – mitochondria-derived reactive oxygen species

NADH – nicotinamide adenine dinucleotide

ND2 – nicotinamide adenine dinucleotide dehydrogenase

NFKB – nuclear factor kappa B

NET – neutrophil extracellular trap

NLR – nucleotide binding domain and leucine-rich repeat

NLRP3 – nucleotide oligomerization domain-like receptor family, pyrin domain containing 3

NOD – nucleotide binding and oligomerisation domain

NOD2 – nucleotide binding and oligomerisation domain-containing protein 2

NSAID – nonsteroidal anti-inflammatory drug

OD – optical density

PAMP – pattern associated molecular pattern

PBMC – peripheral blood mononuclear cell

PCI – percutaneous coronary intervention

PCR – polymerase chain reaction

PDAI – Perianal Crohn's Disease Activity Index

PMI – Precision Medicine Initiative®

pDC – plasmacytoid dendritic cell

PRR – pattern recognition receptor

qPCR – quantitative polymerase chain reaction

RAGE – receptor for advanced glycation end-products

RCT – randomised controlled trial

RNA – ribonucleic acid

rRNA – ribosomal ribonucleic acid

tRNA – transfer ribosomal ribonucleic acid

ROC – receiver operator characteristic

ROS – reactive oxygen species

RPMI – Roswell Park Memorial Institute medium

SCCAI – simple clinical colitis activity index

SEM – standard error of the mean

SIRS – systemic inflammatory response syndrome

SLE – systemic lupus erythematosus

STEMI – ST elevation myocardial infarction

STING – stimulator of interferon genes

T2DM – type 2 diabetes mellitus

TBE – Tris/Borate/EDTA buffer solution

TEM – transmission electron microscopy

TFAM - transcription factor A, mitochondrial

TLR – toll-like receptor.

TLR9 – Toll-like receptor 9

TNF – tumour necrosis factor

TPMT – thiopurine methyltransferase

UC – ulcerative colitis

UK – United Kingdom

UPR - unfolded protein response

WCC – white cell count

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CHAPTER 1:

INTRODUCTION

Introduction Contents

1.1 Inflammatory Bowel Disease

1.1.1 Background to Crohn's disease & ulcerative colitis

The inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are chronic, relapsing and remitting diseases of the gastrointestinal tract. The incidence and prevalence of IBD is increasing, and will affect an estimated 20 million people worldwide by 2025 (Molodecky *et al.*, 2012; Kaplan, 2015). Both conditions are incurable, often diagnosed at a young age and are associated with significant socio-economic costs and morbidity (Baumgart and Sandborn, 2012; Ordás *et al.*, 2012). IBD can severely affect social functioning and cause significant disruption to employment, education, relationships and family life.

There are clear differences between UC and CD (Table 1.1). However, failure to resolve mucosal inflammation (which commonly re-activates upon withdrawal of anti-inflammatory treatments such as glucocorticoids) is a notable shared clinical feature. UC invariably affects the rectum and extends proximally to a variable distance, with characteristically confluent inflammation limited to the mucosal layer of the colon. In contrast, CD can affect any part of the gastrointestinal tract and inflammation is characteristically non-continuous and transmural.

Due to its transmural nature, CD often requires surgery due to fistulas (abnormal connection between two epithelial surfaces) or strictures (narrowing of the intestine that may cause obstruction). Perianal disease (inflammation around the anus) is a particularly disabling manifestation of CD and occurs in up to a third of patients (Schwartz, Loftus and Tremaine, 2002).

	Crohn's Disease	**Ulcerative Colitis**
Anatomical Distribution	May affect anywhere from mouth to anus; commonly affects terminal ileum and colon	Limited to the large intestine; extends from rectum proximally to a variable distance
Type of gut inflammation	Non-continuous, patchy inflammation with skip lesions	Continuous, superficial
Histology	Deep, transmural, focal inflammatory infiltrate. Markedly focal cryptitis, non-necrotizing granulomas, epithelioid granulomas.	Superficial (affecting the mucosa and submucosa) inflammatory infiltrate with loss of crypt architecture, basal plasmacytosis, goblet cell depletion
Main clinical features	Diarrhoea, abdominal pain, fatigue, weight loss	Rectal bleeding, tenesmus, diarrhoea, abdominal pain
Incidence (North American data)	20.2 per 100,000 person-years	19.2 per 100,000 person-years
Peak incidence	Between 20-40 years	Between 20-40 years
Environmental associations	Smoking, western diet, stress, appendectomy	Milder disease with smoking, lower risk with appendectomy
Genetics	Themes involving defective intracellular bacterial killing and innate immunity and de-regulated adaptive immune responses.	Themes involving epithelial integrity, innate immune function, immune regulatory function, and cellular homeostasis in response to endoplasmic reticulum stress.

Table 1.1: Features of Crohn's disease and ulcerative colitis

1.1.2 Pathogenesis of IBD

The last decade has seen remarkable progress in understanding the pathogenesis of IBD. Although the aetiology is complex, the most widely accepted hypothesis purports IBD as an immune mediated condition in genetically susceptible individuals, where disease onset is triggered by environmental factors that perturb the mucosal barrier, alter the healthy balance of the gut microbiota, and abnormally stimulate gut immune responses (Boyapati, Satsangi and Ho, 2015; Colombel and Mahadevan, 2017). IBD is thought to arise from a complex and incompletely understood interplay between these factors (Figure 1.1). Advances in these fields have catalysed a decade of spectacular progress in our understanding of IBD.

The importance of genetic predisposition is clear: first degree relatives of a patient with CD or UC have a 10-fold increased risk of developing the same condition, whilst there is also a significant but less pronounced increased risk of developing the other of the two diseases (Orholm *et al.*, 1991). Meta-analysis now involving 50,000 IBD individuals implicates more than 200 susceptibility loci (Jostins *et al.*, 2012; J. Z. Liu *et al.*, 2015) . This information provided new hitherto unknown insights into disease mechanisms and biological pathways. An aberrant immune response is a key feature of IBD; genetic studies have most strongly and consistently implicated themes involving epithelial integrity, innate immune function, endoplasmic reticulum (ER) stress, defective intracellular bacterial killing and de-regulated adaptive

immune responses (Boyapati, Satsangi and Ho, 2015; G-T. Ho, Boyapati and Satsangi, 2015).

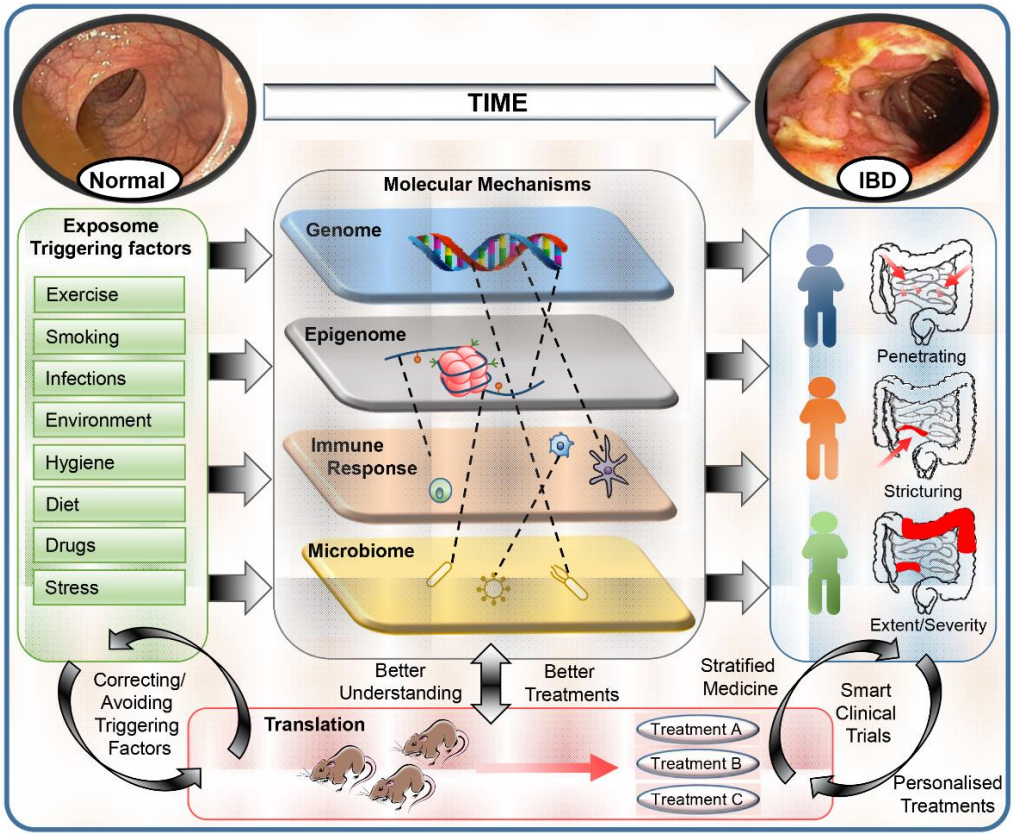

Figure 1.1: Complex interplay of various factors in IBD pathogenesis. IBD pathogenesis involves a complex interplay over time between genetic, epigenetic, immunological, and microbiological mechanisms affected by exposure to triggering factors. Individual patients with IBD have a unique pathogenic signature comprised of different contributions from each of these factors. Stratification of patients based on these signatures may lead to more focused, personalized, and successful therapies. Therapeutic translation is grounded on a greater understanding of these genetic and molecular pathways. Furthermore, correcting and avoiding triggering factors related to the exposome are areas of considerable interest. ‘Smart’ clinical trials with simultaneous mechanistic studies may allow improved understanding even in the case of therapeutic failures (Boyapati, Satsangi and Ho, 2015).

Beyond genetics, epigenetics is emerging as a further tier of information that could complement genome wide association studies (GWAS) (Ventham *et al.*, 2013). Several epigenome wide studies have been published in IBD and other diseases (Nimmo *et al.*, 2012; Adams *et al.*, 2014; Dick *et al.*, 2014; Yuan *et al.*, 2014; McDermott *et al.*, 2016). These studies identify epigenetic mechanisms as a potential interface between genetics and disease. The microbiome or 'other genome' – the collective genome of the gut microbiota – represents a further giant dimension in big data in IBD and other complex multifactorial conditions such as diabetes and obesity (Jostins *et al.*, 2012; Qin *et al.*, 2012; Le Chatelier *et al.*, 2013; Zeevi *et al.*, 2015) enabled by advances in culture independent sequencing technologies.

Although some environmental factors have been established (e.gs smoking, stress and appendectomy), it is likely that many more environmental 'triggers' are relevant but unidentified (e.g. diet). These are particularly difficult research areas and most available evidence relies on case-control studies which suffer from important limitations such as recall bias. There are current efforts recruiting into large prospective cohorts investigating how the environment may contribute to the onset of IBD (e.g. http://www.gemproject.ca) and trigger flares in patients who have IBD (e.g. http://www.predicct.co.uk).

1.1.3 Clinical overview of IBD

1.1.3.1 Diagnosis

The diagnosis of IBD can be difficult, as there is currently no accurate, quick and non-invasive diagnostic test. Furthermore, several other conditions are symptomatically similar including gastrointestinal infection and irritable bowel syndrome (IBS) (Table 1.2). Some patients are symptomatic for many years before a diagnosis is made (Pimentel *et al.*, 2000). Diagnosis usually encompasses clinical, laboratory, radiological, endoscopic and histological findings to establish the disease type, extent and severity.

Differential diagnosis	Features
Bacterial dysentery	Shigellosis; salmonellosis; *Campylobacter* infection
Amoebic dysentery	Watery bloody diarrhoea, recent travel to endemic area
Cytomegalovirus	Immunocompromised patients (e.g. HIV)
Clostridium difficile	Co-exists with UC in ~5–10% of refractory cases
Ischaemic colitis	'Watershed' areas (e.g. deep ulceration at distal transverse colon and splenic flexure): present with bloody diarrhoea
Irritable bowel syndrome	Abdominal pain / cramping, bloating and alternating constipation / diarrhoea in the absence of inflammation on investigation

Table 1.2: Differential diagnosis of IBD

1.1.3.2 Clinical features

IBD is a relapsing and remitting condition that follows an unpredictable pattern. Although broadly classified into CD and UC, the disease is highly individual in terms of symptomatology, prognosis and response to treatment. Clinical features may include diarrhoea, colicky abdominal pain, rectal bleeding, urgency and systemic manifestations such as anorexia, malaise, weight loss and fever. Patients may also have extra-intestinal manifestations (EIM) of IBD such as axial or peripheral arthropathy, erythema nodosum, pyoderma gangrenosum, episcleritis, uveitis, primary sclerosing cholangitis and Sweet's syndrome (Vavricka *et al.*, 2015). Although specific therapies are sometimes required, treatment of the underlying intestinal inflammation often leads to resolution of EIMs.

1.1.3.3 Treatment

Disease flares require rapid diagnosis and institution of active management to minimise the impact of relapse on the patients' wellbeing and to avoid hospitalisation. Complete mucosal healing, the strongest predictive factor for long lasting remission, is difficult to achieve. A summary of the medical therapies used in IBD and indications for surgery are provided in Table 1.3**.**

	Crohn's Disease	Ulcerative colitis
Medical therapy		
Induction of remission	Corticosteroids	5-aminosalicylates (topical and/or oral)
	Anti-TNF therapy	Corticosteroids (topical and/or oral)
	Anti-integrin therapy (vedolizumab)	Ciclosporin (acute severe UC)
	Anti-IL-12/23 therapy (ustekinumab)	Anti-TNF therapy
		Anti-integrin therapy (vedolizumab)
		Anti-IL-12/23 therapy (ustekinumab)
Maintenance of remission	Azathioprine/6-mercaptopurine	5-aminosalicylates
	Methotrexate	Azathioprine/6-mercaptopurine
	Anti-TNF therapy	Methotrexate (evidence less strong)
	Anti-integrin therapy (vedolizumab)	Anti-TNF therapy
	Anti-IL-12/23 therapy (ustekinumab)	Anti-integrin therapy (vedolizumab)
		Anti-IL-12/23 therapy (ustekinumab)
Surgical indications	Failed medical therapy (commonest)	Failed medical therapy (commonest)
	Perforation	Massive haemorrhage
	Corticosteroid dependency	Toxic dilatation
		Perforation
		Corticosteroid dependency
		Malignancy/dysplasia

Table 1.3: Summary of medical therapy and surgical indications for IBD

1.1.3.4 Clinical assessment of disease severity

There are many IBD clinical disease activity assessment tools used in research and clinical practice. For UC, the main clinical indices are the Simple Clinical Colitis Activity Index (SCCAI), the Partial Mayo Index and the Truelove and Witts' Severity Index (for acute severe UC). For CD, the main clinical indices are the Crohn's Disease Activity Index (CDAI), Harvey-Bradshaw Index (HBI) and the Perianal Crohn's Disease Activity Index (PDAI).

A summary of the main strengths and weaknesses of the respective scoring systems for UC (Table 1.4) and CD (Table 1.5) is provided.

Ulcerative colitis			
Index name	**Strengths**	**Weaknesses**	**Reference**
SCCAI	- Can be completed by patient - Reliable, valid, responsive and feasible		(Walmsley *et al.*, 1998)
Partial Mayo Index	- Widely used - Discriminates remission from active disease	- Relies on subjective Physician Global Assessment	(Schroeder, Tremaine and Ilstrup, 1987; Sandborn *et al.*, 2003)
Truelove & Witts' severity Index	- Objective criteria for ASUC - Provides prognostic information	- Only for ASUC	(Truelove, 1955)

Table 1.4: Disease activity assessment indices used in clinical practice for UC (adapted from Walsh et al. 2016)

Crohn's Disease			
Index name	***Strengths***	***Weaknesses***	***Reference***
CDAI	- Widely used	- Complex calculation including a 7-day diary - High variability - Low contribution to total score for perianal disease	(Best *et al.*, 1976)
HBI	- Simpler, less cumbersome - Correlates well with CDAI	- Low contribution to total score for perianal disease	(Harvey and Bradshaw, 1980)
PDAI	- Easy to use	- Only for perianal disease - Fistula severity not included	(Irvine, 1995)

Table 1.5: Disease activity assessment indices used in clinical practice for CD (adapted from Walsh et al. 2016)

The scoring for indices used in this thesis are provided in Appendix A (HBI for CD; SCCAI for UC, and modified Truelove & Witts' criteria for acute severe UC).

1.1.3.5 Severe disease

In CD, most patients will encounter a disabling disease course and approximately half will require surgery within 10 years of diagnosis (Beaugerie *et al.*, 2006; Peyrin-Biroulet *et al.*, 2010). In UC, 15% will develop acute severe UC (ASUC) where there is significant colonic epithelial damage and treatment is with intravenous steroids (Gwo-Tzer Ho, Boyapati and Satsangi, 2015). ASUC is defined by the modified Truelove & Witts' criteria (Appendix A). For patients with ASUC who do not respond to intravenous steroids, rescue therapy with either infliximab or ciclosporin is standard of care but the failure rate of medical therapy is high (~30% requiring surgical removal of the colon) (Turner *et al.*, 2007) (Figure 1.2).

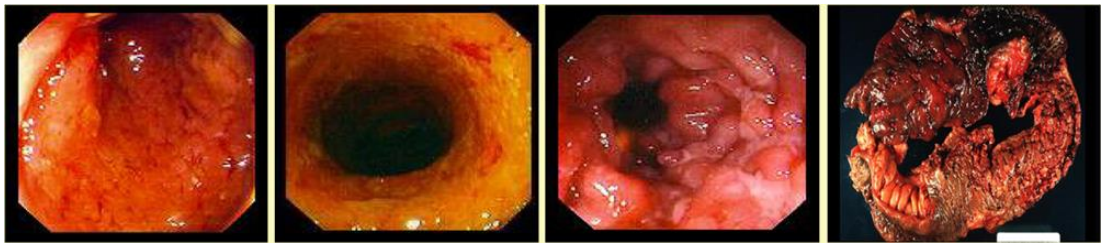

Figure 1.2: (L to R) Endoscopic appearance of mild, moderate and severe UC. Far right image is colonic resection specimen of severe UC.

1.1.4 IBD biomarkers in current practice

There are a handful of biomarkers that have established roles in current clinical practice: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), albumin, faecal calprotectin, anti-TNF antibodies and thiopurine methyltransferase (TPMT) activity measurements. In addition, general blood parameters such as white cell count (WCC) and platelet count may provide information about general inflammation.

CRP

Blood based disease activity assessment in IBD has been dominated by CRP which is induced by IL-6, TNFα and IL-1β. CRP is a hepatocyte derived acute phase reactant protein and detected in the blood. CRP has a short half-life, and serum levels of CRP correlate with disease activity in CD (Vermeire, Van Assche and Rutgeerts, 2004). However, CRP is not specific to gut inflammation, and a subgroup of IBD patients will not mount a CRP response despite having significant inflammation (Boirivant *et al.*, 1988).

Erythrocyte sedimentation rate

ESR measures the vertical distance that erythrocytes fall in one hour. A number of factors influence ESR including erythrocyte characteristics (size, shape, number) and patient factors (age, gender, pregnancy, presence of blood dyscrasias). Elevated levels are included as one of the criteria in the Truelove & Witts' criteria for ASUC. In clinical practice, CRP is used more frequently as it is a better indicator early in the acute phase response.

Albumin

Albumin is a negative acute phase reactant protein and low albumin levels is associated with inflammation. However, hypoalbuminaemia is also associated with non-inflammatory disease states such as malnutrition and malabsorption.

Faecal calprotectin

Faecal calprotectin is a screening tool for gut inflammation (van Rheenen, van de Vijver and Fidler, 2010) and to measure disease activity in IBD (Lin *et al.*, 2014). More recently, the potential to use faecal calprotectin in innovative ways has been explored including as a predictive tool e.g. to identify disease recurrence in post-operative CD (Wright *et al.*, 2014) and as a secondary end-point in IBD clinical trials.

Anti-TNF antibodies

Detection of anti-TNF antibodies allows for expedient switching to an alternative drug (Nanda, Cheifetz and Moss, 2013) and avoids conventional dose escalation which is often futile, expensive and potentially hazardous (Steenholdt *et al.*, 2014). A randomised controlled trial (RCT) in the setting of secondary loss of response to infliximab compared conventional dose intensification with an algorithm based approach based on serum infliximab levels and antibodies (Steenholdt *et al.*, 2014). Here, management dictated by drug levels and antibodies was found to be cost effective with no reduction in clinical efficacy. The current case of anti-TNF however, is instructive and highlights the difficulties ahead in developing a biomarker based decision-

making model. Although therapeutic drug monitoring and antibody testing has been an important recent advance, there is currently no clinically useful predictor of anti-TNF response prior to initiation.

Thiopurine methyltransferase (TPMT)

TPMT measurement can screen for those who are likely to experience life threatening leukopenia from thiopurines (Lennard, 2002) and those who would benefit from a reduced initial dose. Although the routine use of TPMT measurement has been questioned based on cost-effectiveness, this test has been incorporated into standard clinical practice.

Although of some clinical benefit, these current examples demonstrate the lack of accurate biomarkers in clinical practice for help in precise diagnosis, prognostication, monitoring of disease activity and monitoring of response to therapies for individuals with IBD.

1.1.5 Towards personalised biomarkers in IBD

Given the highly individual nature of IBD, clinicians long for a future where a newly diagnosed patient can have his/her genetic, microbiome and immune profile measured at the outset; then matched to the most appropriate biologic or immunosuppressive treatment based on the likelihood of response/adverse effects. IBD individuals will be given information of what 'exposome' to modify and report on their disease activity using the set of optimal biomarkers. At all levels, one can expect a continuous feedback of new data from respective patients, which will further improve the dataset for biomarker discovery in this context. This future may seem farfetched, and many challenges remain, but multiple lines of evidence show progress towards 'precision medicine' in IBD (Boyapati, Kalla, *et al.*, 2016). Key to progress in this area is the ability of researchers to identify novel biomarkers to help personalise therapy.

DAMPs represent underexplored but potentially important pathogenic stimuli that may contribute to maintaining the state of abnormal mucosal inflammation in IBD (Boyapati, Rossi, *et al.*, 2016). Although relatively underexplored in IBD, it is salutary to note that the most useful clinical biomarker used in IBD currently (faecal calprotectin) is a DAMP that was developed from hypothesis-based investigation.

1.2 Damage-associated molecular patterns

1.2.1 Recognising danger and promoting inflammation

The inflammatory response is essential to host defence, promoting microbial containment and clearance. This sentinel function of the innate immune system rapidly and precisely distinguishes between 'self' and 'non-self' by recognizing microbial invariant molecular patterns (pattern associated molecular patterns, PAMPs) through a system of germline encoded pattern recognition receptors (PRRs) (Akira, Uematsu and Takeuchi, 2006). PRR activation leads to intracellular signalling cascades, transcriptional upregulation of inflammatory genes, production of proinflammatory cytokines, chemokines and type I interferons (IFN), and recruitment of inflammatory cells such as neutrophils.

Similar strong immune responses are seen in the absence of invasive pathogens ('sterile inflammation') such as in autoimmunity, trauma and ischemia. This phenomenon is explained by Matzinger's 'danger hypothesis' in which immune responses are geared towards recognizing danger whether these signals arise endogenously or exogenously (Matzinger, 1994) (Figure 1.3). Endogenous damage-associated molecular patterns (DAMPs) are 'danger signals' or 'alarmins' released during host cellular stress or injury. Along with exogenous PAMPs of microbial origins, DAMPs can initiate and

perpetuate an inflammatory response typically via germline encoded pattern recognition receptors (PRR).

1.2.2 PAMPs, DAMPs and PRRs

In this context, PRRs are activated by both non-self (PAMPs) as well as endogenous molecules released at times of danger to the host (DAMPs) (Matzinger, 2002). The major classes of PRRs are cell surface or endosomal toll-like receptors (TLRs), cytoplasmic nucleotide binding and oligomerisation domain (NOD) like receptors (NLRs) and inflammasomes, C-type leptin receptors, RIG-1 like receptors and absence in melanoma 2 (AIM2)-like receptors (Takeuchi and Akira, 2010)(Blander and Sander, 2012). In addition, the more DAMP-specific receptor for advanced glycation end-products (RAGE) is also a categorized as a PRR (Xie *et al.*, 2008).

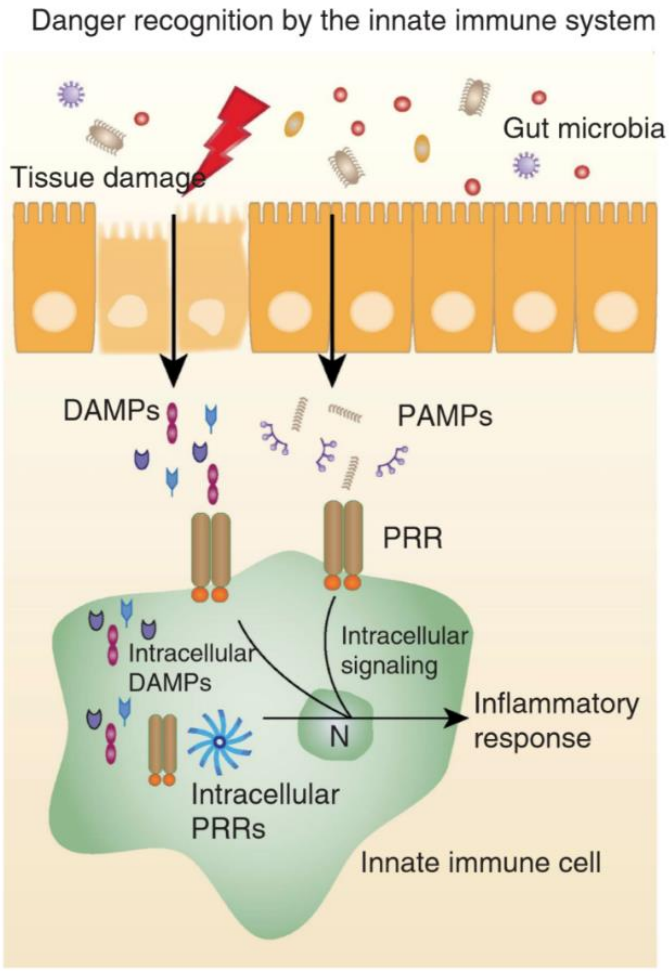

Figure 1.3: Danger recognition by the innate immune system. PRRs such as TLR, NLR, and RAGE sense danger associated with infection via recognition of evolutionarily conserved PAMPs on pathogens or sterile injury via recognition of DAMPs. Activation of cell surface or intracellular PRRs leads to intracellular signalling and inflammatory responses. (Boyapati, Rossi, *et al.*, 2016)

1.2.3 DAMP cellular mechanisms

DAMPs comprise of structurally diverse non-pathogen derived molecules that share a number of characteristics: (1) they bind to and activate PRRs; (2) are passively leaked after plasma membrane rupture following various forms of cell death including necrosis, necroptosis and secondary necrosis; (3) may be actively secreted by stressed cells via non-classical pathways independent of the ER/Golgi apparatus; and (4) may change from a physiological to a proinflammatory function when released into the extracellular milieu (Rock *et al.*, 2008) (Figure 1.4).

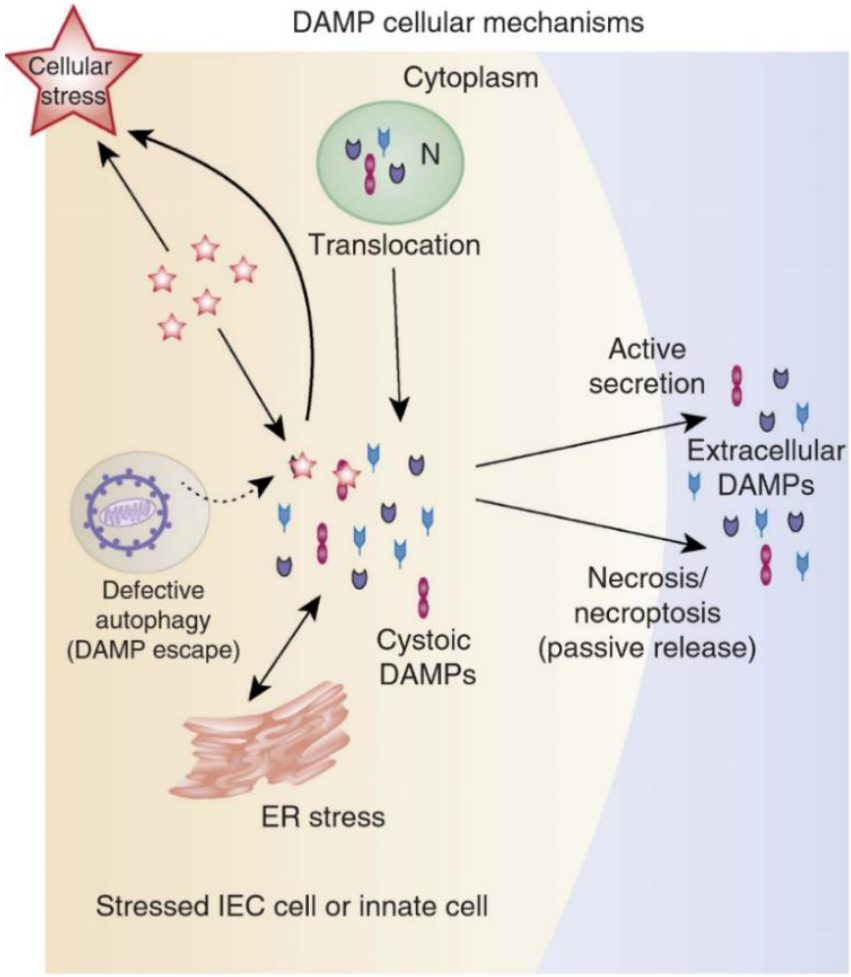

Figure 1.4: DAMP cellular mechanisms. Cellular stress may also lead to damaged cellular components such as reactive oxygen species (ROS) generating mitochondria. Increased ROS production and oxidative stress may have multiple effects including increased translocation and active release of DAMPs and further cellular stress, leading to a vicious cycle. Defects in homeostatic pathways such as autophagy leads to escape of DAMPs such as mtDNA. Intranuclear DAMPs require translocation into the cytosol before active release. Active release (‘‘secretion’’) occurs through nonclassical pathways and cellular membrane rupture after necrosis or necroptosis results in passive release of DAMPs. ER stress contributes to the functional activity of DAMPs e.g. through increased translocation and contributing to its role as an adjuvant; DAMPs can directly lead to increased ER stress. (Boyapati, Rossi, *et al.*, 2016)

Extracellular DAMPs may activate cell surface PRRs or intracellular PRRs after phagocytosis, endocytosis or other mechanisms of internalisation (Schaefer, 2014). DAMPs may originate from any compartment of stressed cells and include intracellular proteins, extracellular matrix (ECM) derived proteins and purinergic molecules. The list of recognized DAMPs is growing rapidly—a list of putative DAMPs and their receptors is provided in Table 1.6.

DAMP	Receptor
HMGB1	TLR2, TLR4, TLR9, RAGE
S100 proteins	TLR4, RAGE, surface heparin sulfate proteoglycan and carboxylated N-glycans
IL-1α	IL-1R
IL-33	ST2 (IL1RL1)
Heat Shock Proteins (HSPs)	TLR2, TLR4, CD91, CD40, CD14
ATP	P_2Y, P_2X, NLRP3
Lactoferrin	TLR4
Mitochondrial DAMPs	mtDNA: TLR9 TFAM: RAGE and TLR9 N-formyl peptides: formyl peptide receptor 1 (FPR1) and FPR2 NLRP3 inflammasome
Histones	TLR2, TLR4, NLRP3, TLR9
Galectins	TLR2
Uric Acid	TLR2, TLR4, NLRP3, CD14
Thioredoxin	Unknown
Cathelicidins	FPR2
Adenosine	A1, A2A, A2B, A3
Defensins	CCR6 and TLR4, TLR1, TLR2
Calreticulin	CD91
RNA	TLR3
Genomic DNA	TLR9, AIM2, NLRP3
Small nuclear RNA	TLR7, TLR8
SAP130	CLEC4E
Extra cellular matrix (ECM) components	
Hyaluronan	TLR2 and TLR4
Biglycan	TLR2, TLR4, P2X4, P2X7, NLRP3
Versican	TLR2, TLR6, CD14
Heparan sulfate	TLR4
Fibronectin (extra domain A)	TLR2, TLR4
Fibrinogen	TLR4
Tenascin C	TLR4
Other ECM components e.g. laminin, elastin and collagen derived peptides	Integrins

Table 1.6: Putative list of DAMPs & receptors (Boyapati, Rossi, *et al.*, 2016)

Under physiological conditions, DAMPs reside intracellularly or are sequestered in the ECM and are thus hidden from recognition by innate immune cells bearing PRRs. In response to perceived danger such as tissue damage, DAMPs are liberated extracellularly serving to signal danger to the host, promoting inflammation and repair processes that are initially beneficial and protective (Schaefer, 2014). However, in the setting of significant and persistent DAMP release, ongoing PRR activation with resultant cytokine and chemokine production may result in deleterious 'collateral damage' and therefore have a central role in pathogenesis. The clearest example is in acute gout, where uric acid crystals directly trigger the NLRP3 inflammasome leading to overwhelming inflammation (Martinon *et al.*, 2006).

The role of DAMPs has been explored in disease models using direct administration of purified or recombinant DAMPs and/or depletion via antagonists or antibodies (Kono and Rock, 2008). DAMP genetic knockout (KO) studies have limitations as they are unable to discriminate between the physiological intracellular and proinflammatory extracellular functions of DAMPs. In the first study to demonstrate how DAMP administration can cause inflammation *in vivo*, Johnson et al. observed a systemic inflammatory response syndrome (SIRS)-like response after administration of the DAMP soluble heparin sulfate (Johnson, Brunn and Platt, 2004). Systemic administration of a recombinant form of the DAMP high-mobility group box 1 protein (HMGB1) in mice is lethal (Wang *et al.*, 1999), with gut epithelial barrier dysfunction a notable feature (Sappington *et al.*, 2002).

In sepsis, initial PAMP mediated activation of PRRs may be followed by cellular damage, DAMP release and subsequent DAMP-PRR inflammatory signalling. In a study illustrating this concept, lethal anthrax challenge in baboons was associated with only transiently elevated bacterial DNA whilst mitochondrial DAMP levels remained elevated until death (Lindberg *et al.*, 2013). The authors used activated protein C treatment as an approach to suppress innate immunity which led to animal survival and no such persistent DAMP elevation. This suggests that endogenous DAMPs may have critical pathogenic roles even in conditions traditionally felt to be PAMP mediated (such as IBD).

1.3 Mitochondria & mitochondrial DAMPs

1.3.1 Introduction to mitochondria

Mitochondria are intracellular double-membrane bound organelles ('cellular powerhouses') with many essential physiological roles in energy production, programmed cell death, calcium homeostasis and the synthesis of lipids, amino acids and haem. In addition, they are involved in antibacterial, antiviral, and stress responses to hypoxia and tissue injury (West, Shadel and Ghosh, 2011; Nunnari and Suomalainen, 2012). Each nucleated cell has a varying number of mitochondria depending on the metabolic requirements of the cell (up to thousands per cell).

Mitochondria are evolutionarily derived from energy producing alpha-bacteria, engulfed by archezoan cells approximately 2 billion years ago leading to a symbiotic relationship that forms the basis of the eukaryotic cells (Dyall, Brown and Johnson, 2004). The mitochondria share several features with their bacterial ancestors, including the double-membrane structure, an independently replicating circular genome rich in hypomethylated CpG motifs and the synthesis of *N*-formylated proteins (Galluzzi *et al.*, 2012).

1.3.2 Mitochondrial DAMPs

As the innate immune system recognizes conserved bacterial molecules, mitochondrial constituents are similarly immunogenic acting as DAMPs when released into the cytosol and extracellular environment, triggering innate immune responses and promoting inflammation (Boyapati, Rossi, *et al.*, 2016).

Cellular stress and necrosis leads to extracellular release of intramitochondrial components such as mitochondrial DNA (mtDNA), N-formyl peptides, transcription factor A mitochondrial (TFAM), ATP and mitochondrial lipids as DAMPs. In addition to roles in activating PRRs (Table 1.7), mitochondrial DAMPs have additional roles such as with chemotaxis – for example, mitochondrial formylated peptides can recruit and activate neutrophils (Dorward *et al.*, 2015).

Mitochondrial DAMPs	**Receptor(s)**
mtDNA	TLR9, NLRP3 inflammasome
TFAM	RAGE and TLR9
N-formyl peptides	FPR1 and FPR2

Table 1.7: Mitochondrial DAMPs and their receptors

1.3.3 Mitochondrial DNA

The mitochondrial DAMP which has thus far attracted the most attention is mtDNA. MtDNA is present in multiple copies within each nucleated cell (each mitochondrion is estimated to contain between 2-10 mtDNA copies (Wiesner *et al.*, 1992)). It is a relatively small, circular, double stranded DNA molecule consisting of 16,569 base pairs (Figure 1.5) enriched with inflammatogenic unmethylated CpG motifs resembling bacterial DNA.

mtDNA comprises of 13 protein coding genes encoding for proteins of the electron transport train within the inner mitochondrial membrane (Figure 1.5) as well as 22 transfer ribonucleic acids (RNAs) coding genes and 2 ribosomal RNAs (12S and 16S rRNAs) which are vital for the processes of translation and peptide synthesis (Taanman, 1999).

Genetic defects in mtDNA may be inherited through maternal transmission and may lead to a wide range of rare debilitating multi-organ system diseases and syndromes (Taylor and Turnbull, 2005).

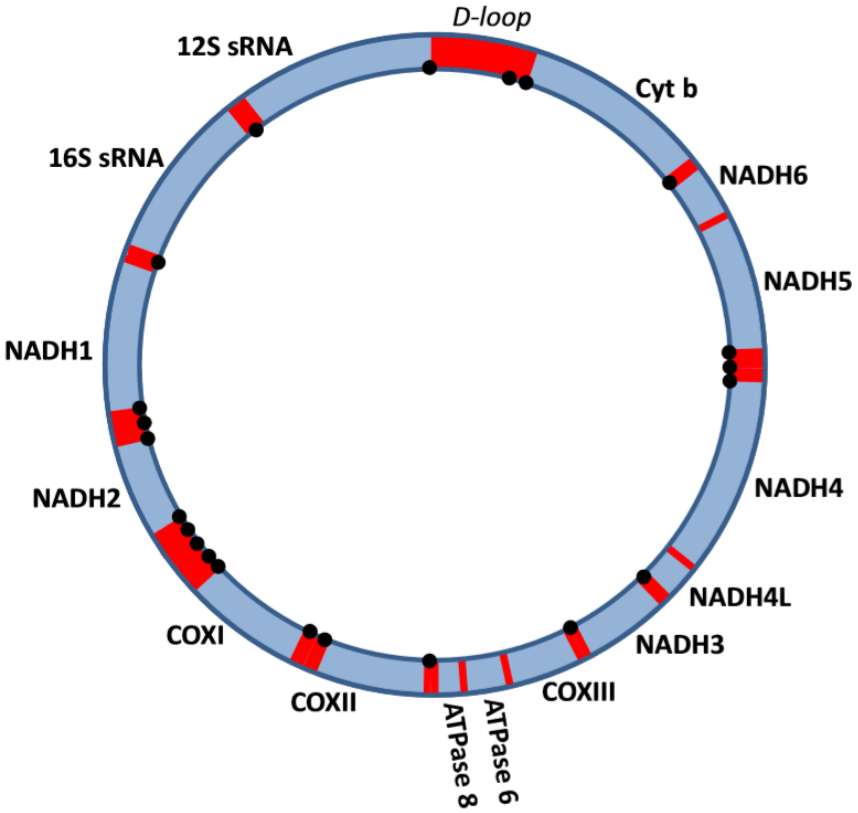

Figure 1.5: Structure of mtDNA. The mitochondrial genome contains 13 protein-coding genes, two rRNA genes (12S and 16S) and 22 tRNA genes (denoted by black circles) alongside a D-loop from where the initiation of replication and transcription occurs. Adapted from Taanman et al. 1999 with permission from Dr David Dorward.

1.3.4 Detecting and measuring circulating mtDNA

I performed a review of the literature around sampling, processing and quantification protocols for mtDNA to inform the study design. Quantification of mtDNA from a sample varies considerably depending on various factors, discussed in full in Section 2.4.1. In brief, variation occurs based on the type of blood fraction used (serum vs. plasma), time to sample processing, consistency in sample processing, centrifugation protocol (whether a second-high speed spin is used) and qPCR (quantitative polymerase chain reaction) protocol.

Therefore, to ensure a robust and accurate assessment of the differences in the levels of circulating mtDNA between IBD and non-IBD cohorts, the following was implemented as part of this thesis:

- A prospective design
- Strict sampling methodology including minimal delays in processing samples.
- Second, high speed centrifugation step employed to remove mtDNA-plasma.
- Both plasma and serum obtained
- qPCR protocols based on sentinel reports (Chiu *et al.*, 2001; Zhang *et al.*, 2010) and then developed and optimised at QMRI, Edinburgh (Dorward *et al.*, 2017).

1.3.5 Circulating mtDNA in inflammatory diseases

Freely circulating mtDNA can be detected, with over 60 studies quantifying mtDNA by qPCR in plasma and serum in human diseases (listed in Table 1.8), with many studies suggesting circulating mtDNA may be a potential biomarker.

Uncontrolled mtDNA release is evident during conditions associated with acute tissue injury such as systemic inflammatory response syndrome (SIRS), fulminant liver failure, trauma, acute myocardial infarction, and sepsis; and in chronic inflammatory states such as systemic lupus erythematosus (SLE) (Boyapati *et al.*, 2017) . This implicates major cellular stress and uncontrolled cell death as key factors in the release of mtDNA (Figure 1.6). In cancer, where its role as 'liquid biopsies' is a topic of considerable interest, the pattern is less clear with relatively lower circulating levels found in some cancers (Lee *et al.*, 2004).

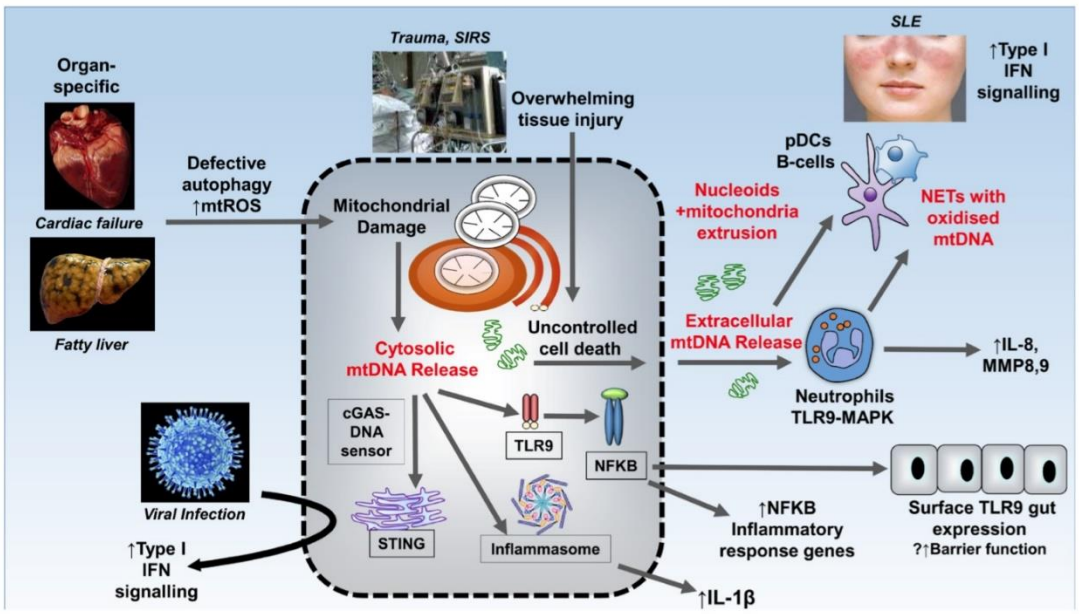

Figure 1.6: The contribution of mtDNA to disease pathogenesis. Medical conditions are in italics. Where and how mitochondria are released are indicated in red. Box in dotted line frames mtDNA sensor target (Boyapati *et al.*, 2017).

Given the significant tissue injury burden typically observed in active IBD, we hypothesised that such pathogenic release is present and that mtDNA can act as a pro-inflammatory DAMP potentiating and perpetuating the abnormal inflammatory response.

Table 1.8: Circulating mitochondrial DNA in human disease

Disease Category	Disease	Blood Fraction	Finding	Reference(s)
Trauma				
	Trauma	Plasma	High mtDNA levels in trauma compared to healthy controls, and correlated with injury severity	(Lam *et al.*, 2004)
	Trauma	Plasma	High mtDNA levels in trauma	(Zhang *et al.*, 2010; Zhang, Itagaki and Hauser, 2010)
	Trauma with MODS	Plasma	Higher levels of mtDNA had higher relative risk for mortality Higher levels of mtDNA in those with SIRS / MODS compared to those without	(Simmons *et al.*, 2013)
	Trauma and severe sepsis	Plasma	mtDNA higher in patients with trauma compared to healthy controls on day 1 mtDNA correlates with injury severity scores in trauma patients mtDNA higher on day 1 in non-survivors compared to survivors	(Hsu *et al.*, 2013)
	Post-Traumatic SIRS	Plasma	mtDNA is an independent predictor for post-traumatic SIRS	(Gu *et al.*, 2013)
	Trauma	Plasma	mtDNA higher in trauma patients with correlation with injury severity	(Nicole Y.L. Lam, Timothy H. Rainer, Rossa W.K. Chiu and Lo, 2004)
	Trauma (femur fracture)	Plasma	mtDNA higher in trauma patients than healthy controls	(Hauser *et al.*, 2010)
	Trauma	Plasma	mtDNA higher in trauma patients compared to healthy controls at two-time points (pre-hospital and day 1)	(Timmermans *et al.*, 2016)

	Trauma	Plasma	mtDNA higher in trauma patients than healthy controls mtDNA higher in non-survivors compared to survivors	(Prikhodko *et al.*, 2015)
	Hip fracture	Plasma	mtDNA levels were higher in hip fracture patients compared to healthy controls, and even higher in the lung injury subgroup compared to lung injury absent subgroup	(Zhang *et al.*, 2017)
Sepsis				
	Severe sepsis	Plasma	mtDNA higher in patients with severe sepsis compared to healthy controls No significant difference in mtDNA between non-survivors and survivors in severe sepsis	(Hsu *et al.*, 2013)
	Severe sepsis in the ED	Plasma	mtDNA higher on admission in severe septic patients than healthy controls; mtDNA higher in non-survivors than survivors, increased initially then gradual decrease after antimicrobial therapy, independent predictor of fatality.	(Kung *et al.*, 2012)
	Sepsis	Plasma	mtDNA higher in septic patients compared to healthy controls	(Bhagirath, Dwivedi and Liaw, 2015)
	Septic shock	Plasma	mtDNA higher levels in patients with septic shock	(Timmermans *et al.*, 2015)
	Adult community-acquired bacterial meningitis	Plasma	mtDNA levels were higher in patients with aseptic and in bacterial meningitis compared to healthy controls mtDNA levels fall during course of admission High mtDNA levels associated with poorer outcome in adult community-acquired bacterial meningitis	(Lu *et al.*, 2010)
	Infectious SIRS	Plasma	mtDNA higher in septic patients compared to healthy controls	(Garrabou *et al.*, 2012)
	Paediatric sepsis	Plasma	mtDNA higher in septic patients compared to critically ill non-septic and healthy control patients	(Di Caro *et al.*, 2015)

	Severe sepsis in the ED	Plasma	No significant difference in mtDNA between sepsis and healthy control cohorts	(Puskarich *et al.*, 2012)
Critically unwell patients				
	ICU patients	Plasma	Increased mtDNA levels associated with ICU mortality	(Nakahira *et al.*, 2013)
	Critically Ill Patients (in ICU)	Plasma	Patients with highest quartile of mtDNA in plasma had higher risk of dying. When stratified by TLR9 expression, only patients with high expression of TLR9 had an association between with mortality and mtDNA level.	(Krychtiuk *et al.*, 2015)
	Out of Hospital Cardiac Arrest	Plasma	Significantly higher levels in non-survivors than survivors	(Arnalich *et al.*, 2012) %
Liver Failure				
	Acetaminophen induced acute liver failure	Serum	mtDNA higher in acetaminophen induced acute liver failure patients compared to healthy controls mtDNA higher in in non-survivors compared to survivors	(McGill *et al.*, 2014)
	Acetaminophen induced acute liver injury	Plasma	mtDNA higher in patients with acetaminophen overdose with abnormal liver function tests compared to healthy controls and those with acetaminophen overdose but normal liver function tests	(McGill *et al.*, 2012)
	Fulminant liver failure	Serum	Higher during acute liver injury	(Marques *et al.*, 2012)
Heart disease				
	AMI	Plasma	Significantly higher mtDNA in STEMI patients than stable angina pectoris patients (reducing rapidly to similar levels 3 days after PCI)	(Bliksøen *et al.*, 2012)
	AMI	Plasma	Significantly higher levels in AMI patients compared to healthy controls.	(L. L. L. Wang *et al.*, 2015)

			Levels dropped to normal immediately post PCI.	
	AMI	Plasma	Significantly higher levels in acute AMI compared to healthy controls on admission	(Qin *et al.*, 2016)
	T2DM with CAD	Plasma	Significantly elevated levels in T2DM compared to healthy controls. Higher levels in those with DM & CAD compared to those without CAD. mtDNA levels correlated with CRP in patients with CAD.	(J. Liu *et al.*, 2015)
	Heart failure	Plasma	Higher levels of mtDNA in heart failure patients compared to age and sex matched healthy controls; no association with disease severity	(Dhondup *et al.*, no date)
	Heart failure	Plasma	Higher levels of mtDNA in acute vs. chronic heart failure; in acute heart failure, mtDNA predicted mortality	(Krychtiuk *et al.*, 2017)
Stroke				
	Acute ischaemic stroke	Plasma	mtDNA levels higher in acute cerebral infarction than healthy controls No significant difference in mtDNA between good vs poor outcome cohorts	(Lakra *et al.*, 2011)
	Subarachnoid haemorrhage	Plasma	No significant difference in mtDNA between subarachnoid haemorrhage and healthy control groups	(Wang *et al.*, 2013)
	Intracerebral haemorrhage	Plasma	No significant difference in mtDNA between intracerebral haemorrhage and healthy control groups No correlation between mtDNA and disease severity	(Wang *et al.*, 2012)
Malignancy				
	Breast Cancer	Plasma	Reduced levels of mtDNA in benign or malignant breast cancer compared to healthy controls	(Kohler *et al.*, 2009)
	Ovarian Cancer	Plasma & Serum	Plasma: Significantly higher levels of mtDNA in ovarian cancer group compared to healthy controls and ovarian benign tumour group Serum: No significant difference between groups above	(Zachariah, Schmid and Buerki, 2008)

Testicular Germ Cell Cancer	Serum	mtDNA levels were significantly higher in patients with testicular cancer than healthy controls although it did not correlate with any clinicopathological variable of disease status	(Ellinger *et al.*, 2009)
Urological malignancies	Serum	mtDNA were significantly higher in 'urological malignancies' (bladder cell, renal cell and prostate cancer)	(Ellinger *et al.*, 2012)
Prostate Cancer	Serum	mtDNA could not distinguish between benign prostatic hypertrophy and prostate cancer. Patients with early biochemical recurrence post radical prostatectomy have higher mtDNA levels	(Ellinger *et al.*, 2008)
Ewing's Sarcoma	Serum	mtDNA significantly lower in patients with Ewing's sarcoma compared to healthy controls	(Yu *et al.*, 2012)
Lung Cancer	Serum	mtDNA significantly higher in lung cancer patients compared to those with benign lung diseases and healthy individuals and closely associated with TNM stage	(Hou *et al.*, 2013)
Advanced Prostate Cancer	Plasma	mtDNA levels are elevated in advanced prostate cancer patients and is associated with decreased survival.	(Mehra *et al.*, 2007)
Adenocarcinoma of the lung in patients receiving erlotinib	Plasma	Rise in mtDNA levels in patients with partial response; drop in mtDNA levels in those with progressive disease or no response. No correlation with progression free survival.	(Huang *et al.*, 2014)
Exposure to carcinogenic Hal-Alkane-Based Pesticides	Serum	Exposure to these carcinogens was significantly associated with elevated serum levels of circulating mtDNA (case control study)	(Budnik *et al.*, 2013)
Renal Cell Carcinoma	Plasma	Higher levels in metastatic compared to non-metastatic patients and controls.	(Lu *et al.*, 2016)

HIV				
	HIV	Plasma	Higher levels in acute HIV infection, late presenters compared to long term non-progressors and healthy controls. Also correlated with viral load.	(Cossarizza *et al.*, 2011)
	Lipodystrophy in HIV patients treated with HAART	Plasma	Significantly higher levels in HIV infected vs non-infected individuals. Significantly higher levels in those with lipodystrophy compared to those without lipodystrophy at month 24.	(Dai *et al.*, 2015)
	HIV	Plasma	No significant association between HIV disease status and mtDNA	(Lauring *et al.*, 2012)
Inflammatory autoimmune conditions				
	Rheumatoid Arthritis	Plasma	Higher percentage of detectable levels in rheumatoid arthritis compared to controls	(Hajizadeh *et al.*, 2003) $
	Granulomatosis with polyangiitis	Serum	Significantly higher levels in granulomatosis with polyangiitis compared to controls	(Surmiak *et al.*, 2015)
Age & Exercise				
	Age	Plasma	mtDNA levels increased gradually after the fifth decade of life	(Pinti *et al.*, 2014)
	Age	Plasma	No association with age but mtDNA associated with HLA-DR	(Verschoor *et al.*, 2015)
	Aging and 'frailty'	Plasma	Aging: no difference in mtDNA between younger and older subjects Frailty: mtDNA copy number directly correlated with frailty score	(Jylhävä *et al.*, 2013)
	Exercise	Plasma	Reduced mtDNA in response to exercise	(Shockett *et al.*, 2016)
	Male Volleyball Players	Plasma	Lower levels in participants in professional volleyball players compared to healthy nonathlete controls	(Nasi *et al.*, 2015)

Psychiatric disorders				
	Bipolar Disorder	Serum	No difference between bipolar disorder & healthy control groups Higher levels in bipolar disorder group compared to sepsis	(Stertz *et al.*, 2015)
	Suicide attempters	Plasma	Higher plasma levels of mtDNA in suicide attempters compared with healthy controls	(Lindqvist *et al.*, 2016)
	Major depressive disorder	Plasma	Higher plasma levels of mtDNA in major depressive disorder compared to controls	(Lindqvist *et al.*, 2018)
	Major depressive disorder and biopolar disorder	Plasma	Lower plasma mtDNA levels in major depressive disorder and bipolar disorder compared to healthy controls	(Kageyama *et al.*, 2018)
Miscellaneous				
	Corrosive injury (gastrointestinal ingestion)	Plasma	Significantly higher mtDNA in mortality group vs survival group at presentation and after 12 hours	(Chou *et al.*, 2008)
	Pulmonary Embolism	Plasma	Predictor of 15-day mortality	(Arnalich *et al.*, 2013) *
	Autism	Serum	Significantly higher mtDNA in young autistic children compared to healthy controls	(van Rossum *et al.*, 2010)
	Haemodialysis	Plasma	Significantly higher levels in maintenance haemodialysis patients compared to healthy controls	(Cao *et al.*, 2014)
	End-stage renal failure in Han population	Plasma	End-stage renal failure patients had higher mtDNA copy number	(Zhang *et al.*, 2016)

Low levels of ionising radiation	Serum	Higher levels in interventional cardiologists exposed to low levels of ionizing radiation compared to controls	(Borghini *et al.*, 2015)
Friedreich's ataxia	Plasma	Significantly reduced mtDNA in Friedreich's ataxia patients compared to healthy controls	(Dantham *et al.*, 2016)
Non-haemolytic transfusion reaction	Platelet concentrates	Higher mtDNA copy number in non-haemolytic transfusion reaction platelet concentrate vs normal platelet concentrate	(Yasui *et al.*, 2016)
Cardiopulmonary bypass surgery	Plasma	Higher mtDNA post cardiopulmonary bypass; patients with post-operative atrial fibrillation had greater increases in mtDNA post-surgery	(Sandler *et al.*, 2018)

Note: this table lists studies reporting mtDNA analysed by PCR on serum or plasma (i.e. circulating as a DAMP) in human diseases

* earlier study in 2010 not included

% letter

conference abstract only

$ PCR rather than qPCR used

1.4 Hypothesis and aim

Aim

The aim of this thesis is to investigate the role of mtDNA in IBD.

Hypotheses

Primary hypothesis: Circulating mtDNA is elevated in IBD. *(Chapter 2)*

Secondary hypotheses:

- Other circulating mitochondrial DAMPs are elevated in IBD *(Chapter 2)*
- Circulating mtDNA can be used as a novel biomarker in IBD *(Chapter 3)*
- mtDNA is released locally at sites of inflammation in IBD *(Chapter 4)*

CHAPTER 2:

CIRCULATING MITOCHONDRIAL DAMPS IN IBD

Chapter 2 Contents

2.1 Introduction

The pathogenesis of IBD is complex and remains to be fully elucidated. One area of limited exploration thus far has been the role of DAMPs in instigating and/or propagating sterile inflammation which has been found to be relevant in several other inflammatory diseases. Mitochondrial DAMPs are of particular interest given their high inflammatory potential.

The mitochondrial DAMP that has attracted the most interest thus far is mtDNA, which is highly enriched with hypomethylated CpG repeats resembling bacterial DNA. A further shared feature of the mitochondria with bacteria is the production of short *N*-formylated peptides. Bacterial and mitochondrial proteins are the only source of *N*-formylated peptides in nature and mitochondrial formylated peptides (also released as mitochondrial DAMPs post cellular rupture) have been found to have important roles in sterile inflammation especially as chemoattractants.

Mitochondrial DAMPs can be detected in the circulation and have been found to be raised in a number of inflammatory diseases. These include diseases of acute and chronic inflammation such as trauma, sepsis, AMI, T2DM and stroke. Although many DAMPs are elevated in IBD, there has been no data to date demonstrating elevated levels of mitochondrial DAMPs in IBD. Mitochondrial DAMPs are of particular interest in IBD given the recent findings by our group of gut mitochondrial dysfunction leading to loss of epithelial barrier function and development of colitis (Ho *et al.*, 2018).

This chapter deals with the primary aim of this thesis which is to test the hypothesis that patients with active inflammatory bowel disease have higher levels of circulating mitochondrial DAMPs compared to non-IBD controls. For the reasons previously outlined, it was determined that a well characterised, prospectively recruited cohort of IBD patients was required. The recruitment inclusion criteria were kept deliberately broad given the role of mitochondrial DAMPs in IBD has yet to be defined.

mtDNA was chosen as the primary mitochondrial DAMP for investigation for several reasons. A paper published in *Nature* in 2010 (Zhang *et al.*, 2010) observed elevated levels of mtDNA in patients with SIRS and sparked significant interest and further research in this area as a potentially functional and clinically useful biomarker (Boyapati *et al.*, 2017). Furthermore, colleagues at the Centre for Inflammation Research (Edinburgh) had recently developed and optimised the qPCR protocol for quantification of mtDNA (Dorward, 2014) based on the method initially described years previously (Chiu *et al.*, 2003) and subsequently used by Zhang *et al*.

To show corroborative evidence of mitochondrial DAMPs being released into the circulation, a second mitochondrial DAMP (*N*-formylated peptides) was also measured in some patients.

2.2 Materials & Methods

2.2.1 Study design

This was a single centre, prospective cohort study.

2.2.2 Funding and ethics

This work was supported by MRC grant G0701898, Crohn's and Colitis United Kingdom (UK) M16-1 to Gwo-tzer Ho; Edinburgh Gastrointestinal Trustees Grant (2014) to Ray Boyapati and Wellcome Trust grant WT096497 to David Dorward and Adriano Rossi.

All clinical and biological material/data acquisition were carried out under Lothian Bioresource ethics approval 15/ES/0094.

2.2.3 Recruitment of study participants

Individuals were recruited from outpatient and inpatient settings from the Gastrointestinal Unit, Western General Hospital, Edinburgh between April 2014 and November 2015. Patients with IBD of either major type (CD or UC) and with a range of severity (from quiescent disease in the outpatient clinic through to severe disease requiring intravenous steroids on the inpatient wards) were considered eligible for inclusion. In addition, individuals with IBS or with no history of IBD and no gastrointestinal symptoms were recruited as

non-IBD symptomatic controls. A call was made for healthy control volunteers at the Queens Medical Research Institute, Edinburgh (those with no history of gastrointestinal illness/diagnosis or other known chronic health conditions). Given the prospective design of our study, we set out to carry out sex- and age-matching. Healthy controls were recruited between January 2015 and June 2015. Individuals were excluded if they were younger than 18 or were unable to give written consent.

For the IBD cohort, recruited patients fulfilled the criteria of CD or UC based on clinical, endoscopic and histological criteria (Lennard-Jones, 1989). IBS individuals (non-IBD symptomatic controls) had altered bowel habit and were defined following normal ileo-colonoscopy, stool calprotectin and blood parameters.

2.2.4 Information/samples collected

At initial contact, patients were provided with a patient information sheet and provided time to consider participation in the study. If the patient agreed to participate, two consent forms (one for use of questionnaire data and one for use of samples) and a comprehensive IBD questionnaire (Appendix B) was completed at first recruitment. The comprehensive IBD questionnaire included information on ethnic origin, country of birth, social history, smoking/alcohol history, childhood exposures, family and medical history, IBD history, medication history and pregnancy status.

An additional clinical activity form (Appendix C) was completed with the assistance of the researcher at the time of each sample. This form included information on weight, temperature, heart rate, current medications, and information on clinical assessment of disease activity as per two validated scoring indices: the Simple Clinical Colitis Activity Index (SCCAI) for UC (Walmsley *et al.*, 1998) and Harvey Bradshaw Index (HBI) for CD (Harvey and Bradshaw, 1980) (Appendix A). Completed forms were stored securely for entry into a database.

Where appropriate, clinical bloods (full blood examination, albumin and CRP) were collected at the time of recruitment and blood sampling. Where multiple samples were taken for a single patient, the mean values were used in any per-patient analysis.

Overall, the aim was to collect clinical and biochemical data to measure disease type, activity, severity and extent. A further aim was to collect some longitudinal data to assess for variation in mtDNA over short and long time frames and thus, for some patients, multiple sample points were taken.

2.2.5 Clinical disease activity stratification

For UC and CD, disease activity was classified using the SCCAI and HBI respectively (Appendix A). Clinical remission was defined as SCCAI of less than or equal to 2 for UC patients, and HBI of less than 5 for patients with CD.

Patients with clinical active disease (i.e. not in clinical remission) were further classified into severe disease if they required intravenous steroids for CD or fulfilling the modified Truelove & Witts' criteria for ASUC (Appendix A).

2.2.6 Plasma and serum sampling & processing

Venepuncture was performed by a trained clinician with a 21-gauge butterfly needle. 12-18mL of blood including at least 9mL in an ethylenediaminetetraacetic (EDTA) acid tube (Vacuette ®) for plasma and 1 x serum tube (Vacuette ®) was collected and processed within 2 hours of collection.

EDTA blood was centrifuged at 1000g for 10 minutes at 4^{o}C and the plasma fraction was transferred to a 15mL Falcon ® tube; this was then centrifuged at 5000g for 10 minutes at 4^{o}C to remove platelets and microparticles in order to achieve 'cell free plasma'.

Serum was stored for 1-hour storage at 4^{o}C for clotting and then centrifuged at 2,500g for 10 minutes at 4^{o}C. Both serum and plasma fractions were divided into 0.5mL aliquots and stored at -80^{o}C until further use.

At the same venepuncture, bloods requested by clinicians caring for the patient (in the IBD or IBS cohorts) were taken and sent to the Western General Hospital (Edinburgh) clinical laboratory for full blood examination, albumin and CRP.

2.2.7 Mitochondrial DNA

The methods described below were largely developed and optimised by Dr David Dorward at the MRC Centre of Inflammation Research (Edinburgh) as part of his research into the contribution of mitochondrial formylated peptides to the pathogenesis of acute lung injury (Dorward, 2014; Dorward *et al.*, 2017). The methods with amendments are reproduced here with permission.

2.2.7.1 Isolation of DNA from serum & plasma

DNA was isolated from serum and plasma using an automated robotics platform (QIAcube, Qiagen Qiagen, Valencia CA, USA) at the same time to ensure uniformity. QIAamp DNA Blood mini kits were used as per manufacturer's instructions (blood and body fluids protocol, Qiagen) as described previously (Chiu *et al.*, 2003; Zhang, Itagaki and Hauser, 2010).

Plasma and serum samples were initially centrifuged at 9,500g for 10 minutes and 200µL of sample added to 20µL QIAGEN protease in a 1.5mL Eppendorf tube. After the addition of 200µL of Buffer AL samples were vortexed for 15 seconds and incubated at 56°C for 10 min. Ethanol was then added (200µL), samples vortexed, transferred to a QIAamp mini spin column and centrifuged at 6,000g for 1 min. The collection tube and elute were discarded and the column placed in a clean tube. 500µL of Buffer AW1 was then added, samples centrifuged again at 6,000g for 1 minute and flow through discarded. Following the addition of 500µL Buffer AW2 columns were centrifuged at 16,000g for 3

minutes and then again for a further 1 minute to ensure removal of any ethanol carry-over. Spin columns were transferred to clean 1.5mL Eppendorf tubes and 50µL of Buffer AE added to each filter. Following 5 minutes incubation at room temperature samples were centrifuged at 6,000g for 1 minute and the elute stored at -20°C until analysed.

2.2.7.2 Developing standard curves for qPCR

Isolation of mitochondria

Whole mitochondria were extracted from cultured HepG2 cells selected due to their high metabolic capacity and abundant mitochondria. Mitochondria were extracted from cultured HepG2 cells using the Mitochondrial Isolation kit for cultured cells (Sigma) using the manufacturer's instructions.

Adherent HepG2 cells were detached from their cell culture flasks with trypsin-EDTA and RPMI with 10% FCS was added. Cells were pelleted at 600g for 5 minutes at 2°C and then washed (cells were resuspended in ice cold PBS and counted using a haemocytometer, then pelleted at 600g for 5 minutes). 2x107 cells were used for each mitochondrial preparation. The wash step was then repeated without counting the cells. 2mL of the prepared Extraction Buffer A was added and incubated on ice for 15 minutes. Cells were then homogenised using a Dounce homogenizer. Homogenisation was performed gradually and followed by staining an aliquot with trypan blue and counting the cells under a

microscope, diluting if necessary to count the cells. An additional 5 strokes were performed until at least 50% of cells were damaged.

The homogenate was then centrifuged at 600g for 10 minutes at 2°C. The supernatant was then transferred to a fresh tube and centrifuged at 11,000g for 10 minutes at 2°C. The supernatant was removed, and the pellet was resuspended in 200 µL of CelLytic M Cell Lysis Reagent with Protease Inhibitor Cocktail (1:100 [v/v]). Isolated mitochondria were stored at -20^{o}C until further use.

Extraction of DNA from isolated mitochondria

DNA was extracted from isolated mitochondria using the QIAamp DNA Micro Kit (Qiagen, Valencia CA, USA) as per the manufacturer's instructions.

50µL of media containing isolated mitochondria was added to 50µL of Buffer ATL in a 1.5mL Eppendorf tube. 10µL of proteinase K and 100µL of Buffer AL was then added, and mixed by pulse-vortexing for 15 seconds and incubated at 56°C for 10 min. 50µL of ethanol (96-100%) was then added, vortexed, and incubated at room temperature (15-25°C) for 3 minutes. The entire lysate was then transferred to a QIAamp mini spin column and centrifuged at 6,000g for 1 minute. The collection tube and elute were discarded and the column placed in a clean tube. 500µL of Buffer AW1 was then added, centrifuged again at 6,000g for 1 minute and column placed in a clean tube. Following the addition of 500µL Buffer AW2 columns were centrifuged at 6,000g for 1 minute and

then the column was placed in a clean tube. This was then centrifuged at 20,000g for 3 minutes to dry the membrane completely. Spin columns were transferred to clean 1.5mL Eppendorf tubes and 50µL of Buffer AE added to each filter. Following 5 minutes incubation at room temperature, samples were centrifuged at 20,000g for 1 minute and the elute stored at -20°C until analysed.

Purity of the DNA was determined using Nanodrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) and all DNA samples had OD260/OD280 values of 1.7-2.0.

Amplification of mtDNA primer products

Conventional PCR was performed using primers against mtDNA coding for nicotinamide adenine dinucleotide dehydrogenase (ND2) and cytochrome C oxidase subunit III (COXIII). COXIII and ND2 primers were selected as primer sequences (Appendix D) as they have been previously described for quantification of mtDNA (Lu *et al.*, 2010; Zhang *et al.*, 2010) and are specific for human mtDNA. Primers were blasted against human genome as well as known bacteria to ensure selectivity for human mtDNA (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

mtDNA primer products were amplified from isolated mtDNA by conventional PCR.

The reaction mix contained:

- 2x PCR master mix 50µl
- 100µM forward primer 1µl
- 100µM reverse primer 1µl
- DNA 10µg
- ddH_2O Added for final volume of 100µl

PCR conditions were

- Stage 1 95^oC for 2 minutes
- Stage 2 40 cycles of 95^oC for 30 seconds,
  58^oC for 30 seconds
  72^oC for 30 seconds
- Stage 3 72^oC for 5 minutes

Primer validation

To confirm the PCR product size was as expected, the PCR product was run on a 4% agarose gel (2g of agarose resuspended in 50mL 1x TBE with 5µl Gel Red added to the solution once the agarose dissolved). 10µl of PCR product was used and samples run next to a 100bp DNA ladder. The gel was run at 120V for 55-60 minutes until the leading edge reached the distal gel edge with products subsequently visualised using an ultraviolet illuminator (UV Transilluminator, UVP Inc). PCR product size was as expected (103bp for COXIII, 90bp for ND2) (Figure 2.1).

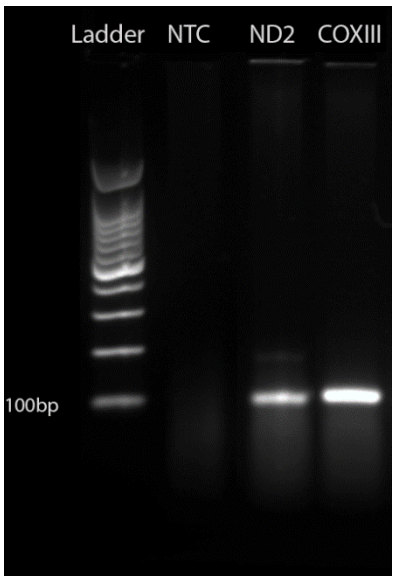

Figure 2.1: Gel confirming amplified PCR primer products

In addition, ND2 and COXIII mtDNA primer specificity has been previously demonstrated within biological systems by demonstrating minimal PCR products seen in mitochondrial depleted cells (Dorward, 2014). In brief, A549 and Beas 2B cells were cultured with ethidium bromide for 6 weeks to deplete mitochondria as described (King and Attardi, 1989; Crouser *et al.*, 2009) after which DNA and protein were extracted. Primer specificity was demonstrated with minimal PCR products seen in mitochondrial depleted (Ro) cells following agarose gel electrophoresis relative to untreated control cells cultured for the same duration. Furthermore, there was no observable loss of nuclear DNA content as assessed by qPCR.

Generation of Standard Curves

Standard curves for absolute quantification of circulating free mtDNA were generated using the initial method described for quantification of plasma mtDNA (Chiu *et al.*, 2003). The QIAgen PCR Purification kit (Qiagen) was used on amplified primer products (as described above) in accordance with manufacturer's instructions. In brief, 500µL of Buffer PB was added to 100µL PCR product, transferred to a spin column and centrifuged at 6,000g for 1 minute before 750µL Buffer PE was added and the column centrifuged. The DNA was then eluted with 50µL Buffer EB into a sterile 1.5mL Eppendorf and stored at -20°C until use. Isolated DNA was quantified by nanodrop (ThermoScientific) and serially diluted. mtDNA concentration in copies/µL was determined using the formulae described below in Material and Methods section 2.2.7.3.

2.2.7.3 qPCR protocols for absolute quantification

Primers (MWG Eurofins) were suspended at 100µM stock solution with DEPC-treated water and stored at -20°C prior to use. Subsequently, 20x primer solution (1.8µM) was made (3.6µL forward, 3.6µL reverse, 192.8µL DEPC-H_2O). In MicroAmp® Optical 384-Well Reaction Plates (Applied Biosystems) 7µl of master mix containing 5µL 2x SYBR Green Fast mix (Applied Biosystems), 0.5µL 20x primer mix and 1.5µl DEPC-treated water was mixed with 3µl of isolated DNA sample or standard. All reactions were carried out in duplicate, and discordant results retested. All plates contained wells with no DNA as a negative, no template control.

qPCR reactions were conducted in an ABI7900 Fast Real-Time PCR System (Applied Biosystems) with the following settings:

- Stage 1 — 95°C for 20 seconds
- Stage 2 — 40 cycles of 95°C for 3 seconds & 60°C for 30 seconds
- Melt curve — 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, 60°C for 15 seconds

Absolute quantification of mtDNA was determined relative to the standard curve based on the following equation (as described by Chiu *et al.* (Chiu *et al.*, 2003)): $C = Q \times V_{DNA}/V_{PCR} \times 1/V_{ext}$

- C — target concentration in plasma or serum (copies per millilitre)
- Q — target quantity (copies) determined by sequence detector in PCR
- V_{DNA} — total volume of DNA obtained after extraction, typically 50µl per extraction
- V_{PCR} — volume of DNA solution used for PCR, typically 3µl
- V_{ext} — volume of plasma, typically 200µl

Amplification efficiency of between 90 and 110% was taken as acceptable where slope refers to the gradient of the standard curve: (Efficiency = $10^{(-1/slope)}$ – 1). The coefficient of determination value was also calculated with $r^2>0.985$. SYBR green analysis meltcurves were run to identify the presence of any primer dimer peaks.

2.2.8 Mitochondrial formylated peptides

Mitochondrial *N*-formylated peptides are functionally similar to their bacterial counterparts acting as primarily as neutrophil chemoattractants (Carp, 1982). To provide further corroborative evidence to our mtDNA data, we employed a mass spectrometric approach to identify and quantify *N*-mitochondrial formylated peptides in a subset of 5 acute severe UC vs. 5 non-IBD controls. The 10 patient samples for analysis were picked at random from the recruited patient population of ASUC (5 samples) and non-IBD (5 samples).

Mass spectrometry was performed by Dr Mary Doherty and Professor Phil Whitfield at the University of the Highland and Islands using their previously optimised protocol as outlined in (Dorward *et al.*, 2017). The methods are reproduced here with permission.

Synthetic peptides

N-formylated hexapeptides were identified on the basis of their accurate mass, retention times and characteristic fragmentation patterns compared to custom synthesised standards (Peptide Protein Research Ltd, Fareham, UK). The hexapeptide standards of the N-terminal fragments of each of the 13 mitochondrial formylated peptides were based on sequences previously described (Rabiet, Huet and Boulay, 2005) (Appendix E). They were resuspended in methanol:H_2O (1:1) to a 1mg/mL stock solution, serial dilutions using 0.5% acetic acid made and analysed by LC MS/MS.

Mass Spectrometry analysis of standard peptides

The standards were first infused into the mass spectrometer to determine the molecular ion and then each fragmented using both collision induced dissociation (CID) and higher collision dissociation (HCD). A 5µL aliquot of each sample was analysed by LC-MS/MS using a Thermo LTQ-Orbitrap XL LC-MS^n mass spectrometer equipped with a nanospray source and coupled to a nanoUPLC (Waters nanoAcquity) The peptides were loaded onto a Symmetry C18 trap column (5µm particle size, 180µm x 20mm, Waters) and then were separated using a BEH130 C18 analytical column (1.7µM particle size, 75µm x 250mm) with an acetonitrile/water gradient and MS spectra collected using data-dependent acquisition in the range m/z 400-1300. Peptides were fragmented using both CID and HCD. A mixture of the standard peptides was also prepared and analysed in the same way. The analyses were repeated at a range of concentrations and standard curves plotted for the peptides analysed individually (500fg-500pg) and as the combined samples (250fg-250pg on column).

Separation of protein and peptide containing fractions from patient plasma

Plasma samples were each centrifuged at 13,000rpm for 1 minute to remove any residual cellular debris. To precipitate proteins 100µL sample was added to 1000µL acetone and incubated at -20°C for 1 hr. Following centrifugation at 13,000 rpm for 1 minute the peptide-rich acetone layer was aspirated and acetone then evaporated at 40°C in a Speed Vac for 1 hr until approximately

50µL of acetone remained. Peptide samples were diluted either 10- or 100-fold in 0.5% acetic acid then centrifuged at 13,000rpm for 4 minutes prior to analysis as described for the standard peptides.

Analysis of protein-derived peptides by LC-MS/MS

Plasma samples (100µL) were acetone precipitated, dried down under vacuum and reconstituted in 0.5% acetic acid. Peptides were then analysed by LC-MS/MS in positive ion mode using a Thermo LTQ-Orbitrap XL mass spectrometer (Hemel Hempstead, UK) coupled to a Waters nanoAcquity UPLC system (Manchester, UK) with a linear gradient over 39 minutes (mobile phase A: 0.5% acetic acid in water; mobile phase B: 0.5% acetic acid in acetonitrile). Quantification was achieved using a corresponding stable isotope labelled internal standard and calibration curve for each *N*-formylated hexapeptide.

The peptides were separated using BEH130 C18 trapping and analytical columns with an acetonitrile/water gradient and MS spectra collected using data-dependent acquisition in the range m/z 400-1300. Peptides were fragmented using both CID and HCD and the data analysed using ProteomeDiscoverer for identification MaxQUANT for relative quantification.

2.2.9 Statistical Analysis

Data are presented as numbers, percentages, means ± standard error of the mean (SEM) and medians ± interquartile range (IQR) for parametric and non-parametric data respectively. Student t- and Mann-Whitney statistics were used for parametric and non-parametric data respectively. The sensitivity, specificity, and likelihood ratio for mtDNA, CRP and albumin levels to predict the need for colectomy in ASUC were calculated using receiver operator characteristic (ROC) curve analysis. Area under ROCs curves (AUROCs) were calculated for each biomarker, and differences between AUROCs compared using the DeLong method. Kaplan – Meier survival analysis was used to compare the course of disease between patients with high and low mtDNA levels.

Multivariate logistic regression was performed to assess variables predictive of high mtDNA. Wilcoxon matched-pairs signed rank test was used to determine the difference in matched pre- and post-colectomy mtDNA levels and matched faecal mtDNA levels using two protocols. The Delong method was used to compare AUROCs for ability to predict colectomy. Spearman's correlations were calculated to evaluate the relationship between mtDNA level and other biochemistry, and between *COXIII* and *ND2* qPCR results.

Statistical analyses were performed using Graphpad Prism version 7 (Graphpad Software, San Diego, California, USA) and SPSS version 22 (IBM Corp., Chicago, USA). Two-sided p values of <0.05 were considered statistically significant.

2.3 Results

2.3.1 Study recruitment

97 IBD patients (67 UC and 30 CD patients), and 40 non-IBD controls (20 healthy [HC] and 20 IBS controls) were prospectively recruited to the study. The first participant was recruited in August 2014 and the final participant in June 2015 (Figure 2.2)

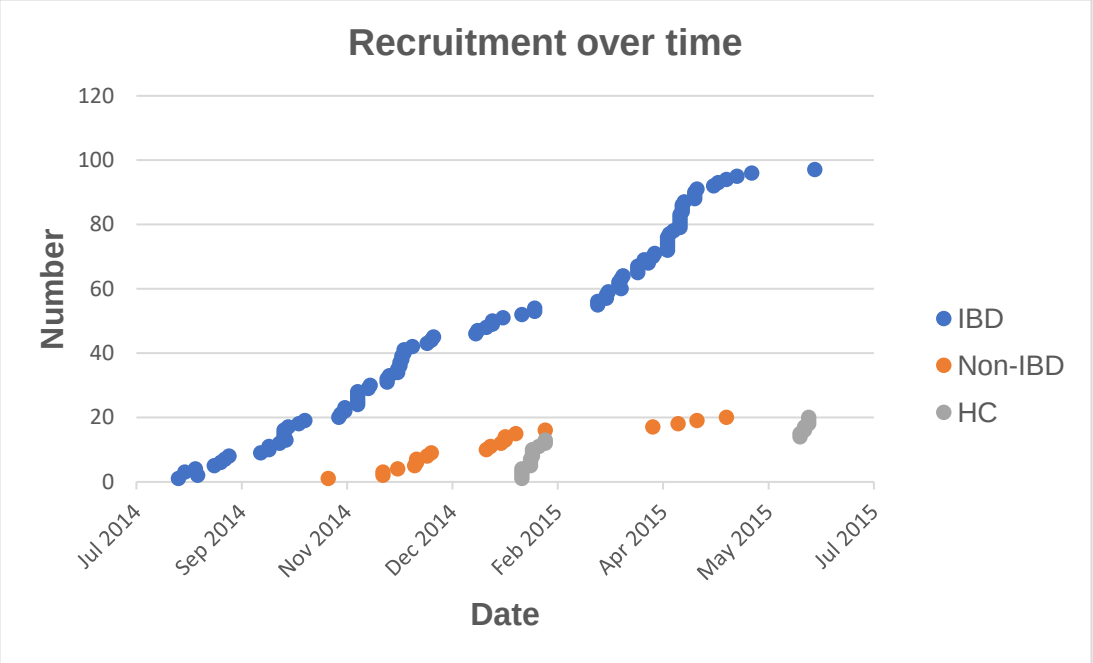

Figure 2.2: Recruitment of IBD, non-IBD and HC cohorts over time

In total, there were 160 sample points for the 97 IBD patients. 58 patients had one sample point, 27 patients had two sample points, 5 patients had three samples points, 2 patients had four sample points and 5 patients had five sample points. Each healthy control and IBS control had one sample point.

2.3.2 Baseline characteristics

The baseline characteristics of the IBD (CD and UC) and Control (HC and IBS) cohorts are summarised in Table 2.1. There were no statistically significant differences between the cohorts based on age, sex or smoking status.

	IBD		**Controls**		**p value**
	CD	**UC**	**HC**	**IBS**	
n	30	67	20	20	
Age	37 (27-44)	36 (28-51)	36 (32-46)	33 (27-42)	NS
M / F	17 / 13	44 / 23	10 / 10	13 / 7	NS
Current smoker	26%	21%	15%	25%	NS

Table 2.1: Demographic details of CD, UC, HC and IBS cohorts; all $p > 0.05$ (one-way ANOVA). NS: not significant

Ambulatory (outpatients) were divided into remission and active disease based on the HBI for CD and the SCCAI for UC (Appendix A). Patients were further categorised into those with severely active disease (hospitalised and receiving intravenous steroids for CD; and hospitalised, receiving intravenous steroids and meeting modified Truelove and Witts' criteria [Appendix A] for UC). The baseline biochemistry and HBI/SCCAI of the IBD cohort based on IBD type and activity is presented in Table 2.2**.**

Crohn's Disease			
	Clinical Remission & Ambulatory	**Clinically Active & Ambulatory**	**Severely active (hospitalised, IV Steroids)**
Hb (g/dl)	145 (137-151)	156 (138-158)	130 (125-137)
WCC (x10^9/L)	7.4 (5.4-15.1)	10.9 (7.7-11.9)	8.75 (7.4-11.6)
Platelets (x10^9/L)	253 (218-295)	509 (300-414)	334 (279-378)
CRP	3.5 (1-7.5)	5 (3.5-8)	26 (12-62)
Albumin	38 (33-40)	38 (37-38)	29 (27-34)
HBI	1 (0-2)	7 (6-9)	7 (4-14)
Ulcerative Colitis			
	Clinical Remission & Ambulatory	**Clinically Active & Ambulatory**	**Severely active (hospitalised, IV Steroids)**
Hb (g/dl)	136 (128-151)	131 (123-146)	114 (104-130)
WCC (x10^9/L)	6.3 (4.7-7.5)	7.7 (6.3-8.8)	11.3 (8.3-14.6)
Platelets (x10^9/L)	292 (252-303)	305 (256-335)	414 (288-501)
CRP	2 (2-5)	3 (2-17)	21 (10-54)
Albumin	39 (38-40)	38 (35-40)	30 (25-34)
SCCAI	0 (0-0)	6 (4-8)	7 (4-10)

Table 2.2: Baseline characteristics of IBD cohorts by IBD type & activity. Data presented as median (±IQR).

As expected, values for biochemistry and clinical activity indices differed between various cohorts as stratified by clinical activity (using Mann-Whitney tests for comparison of non-parametric data). Groups are referred to as 'clinical remission', 'clinically active' and 'severely active' below.

Haemoglobin was significantly lower in severely active CD patients compared to those in clinical remission ($p = 0.04$). For UC patients, haemoglobin was significantly lower in severely active patients compared to clinical remission ($p=0.0007$) and clinically active disease ($p=0.0006$). WCC was significantly higher in severely active UC patients compared to clinical remission ($p=0.0006$) and clinically active disease ($p=0.0006$).

CRP levels in severely active CD disease was significantly higher than those in clinical remission ($p=0.0029$). Severely active UC patients had significantly higher CRP values than those in clinical remission ($p=0.039$) and active disease ($p<0.0001$). CD and UC patients with severely active disease had significantly lower albumin levels than those in clinical remission (CD $p=0.017$, UC $p<0.0001$) and active disease (CD $p=0.0029$; UC $p<0.0001$). Platelets were higher in the UC severely active disease group compared to clinical remission ($p=0.0057$) and active ambulatory disease (0.0022)

As per stratification, HBI was lower in CD patients with remission compared to clinically active disease ambulatory ($p=0.0003$) and severely active disease ($p<0.0001$) but not different between clinically active and severely active (hospitalised) groups. SCCAI was lower in UC patients with remission compared to clinically active disease ambulatory ($p<0.0001$) and severely active disease ($p<0.0001$) but not different between clinically active and severely active (hospitalised) groups.

2.3.3 Reproducibility of the mtDNA assay

Duplicates measurement of mtDNA from plasma using COXIII primers were analysed on the same PCR plate. The coefficient of variation was calculated to be 7.7% (95% CI, 6.9%-8.6%).

2.3.4 Plasma and serum mtDNA correlation

mtDNA levels from paired serum and plasma samples (i.e. from the same patient at the same time) were compared in a subset of samples (n=114). There was a moderate correlation between plasma and serum mtDNA data for COXIII (Figure 2.3, Spearman's r=0.46) and ND2 genes (Figure 2.4, Spearman's r=0.52).

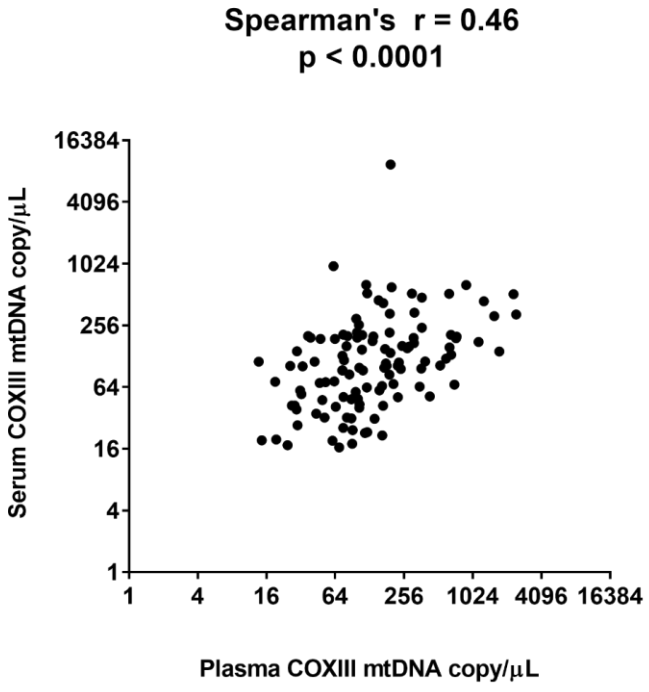

Figure 2.3: Correlation between serum and plasma mtDNA levels for analysed samples using COXIII primers (r=0.46, p<0.0001).

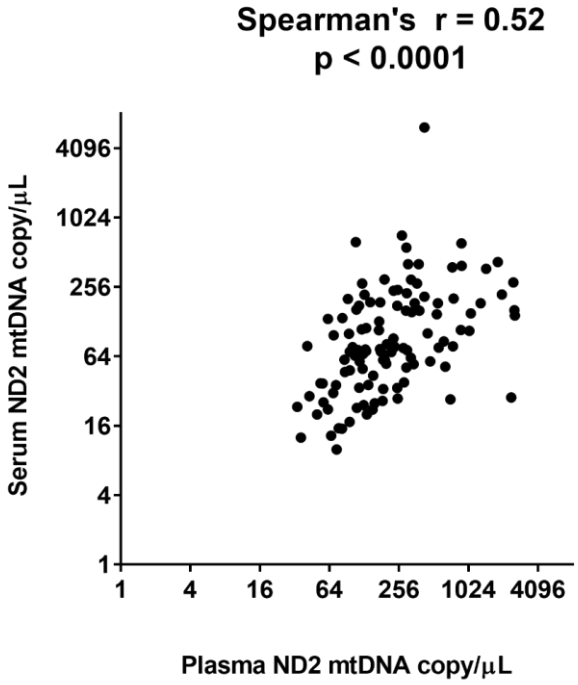

Figure 2.4: Correlation between serum and plasma mtDNA levels for analysed samples using ND2 primers (r=0.52, p<0.0001).

Plasma was chosen as the sample for main analysis due to reasons discussed in detail in Section 2.4.1.1. In brief, most published studies (including the major studies in this area) examining the role of mtDNA in circulation use plasma, and there is a risk of post-sampling liberation of DAMPs with leukocyte rupture in serum processing.

Herein, unless otherwise specified, mtDNA relates to levels detected in plasma.

2.3.5 Correlation between mtDNA genes in plasma

qPCR was performed using 2 sets of primers flanking *COXIII* and *ND2* genes of the mitochondrial genome. *COXIII* and *ND2* data were highly correlated in plasma (r=0.84; p<0.0001) (Figure 2.5) and serum (r=0.93; p<0.0001) (Figure 2.6).

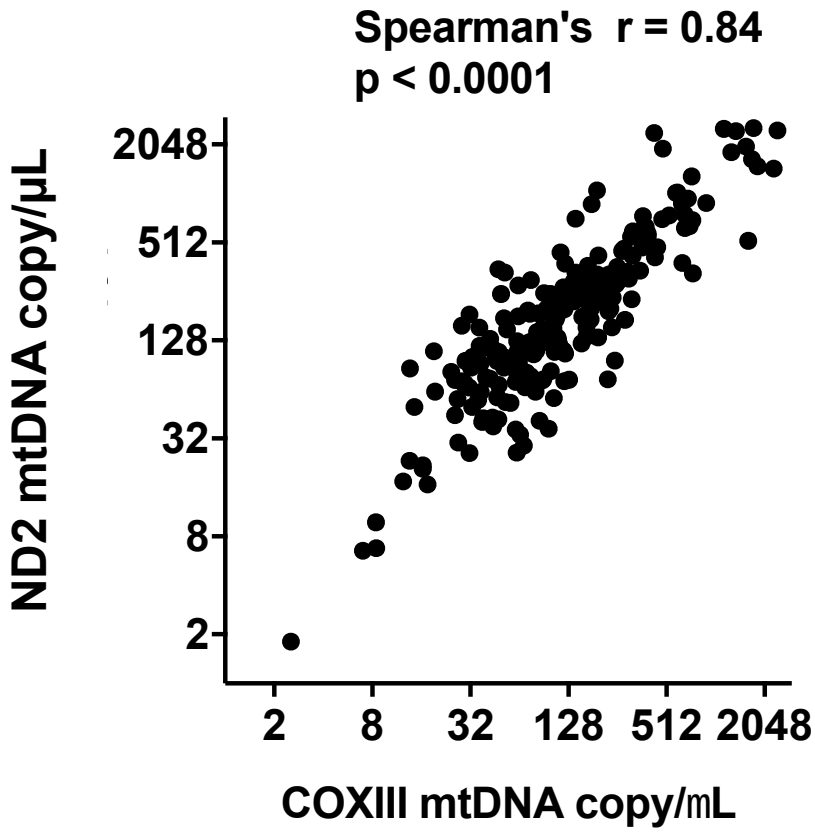

Figure 2.5: Correlation between plasma mtDNA (copy/µL) for all samples using 2 different primers *COXIII* and *ND2* (r=0.84, p=<0.0001).

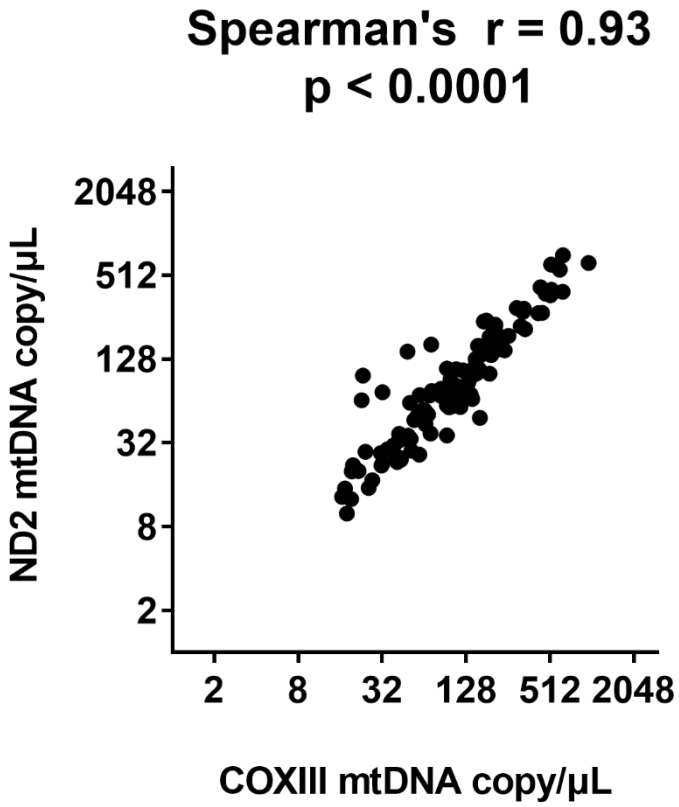

Figure 2.6: Correlation between serum mtDNA (copy/µL) for all samples using 2 different primers *COXIII* and *ND2* (r=0.93, p=<0.0001).

COXIII amplification efficiency as assessed by qPCR analysis of serial dilutions of DNA is slightly higher than ND2 (95% vs 96.4%)(Dorward, 2014). Given the strong correlation between COXIII and ND2 genes, COXIII data was chosen for further analysis and is presented herein.

2.3.6 Higher circulating mtDNA plasma levels in CD and UC

Overall, we found significantly higher levels of circulating cell-free plasma mtDNA in IBD (167.8 copies/µL [IQR 78.06-387.2]) compared to HC (64.6 copies/µL [IQR 51.6-104]) (p=0.0002) and IBS (44.6 copies/µL [IQR 27.9-134.7]) (p<0.0001). There was no difference between HC and IBS, and these groups were combined as non-IBD controls in further analysis (Figure 2.7).

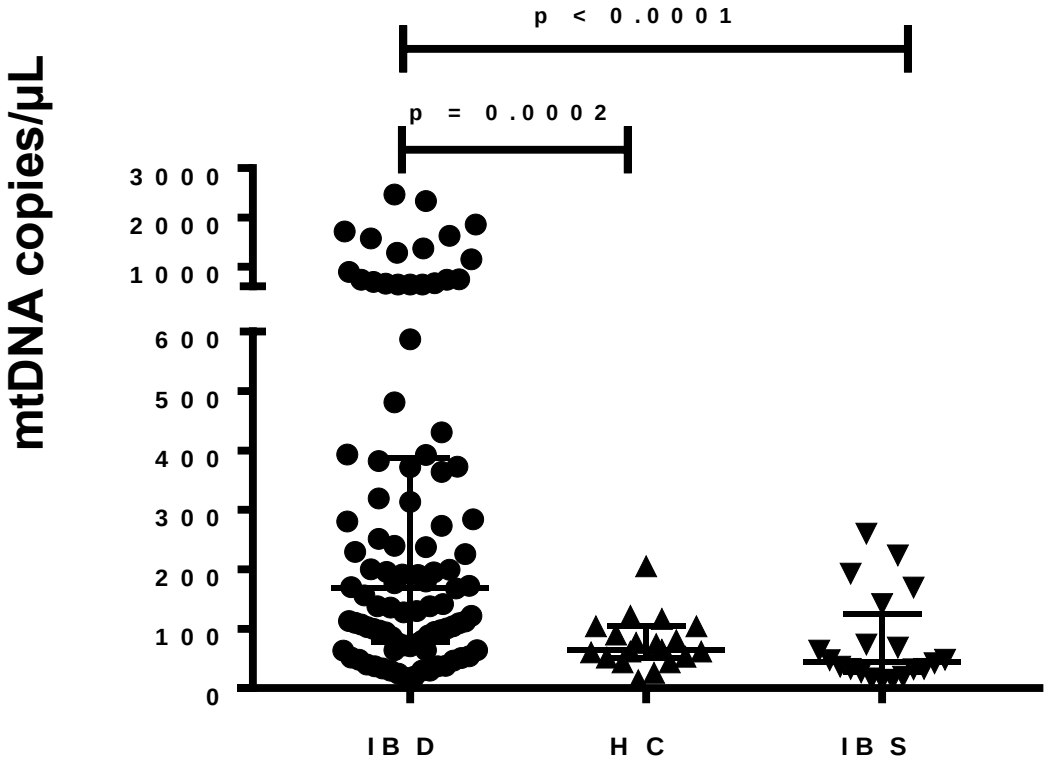

Figure 2.7: Plasma mtDNA (copy/µL) in IBD, HC and IBS (n= 97, 20 and 20 samples respectively). Median ± IQR.

Plasma mtDNA levels were significantly higher in both UC (172.3 copies/µL [IQR 74.4-393.2]) ($p<0.0001$) and CD (136.7 copies/µL [IQR 88.0-370.9]) ($p<0.0001$) compared to non-IBD controls (61.5 copies/µL [IQR 32.8-104]) (Figure 2.8).

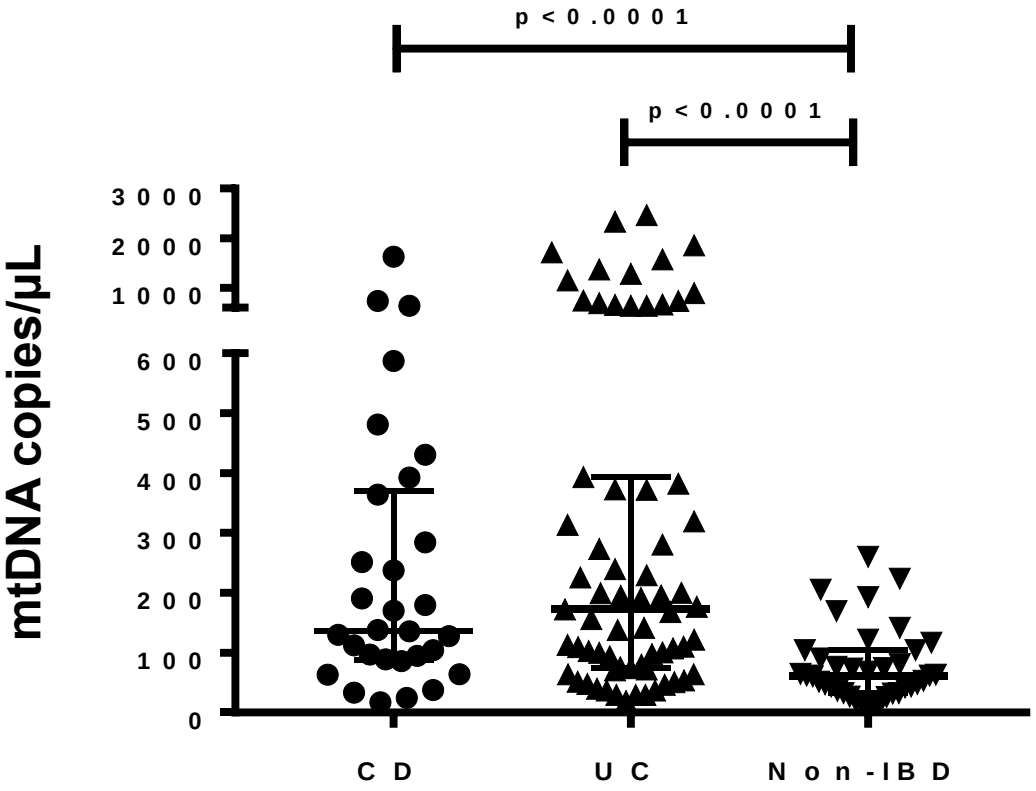

Figure 2.8: Plasma mtDNA (copy/µL) in CD, UC and non-IBD (n= 30, 67 and 40 samples respectively). Median ± IQR.

2.3.7 Mitochondrial *N*-formylated peptides increased in ASUC

A screen for the free *N*-terminal hexapeptides of the thirteen-known mitochondrial encoded proteins (Appendix E) confirmed the presence of five *N*-formylated termini (fMMYALF, fMTPMRK, fMNPLAQ, fMNFALI and fMTMHTT) in acute severe UC plasma samples which were not detected in non-IBD controls. When quantified with synthetic standards, we found that the concentrations of each of these formylated peptides was significantly elevated ($p<0.01$) in acute severe UC (Individual values: Figure 2.9; Summary data: Figure 2.10).

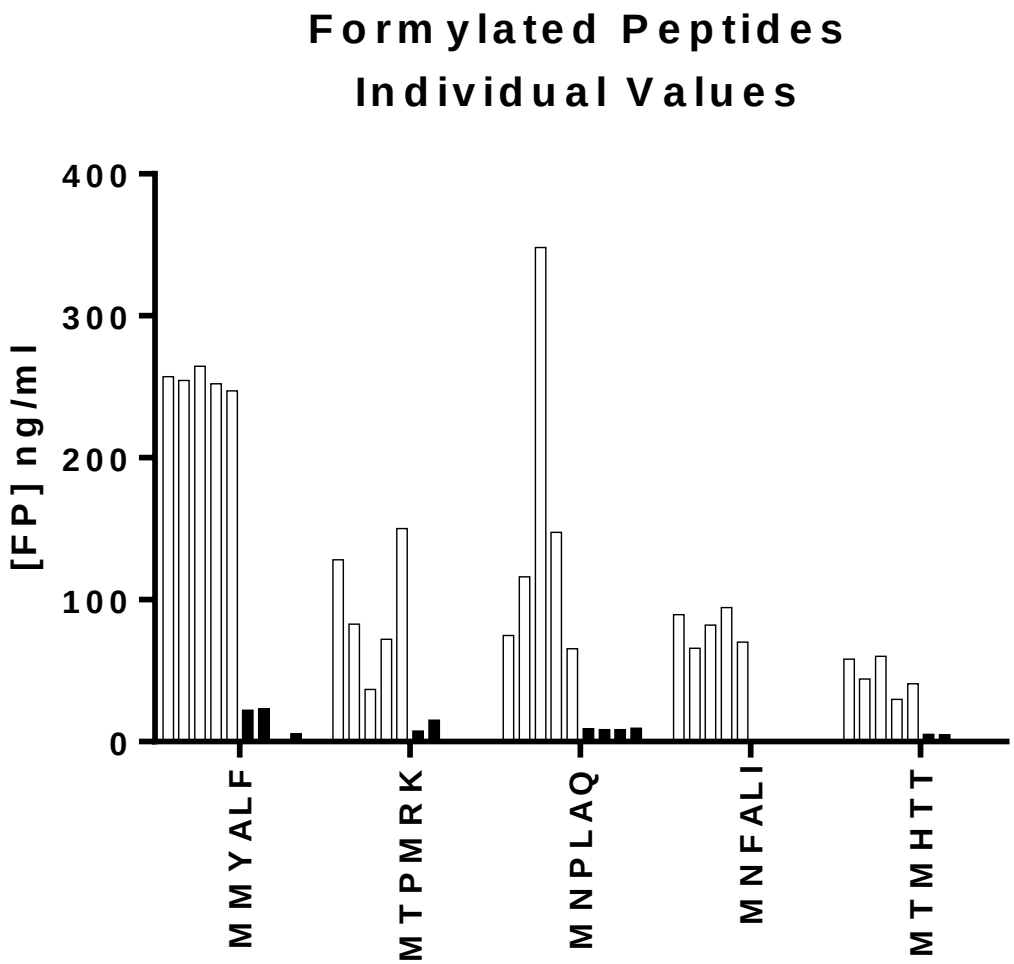

Figure 2.9: Individual values of mitochondrial formylated peptide quantification in 5 UC (white bars) vs. 5 non-IBD controls (black bars) for fMMYALF, fMTPMRK, fMNPLAQ, fMNFALI and fMTMHTT).

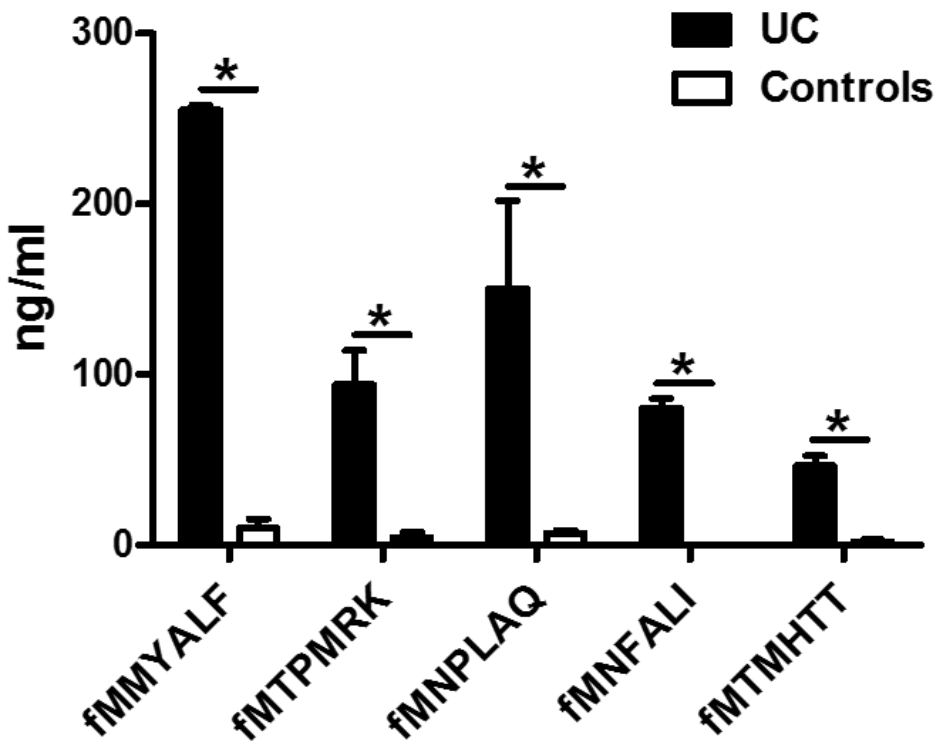

Figure 2.10: Summary data of mitochondrial formylated peptide quantification in 5 UC vs. 5 non-IBD controls (*p=<0.01 for fMMYALF, fMTPMRK, fMNPLAQ, fMNFALI and fMTMHTT). Mean ± SEM.

2.3.8 Summary of results

- The study prospectively recruited 97 IBD patients (67 UC and 30 CD) and 40 non-IBD controls (20 HC and 20 IBS).

- We have for the first time shown that:

    - mtDNA is elevated in the circulation of patients with IBD (in both CD and UC) when compared to non-IBD controls.

    - *N*-formylated peptides (a second mitochondrial DAMP) is elevated in patients with ASUC compared to controls.

- We found a strong correlation between two mitochondrial specific genes (ND2 and COXIII) in mtDNA quantification

- There was a moderate correlation between plasma and serum mtDNA

2.4 Discussion

2.4.1 Sample type, processing and quantification of mtDNA

2.4.1.1 Plasma or serum

Most published studies in inflammatory diseases have used plasma for quantification of circulating mtDNA. In 2003, a sentinel report suggested a standardised quantification method of circulating mtDNA using plasma (Chiu *et al.*, 2003). Serum DNA concentration has been reported as 3 to 24 fold higher compared to plasma (Lo *et al.*, 1998; Lee *et al.*, 2001; Lui *et al.*, 2002; Zhong *et al.*, 2007). One study reported circulating cell free nuclear DNA as being even up to 45 times as high in serum than in plasma (Xia *et al.*, 2009), and mtDNA being 12 fold higher. The higher levels of DNA in serum compared to plasma is likely due to the release of intracellular DNA in the process of fragile cell lysis from clotting (Lee *et al.*, 2001; Thijssen *et al.*, 2002). For this reason, many have suggested that plasma better reflects *in vivo* levels of circulating cell free DNA.

Serum samples appear to produce more variable DNA quantification results compared to plasma (Boddy *et al.*, 2005). One contributing factor is likely to be that plasma is less affected by delays in processing than serum. DNA concentrations in serum samples were 2.3 fold higher than plasma when processed immediately compared to between 3.8 to 4.8 fold higher when

stored for 2-8 hours at room temperature and 3 fold higher when stored for 24 hours at 4^{o}C (Jung and Klotzek, 2003).

Umetani *et al.* examined this issue in more detail with a study comparing serum and plasma DNA levels in patients with various cancers (Umetani, Hiramatsu and Hoon, 2006). The authors found serum had six-fold higher circulating DNA levels compared to plasma in the same patients and the amount of DNA in paired serum and plasma specimens was positively correlated ($r=0.72$, $p=0.0002$). It seems likely that both leukocyte cell lysis during serum separation and the unequal distribution of DNA during separation from whole blood contribute to the higher levels of DNA detected in serum compared to plasma although the precise relative contributions remain unclear (Umetani, Hiramatsu and Hoon, 2006). Furthermore, it is possible that different patient populations and diseases may influence how well circulating DNA correlates between serum and plasma.

Given the large differences in size and structure of nuclear DNA to mtDNA (3 billion base pairs compared to 16,569 base pairs; lack of introns and long noncoding sequences in mtDNA), it may be problematic assuming that the data presented above would apply to mtDNA. However, there is limited direct evidence relating to the differences in detected levels of mtDNA in serum and plasma. Lauring *et al.* noticed that in their healthy blood donors, mtDNA levels as determined by qPCR in the serum and plasma was similar, although this data was not presented (Lauring *et al.*, 2012). Conversely, Zachariah *et al.*

performed an analysis of serum and plasma mtDNA levels in patients with ovarian cancer and found that although there was a difference between mtDNA in healthy controls / benign tumours compared to the ovarian cancer group in plasma, this difference was not evident in serum (Zachariah, Schmid and Buerki, 2008). The serum levels of mtDNA were significantly higher compared to plasma possibly due to release of cellular DNA during clotting.

2.4.1.2 Delays in processing of plasma/serum samples

Specific studies assessing the effect of delays in processing of samples on quantification of circulating mtDNA levels are lacking. Jung *et al.* found no effect in the concentration of DNA in plasma samples for up to 24 hours (Jung and Klotzek, 2003). However, DNA detected in serum samples were significantly higher (approximately double) if centrifugation was delayed for two hours at room temperature compared to if processing occurred immediately. Xue *et al.* more recently found that if processing was delayed by more than two hours post venipuncture, significantly higher levels of DNA were detected in plasma (4-25 hours) irrespective of whether samples were kept on ice or at room temperature prior to centrifugation (Xue *et al.*, 2009).

Irrespective of sample type, it is likely that extensive delays between venipuncture and centrifugation is associated with significant increases in the total DNA copy number detected. This effect is presumably due to leukocyte lysis and this effect appears to be more pronounced the longer the delay until centrifugation.

2.4.1.3 Centrifugation protocols for plasma

The concentration of circulating nucleic acids in plasma varies based on the blood processing protocol used. mtDNA copy number after centrifugation at 1,600g as detected by qPCR was found to be significantly higher compared to centrifugation at 1,600g followed by a further 16,000g spin (Chiu *et al.*, 2003). MtDNA exists in plasma in both particle and non-particle associated forms (Chiu *et al.*, 2003). After separation of plasma from whole blood using a slow speed spin, subsequent filtration or a high speed spin is required to remove particles, cell fragments and platelets to achieve 'cell free plasma' (Chiu *et al.*, 2001). The centrifugal force required for the high speed spin is unclear but common protocols in the literature include 5,000g, 9,600g and 16,000g (Chiu *et al.*, 2001)(Zhang *et al.*, 2010).

Of the particles removed in high speed centrifugation, platelet associated mtDNA seem particularly important. Lauring *et al.* attempted to determine whether residual platelets in processed samples would lead to spurious mtDNA results (Lauring *et al.*, 2012). From stored plasma (previously spun twice at 1,000g/10min), platelets were separated with a subsequent centrifugation at 3,000g for 5 minutes. Although there was a correlation between the mtDNA calculated in each sample ($r = 0.46$), there was significantly less mtDNA detected in the sample with the platelet depleting extra centrifugation step.

Other groups have also examined the effect of platelets on mtDNA quantification but in the context of either whole blood, peripheral blood mononuclear cell (PBMC) or buffy coat preparations**.** Urata *et al.* measured mtDNA before and after platelet depletion in PBMCs, concluding that platelet contamination indeed caused large overestimation of mtDNA (Urata *et al.*, 2008). In the same study, platelet depletion decreased variation of mtDNA copy number to about a half indicating that achieving platelet poor preparations is important for reproducible measurement. Similarly, other authors have also found platelets to be an important source of contamination (Banas, Kost and Goebel, 2004).

2.4.1.4 DNA degradation

There is evidence that DNA degrades over time even with storage at -80^{o}C. One study estimated this at 30% per year (Sozzi *et al.*, 2005) – both for plasma and extracted DNA samples – although this has not been specifically assessed in mtDNA. This impacts on comparison cohorts when using biobanks of patient samples that have been collected and processed for other purposes.

2.4.1.5 Quantification method for mtDNA

Review of the literature revealed significant discrepancies in the primer design and qPCR conditions. Work by Dr David Dorward at the MRC Centre of Inflammation Research (Edinburgh) in establishing optimised a mtDNA qPCR protocol was used as the basis of the qPCR methods in this thesis (Dorward, 2014).

2.4.1.6 Recommendations for mtDNA quantification in DAMP research

MtDNA acts as a DAMP after being liberated from cells during non-apoptotic cell death. The aim of quantifying mtDNA in the circulation in inflammatory disease is to quantify freely circulating levels at time of sampling. To achieve this, a rigorous protocol is necessary.

Based on the literature review, I have formulated the following recommendations for any research aiming to quantify mtDNA in the circulation in the context of its role as a DAMP:

- Process samples expediently (within two hours) of venepuncture.
- Preferentially use plasma over serum due to variability in serum mtDNA; quantification and potential leucocyte lysis (which may erroneously increase the DAMPs detected);
- Achieve ‘cell free plasma’ through a two-stage centrifugation process with a second, high speed spin to eliminate cellular debris and particulate associated mtDNA;
- Avoid using samples stored for many years, which may suffer from DNA degradation.

2.4.2 Prospective cohort

2.4.2.1 Decision to recruit prospectively

The initial plan for this thesis was to perform an analysis of previously biobanked serum to test the hypothesis that mtDNA was raised in the circulation of patients with IBD. I made initial enquiries as to the availability of such IBD 'biobanked' samples for this work, which revealed that a large number of (mainly) serum samples had been stored over the last decade and potentially available for such an analysis.

However, as detailed in the previous section (Section 2.4.1), a literature review into this area strongly suggested that precise methodology was critical for accurate mtDNA analysis. Unfortunately, most stored patient samples in available IBD biobanks had large variations in sampling date, sample type and processing with variable documentation available. Furthermore, it became apparent that for any potential assessment of mtDNA as a biomarker of IBD disease activity or prognosis, a well characterised cohort would be required.

Several challenges were identified in the use of biobanked samples. In brief:

- Most biobank samples available were of serum, rather than plasma;
- Most plasma sampled did not undergo a second, high speed centrifugation spin (see Section 2.4.1.3);
- The timing (post-venepuncture) and method of centrifugation varied considerably between samples;

- There was a variable lack of documentation and paired clinical/ biochemical information;
- Many available samples were collected many years previously (see Section 2.4.1.4)

In summary, it appeared from initial investigation that use of biobanked samples were inadequate to accurately test the primary hypothesis of this thesis. Therefore, a prospective study was designed with consistent and validated sampling and processing methodology.

2.4.2.2 Evolution of the prospective cohort

Initially, patients were recruited from inpatient and outpatient services with a range of disease activity levels. However, early data suggested that higher mtDNA levels were present in patients with active disease compared to IBD in remission. This shifted the focus of the recruitment strategy to patients with active disease, to help with assessment of the potential role of mtDNA as a novel biomarker.

2.4.2.3 Limitations of the recruited cohort

Although prospectively recruited patients provided the study with significant strengths, including a strictly enforced and consistent protocol and a well characterised cohort, there are limitations that must be acknowledged.

The patients were recruited from a single-centre (Western General Hospital, Edinburgh). Inherent in a single-centre strategy is the potential for recruitment bias. After adjustment for sample size, single-centre studies tend to show larger treatment effects on survival than do multicenter trials (Dechartres *et al.*, 2011). This may be particularly relevant with the shift in focus in this study from IBD towards 'active IBD'. Indeed, far more UC patients (and in particular ASUC) were recruited compared to CD. This was largely a result of accessibility of ward based hospitalised patients for recruitment. Notification of potential participants was generally from clinicians and nursing staff on the wards, which also contributed to significantly more patients with severe disease being recruited than initially anticipated.

However, this bias towards more severely active disease may also have been influenced by the overwhelming inflammatory response seen in ASUC (i.e. these patients would be the lowest hanging fruits for high mtDNA levels). However, given the exploratory nature of this research and funding restrictions, this was felt to be an appropriate methodology. It is clear that any findings from this exploratory study will require validation in further cohorts.

Although attempts were made to age and sex match controls with IBD patients, this was limited by the pool of controls available for recruitment. Despite this lack of strict age/sex matching, there was no significant differences between the recruited cohorts in terms of demographics.

Given the relatively small number of patients recruited, there is potential for type II error in some of the subgroup analysis performed. In particular, only 30 patients were recruited to the CD cohort. This could lead to type II error especially with assessing mtDNA as a biomarker with subgroup disease activity analysis (Chapter 3).

2.4.3 Reproducibility of the mtDNA assay

There are various statistical methods that can be used to determine a test's test-retest reliability ("reproducibility"). Correlation coefficients can provide an indication of relative reliability, and are a reflection of the way in which two sets of observations follow a straight line (of best fit). However, the main drawback of correlation tests in assessing for repeatability is that it does not provide any insight into systematic errors that may be present. In this way, a high level of correlation may have a marked divergence from the line of complete agreement. Correlation of variation also provides an assessment of relative reliability and assesses the extent of variability in relation to the mean. In the context of duplicate measurements, it provides an indication of the precision or repeatability of a test.

In this study, intra assay coefficient of variation (CV) was 7.7% for mtDNA quantification. This intraassay CV compares favourably to some published studies for mitochondrial DNA quantification of 11.3% (Gahan *et al.*, 2001) and 13.0% (Bhat *et al.*, 2004) and unfavourably with other studies of 1% (Gourlain *et al.*, 2003) and 3.3% (Xia *et al*., 2009). The differences in the reported intra

assay CV rates could be partly explained by the different blood fractions and PCR primers used in the above studies. Collectively, the data suggests good reproducibility of qPCR for mitochondrial DNA quantification.

Data for inter assay reproducibility was not available in this study. Reported inter assay CV rates in the literature for mitochondrial DNA quantification vary between 5.5% and 30% (Gourlain *et al.*, 2003; Kavlik *et al.*, 2011; Bhat *et al.*, 2004).

There are numerous other statistical methods that may be used to assess agreement between two sets of data, including visualisation via a Bland-Altman plot (Bland and Altman, 1986). A Bland-Altman plot is generally used to compare two measurement techniques and plots the absolute difference between the different tests on the vertical axis around the mean difference. It can help detect systemic bias and provide limits where 95% of the differences are expected to lie between limits (if the data points are normally distributed).

2.4.4 Variability in healthy control mtDNA levels

There was a notable variability in the mtDNA levels of healthy controls in this study (median 64.6 copies/µL, range 13.7 - 205.2, IQR 51.6 - 104). There are a number of possible explanations for this variability. Firstly, variation in measurable levels of biomarkers is expected in healthy individuals. For example, the 2.5th, 50th and 97.5th percentile values for CRP was found to be 0.08, 0.64 and 3.11 in healthy donor population with a nongaussian distribution

(Macy *et al.*, 1997). In addition, ageing and exercise may influence circulating cell free mtDNA levels. MtDNA levels were found to be higher in levels of older (Pinti *et al.*, 2014) and correlated with 'frailty' scores (Jylhävä *et al.*, 2013). However, another study found no association between plasma mtDNA levels and age (Verschoor *et al.*, 2015). Exercise has been associated with lower circulating mtDNA levels (Shockett *et al.*, 2016), and lower levels have been found in professional sportspeople compared to nonathlete controls (Nasi *et al.*, 2015). In addition, other conditions not classically associated with inflammation such as depression have been found to have been associated with higher mitochondrial DNA levels (Lindqvist *et al.*, 2018) (Kageyama *et al.*, 2018). There was no association with age or gender and mtDNA in healthy controls in this study, although the numbers were small.

Large, population-based studies are required to determine the distribution of mitochondrial DNA levels in the healthy population. This will help identify 'normal' ranges and 'abnormal' levels as well as determine whether the distribution is gaussian.

2.4.5 Correlation between serum and plasma mtDNA levels

As discussed earlier (Section 2.4.1.1), the two main blood fractions used for circulating mtDNA quantification are plasma and serum. Most studies in inflammation research have used plasma, due to the theoretical risk for serum of intracellular DAMPs (including mtDNA) being released during clotting.

However, only a few studies have examined the correlation between plasma and serum in matched samples. In addition, I am not aware of any published data comparing matched plasma vs. serum for mtDNA quantification (as opposed to nuclear DNA). Given both plasma and serum were collected for participants, we performed comparative matched analysis.

We found a modest positive correlation between paired plasma and serum samples for mtDNA genes COXIII ($r = 0.46$) and ND2 ($r = 0.52$). These findings are in keeping with the existing literature for quantification of nuclear DNA (Boddy *et al.*, 2005; Umetani, Hiramatsu and Hoon, 2006; Zanetti-Dallenbach *et al.*, 2008). Umateni *et al.* found a positive correlation between qPCR detected DNA in serum and plasma ($n=24$, $r=0.72$, $p=0.0002$) (Umetani, Hiramatsu and Hoon, 2006). Zanetti-Dallenbach *et al.* found a positive correlation ($n=107$, $r=0.54$, $p=0.01$) for DNA in serum and plasma in healthy control, and benign and malignant breast cancer participants. Boddy *et al.* found a positive correlation for DNA between two-spin plasma and serum samples in patients with malignant and benign prostatic disease ($n=40$, $r = 0.56$, $p < 0.001$).

The stronger correlation seen in Umetani *et al.* compared to results in this study could be due to various factors. Firstly, the number of paired samples assessed was larger in our sample ($n=114$). Secondly, processing protocols differed significantly with our protocol (and with those of the other two studies mentioned above) with no second high speed spin being performed for plasma

samples; instead, a 13mm filter for both serum and plasma was used. Thirdly, our data reflected mtDNA gene amplification whereas Umetani *et al.* amplified nuclear DNA using a primer set designed to produce an amplicon size of 115bp.

This is the largest correlative comparison (n=114) that I am aware of comparing DNA as quantified by qPCR in cell-free plasma vs. serum. Furthermore, this is the first comparative analysis of mtDNA as quantified by qPCR in cell-free plasma vs. serum.

2.4.6 Mitochondrial DAMPs are elevated in IBD

2.4.6.1 Major findings

Recently, mtDNA as detected by qPCR was recently reported by our group to be higher in the plasma of mice with chemical induced colitis compared to those without colitis (Ho *et al.*, 2018). In this thesis, I present data to show for the first time that significantly increased levels of mtDNA are found in active human IBD. This applies to both UC and CD when compared to non-IBD controls.

I corroborated these findings with a second known mitochondrial DAMP, by demonstrating the presence of *N*-formylated peptides arising from the mitochondria in the plasma of patients with active UC. Of the mitochondrial *N*-formylated peptides detected, fMMYALF was the most abundant. This is highly

relevant as fMMYALF is the most biological active mitochondrial *N*-formylated peptide (Rabiet, Huet and Boulay, 2005).

2.4.6.2 Practical considerations

The supportive *N*-formylated peptide data was exploratory in nature and performed in only a small subset of patients (n=5 ASUC and n=5 HC). Funding and time restrictions prevented further investigation of the role of *N*-formylated peptides in IBD. However, given the biological associations between *N*-formylated peptides and mtDNA, this data provides valuable support to the hypothesis that mitochondrial DAMPs are released during inflammation in IBD. In the future, we seek to perform similar measurements in a larger cohort of IBD patients including CD and mild-moderate UC.

An additional limitation to our data is that we have not studied mtDNA in non-IBD intestinal inflammatory conditions such as infectious colitis or diverticulitis. It is conceivable that high mtDNA release is also present in these conditions, and further investigation is warranted.

2.4.6.3 MtDNA levels in inflammatory disease

Circulating mtDNA levels have been found to be elevated in several other inflammatory conditions (summarised in Section 1.3.5). Here, I put the findings of raised circulating mtDNA levels in IBD into the wider context of inflammatory disease.

Systemic inflammatory response syndrome (SIRS)

SIRS is a serious condition associated with high mortality where affected individuals display progressive signs or symptoms of systemic upset reflecting widespread inflammation, often involving multiple organ dysfunction and failure (e.g. lungs, kidneys, brain). SIRS is often a result of major sepsis but also commonly occurs in the context of injury such as trauma. An early study by Lam *et al.* found that individuals admitted for blunt traumatic injury had increased plasma nuclear DNA and mtDNA levels (Lam *et al.*, 2004). Subsequently, Hauser and colleagues made the seminal observation that it is the freely circulating mtDNA following traumatic injury which possess the distinct ability to trigger and drive the clinical manifestation of SIRS (Zhang *et al.*, 2010). Several studies have now confirmed the observation of elevated plasma mtDNA in trauma and SIRS (Nicole Y.L. Lam, Timothy H. Rainer, Rossa W.K. Chiu and Lo, 2004; Hauser *et al.*, 2010; Zhang, Itagaki and Hauser, 2010; Gu *et al.*, 2013; Hsu *et al.*, 2013; Simmons *et al.*, 2013; Prikhodko *et al.*, 2015; Timmermans *et al.*, 2016).

In sepsis, elevated levels of circulating mtDNA have also been found in multiple studies (Lu *et al.*, 2010; Garrabou *et al.*, 2012; Kung *et al.*, 2012; Hsu *et al.*, 2013; Bhagirath, Dwivedi and Liaw, 2015; Di Caro *et al.*, 2015; Timmermans *et al.*, 2015). The one negative study in sepsis may be explained by numerous factors including a relatively well patient cohort, only one ‘spot’ measurement being taken at presentation and the potentially confounding factor of cellular content/debris (Puskarich *et al.*, 2012).

Acute single organ injury: Liver, heart and brain

High levels of mtDNA are present in the serum and plasma of patients with acute injury to a variety of single organs. Acetaminophen overdose induces massive hepatocyte necrosis and in severe cases can lead to multi-organ failure and remains one of the commonest indications for liver transplantation. Drug induced acute liver failure patients had serum mtDNA levels of up to 10,000 fold higher (Marques *et al.*, 2012) than healthy controls. Serum mtDNA of acetaminophen overdose patients with derangement in the liver enzyme alanine aminotransferase (a marker of hepatocyte damage) is significantly higher than overdose patients who had normal liver enzymes (McGill *et al.*, 2012), suggesting the extent of mtDNA release into the circulation depends on the extent of hepatocyte necrosis.

Similarly, extensive cardiomyocyte necrosis is found in acute myocardial infarction which is also associated with elevated mtDNA in multiple studies (Bliksøen *et al.*, 2012; L. L. L. Wang *et al.*, 2015; Qin *et al.*, 2016) and fall after angioplasty or coronary stent insertion to restore blood flow to the damaged myocardium (Bliksøen *et al.*, 2012; L. L. L. Wang *et al.*, 2015). Patients with diabetes mellitus and coronary artery disease have higher mtDNA levels than those with diabetes but without coronary artery disease (J. Liu *et al.*, 2015). MtDNA is also higher in acute cerebral ischaemia, caused by a reduction in cerebral blood flow by embolus or local thrombosis, and plasma levels gradually drop over time after the initial tissue injury (Lakra *et al.*, 2011). Interestingly, studies by the same group relating to plasma mtDNA in

subarachnoid haemorrhage and spontaneous intracerebral haemorrhage found no significant difference compared to healthy controls, although both were small studies (Wang *et al.*, 2012, 2013).

Overall in these conditions, significant mtDNA release following massive tissue or cellular injury is evident and likely contributes to the uncontrolled inflammatory response.

Chronic inflammatory and immune-mediated diseases

Distinct to conditions relating to injury, the role for mtDNA in immune-mediated inflammatory diseases is now also emerging. In rheumatoid arthritis, a chronic relapsing autoimmune condition affecting the joints, mtDNA was present in the plasma and synovial fluid of most patients but undetectable in healthy controls (Hajizadeh *et al.*, 2003). Similarly, higher plasma mtDNA is found in granulomatosis with polyangiitis, an autoimmune disease whose features include necrotizing granulomatous inflammation and vasculitis (Surmiak *et al.*, 2015).

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease in which hallmarks include excessive type I interferon (IFN) and antibodies against nucleic acids. Caielli *et al.* explored the potential pathogenic importance of oxidised mtDNA in SLE. They showed that there is a defect in mitochondrial clearance which leads to abnormal extrusion of oxidised mtDNA which triggers subsequent interferogenic response (Caielli *et al.*, 2016).

Higher levels of mtDNA have been found in the chronic inflammatory states of HIV (although not in all studies), end-stage renal failure and diabetes mellitus (Table 1.8). In obese individuals with steatohepatitis, mitochondria enclosed in microparticles can also be detected in plasma (Garcia-Martinez *et al.*, 2016). These findings suggest that mtDNA, otherwise a 'self-signal', may be an active component in the aberrant immune or inflammatory response in chronic diseases and in autoimmunity.

2.4.6.4 Other DAMPs raised in IBD

In IBD, the chronic non-resolving and extensively inflamed gut mucosa represents an enriched source of local and systemic DAMPs (Figure 2.8). Unsurprisingly, several other DAMPs are found in abundance during active disease in IBD including the S100A calgranulins (S100A8/9 complex or calgranulin A/B or MRP8/14 or calprotectin; and S100A12), HMGB1 and interleukin-1α/33 (IL-1α and lL-33). The latter group DAMPs are regarded as 'alarmins' (Garlanda, Dinarello and Mantovani, 2013), molecules that possess cytokine-like functions which are stored in cells and released upon uncontrolled cell death.

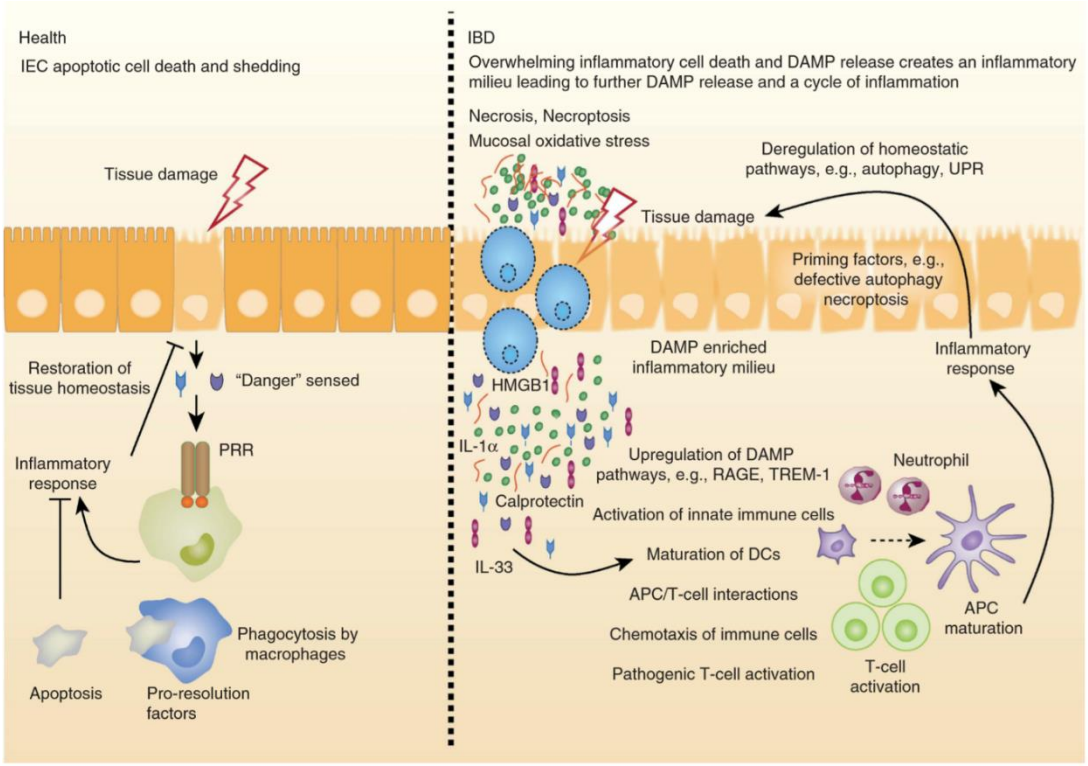

Figure 2.11: Contribution of DAMPs to inflammatory response in IBD. In health, intestinal epithelial cells undergo constant shedding and apoptosis. Tissue damage releases danger signals which initiates a protective inflammatory response to restore tissue homeostasis. In IBD, non-apoptotic cell death, mucosal oxidative stress and deregulation of homeostatic pathways lead to overwhelming release of DAMPs creating a pro-inflammatory milieu. These DAMPs lead to an inflammatory response through a variety of pathways leading to further tissue damage and ongoing intestinal epithelial cell death. (Boyapati, Rossi, *et al.*, 2016)

A comprehensive list of DAMPs implicated in IBD and experimental colitis is provided in Table 2.3 although it is noteworthy that many DAMPs have yet to be studied in the context of intestinal inflammation (for references, please see (Boyapati, Rossi, *et al.*, 2016)).

DAMP/Alarmin	Main source
S100A8/S100A9	Neutrophils, monocytes, epithelium
S100A12	Neutrophils
HMGB1	Predominantly macrophages and monocytes but also NK cells, DC, neutrophils, eosinophils and platelets
IL-1α	Neutrophils, macrophages, IECs
IL-33	Initially via stressed IECs and later via lamina propria cells
Lactoferrin	Neutrophils, brush border cells, macrophages, monocytes, lymphocytes
Heat shock proteins (HSPs) **	Wide variety of cell types
Tenascin-C	Wide variety of cell types
Hyaluronan	Wide variety of cell types
Galectins	Wide variety of cell types
ATP	Wide variety of cell types

** It is controversial as to whether heat shock proteins are DAMPs

Table 2.3: DAMPs implicated in IBD and experimental models of colitis (Boyapati, Rossi, *et al.*, 2016)

The findings of this chapter support the idea that in addition to the DAMPs discussed above, mitochondrial DAMPs are also released into the circulation in IBD. The likely source of mitochondrial DAMPs in IBD is the inflamed gut mucosa where cellular stress and death occur. However, the mechanisms relating to DAMP release in IBD are complex and warrant further discussion. The next section deals with the mechanisms regulating DAMP activity and clearance that are relevant to IBD.

2.4.7 Implications of findings

In this chapter, I have provided the first evidence to show that mitochondrial DAMPs (in particular mtDNA) is released into the circulation in active IBD and that higher levels are associated with more severe disease. I hypothesise that mtDNA is an important pathogenic trigger that maintains the state of abnormal mucosal inflammation. IBD-specific factors which support this hypothesis related to DAMP activity and release are discussed below.

2.4.7.1 The manner of cell death affects DAMP release

Current evidence suggests the load and composition of DAMPs may determine whether their effects become pathogenic, hence re-emphasizing the delicate balance between the protective and pathologic roles of DAMPs. Here, I review the factors that may influence this balance in the context of IBD.

In health, the intestinal epithelium is replaced every 5-7 days; epithelial cells are either shed or die by apoptosis. In active IBD, non-apoptotic cell death (e.g. epithelial necrosis) occurs more commonly (Gunther *et al.*, 2012). More recently, necroptosis or programmed necrosis is increasingly appreciated as an alternative mechanism (Kaczmarek, Vandenabeele and Krysko, 2013) which appears to contribute to intestinal inflammation similar to that found in IBD (Günther *et al.*, 2011; Welz *et al.*, 2011).

Of interest, relevant KO mouse models and some limited evidence in human studies suggest a role for necroptosis in IBD although it remains possible that these clinical phenotypes are primarily driven by loss of barrier and specialised enterocyte function (Paneth cells in this case) rather than mucosal DAMP release (Boyapati, Rossi, *et al.*, 2016). Necroptosis lacks the massive caspase activation seen in apoptosis and this leads to comparative DAMP activation. For example, the lack of caspase-activated deoxyribonuclease (DNase) means DNA is not cleaved, leading to higher molecular weight DNA with greater proinflammatory potential (Martin, Henry and Cullen, 2012).

Similarly, full length IL-33 is released in necroptosis compared to the non-immunological IL-33 in apoptosis which is due to caspase-dependent proteolysis (Lüthi *et al.*, 2009). HMGB1 is oxidised into its non-immunological form during apoptosis by caspase mediated reactive oxygen species (ROS) with irreversible binding to chromatin, but this does not occur in necroptosis (Taylor, Cullen and Martin, 2008). The DAMP-necroptosis link has been

illustrated in several experimental models of necroptosis in skin, brain and systemic inflammation, which have shown higher levels of various DAMPs such as S100A9, IL-33, mtDNA and HMGB1 (Kaczmarek, Vandenabeele and Krysko, 2013).

2.4.7.2 *The influence of the mucosal milieu on the inflammatory pathogenicity of DAMPs*

Increased mucosal oxidative stress is another key feature of active IBD, which can enhance the pro-inflammatory effects of DAMPs. An oxidative milieu modifies various proteins and lipids such as cholesteryl ester hydroperoxides and oxidised phospholipids, activating their role as potent DAMPs causing further inflammation (Imai *et al.*, 2008; Choi *et al.*, 2009). Oxidised mtDNA also becomes significantly more inflammatogenic. Shimada *et al.* found that cytosolic oxidised mtDNA rather than its non-oxidised form, directly activates the NLRP3 inflammasome and IL-1β production (Shimada *et al.*, 2012). Pazmandi *et al.* further showed the increased immunogenicity of oxidatively modified mtDNA on plasmacytoid dendritic cells compared to native mtDNA (Pazmandi *et al.*, 2014).

2.4.7.3 De-regulation of mucosal homeostatic pathways 'prime' the inflammatory potential of DAMPs

Defective autophagy and the unfolded protein response (UPR) regulating ER stress are important in the pathogenesis of IBD (Kaser *et al.*, 2008). A meta-analysis of GWAS has identified the autophagy genes ATG16L1 and IRGM as key susceptibility genes particularly in CD (Jostins *et al.*, 2012). ER stress

related genes have been implicated in IBD by GWAS (ORMDL3) and candidate gene approaches (XBP1 and AGR2) (Zheng *et al.*, 2006; Kaser *et al.*, 2008). The importance of autophagy in endogenous DAMP-mediated inflammation is increasingly appreciated although its role in the clearance of intracellular pathogens ("xenophagy") is established.

From a DAMP perspective, failure to clear proinflammatory damaged mitochondria is a key consequence of defective autophagy and may contribute to enrichment of cells with DAMPs, priming them to be released into the circulation. Dysfunctional, ROS-generating mitochondria (Zhou *et al.*, 2011) and specifically oxidised mtDNA (Shimada *et al.*, 2012) can activate the NLRP3 inflammasome. Other DAMPs such as ECM components biglycan and hyaluronic acid can additionally prime inflammasome activation in this context (Iyer *et al.*, 2009). Nakahira et al. showed that defective autophagy promotes the accumulation of mitochondrial DAMPs and NLRP3 inflammasome activation (Nakahira *et al.*, 2011). Indeed, in ATG16L1-deficiency there is an increased susceptibility to inflammasome mediated release of IL-1β and IL-18 (Saitoh *et al.*, 2008).

A further study showed that defective autophagy can lead to the release of DAMPs and subsequently contribute directly to inflammatory pathology in vivo (Oka *et al.*, 2012). Here, Oka et al. showed that mice deficient in DNase leaked mtDNA and developed a TLR9 mediated proinflammatory state, cardiomyopathy and heart failure (Oka *et al.*, 2012). These studies point to a

failure in autophagy resulting in a higher load of inflammatory intracellular DAMPs. It is noteworthy that in vivo mouse models of ATG16L1 deficiency (chimeric, hypomorphic, human IBD ATG16L1 polymorphism T300A knock-in and epithelial specific ATG16L1-deficiency) do not develop spontaneous colitis but are very susceptible to gut inflammation when subjected additional injurious stimuli (dextran sulfate sodium [DSS], murine norovirus or genetic deficiency of ER-stress) (Boyapati, Rossi, *et al.*, 2016) . Hence, a postulated potentiating rather than initiating role in gut inflammation.

2.4.5.4 Summary

In the setting of overwhelming DAMP release in IBD, the inflammatory milieu may tip the balance from a protective to pathological inflammatory state. Furthermore, the local mucosal inflammatory state may help prime DAMPs such as mtDNA to become more inflammatogenic.

It is important to emphasise that the observation of elevated circulating mtDNA levels in patients with IBD does not in itself imply any mechanistic effects of mtDNA in IBD. Raised mitochondrial DAMPs may be a hallmark of inflammation associated with IBD or alternatively, it may be an important factor in perpetuating inflammation in IBD. The IBD-related factors associated with the higher potential inflammatory effect of DAMPs (as discussed above) supports mtDNA as an active player, but substantial further investigation (discussed in Chapter 5).

Many inflammatory conditions are associated with raised levels of mtDNA, suggesting that release is unlikely to be disease-specific. However, this lack of disease-specificity does not preclude a potentially important role for mtDNA in propagating inflammation associated with IBD. Separate from the mechanistic discussion is the potential role of mtDNA as a biomarker. It is notable that the main clinically useful biomarkers in IBD (faecal calprotectin, CRP and albumin) are not specific for IBD-related inflammation. The next chapter deals with the potential role of mtDNA as a novel biomarker in IBD.

CHAPTER 3:

MITOCHONDRIAL DNA AS A BIOMARKER IN IBD

Chapter 3 Contents

3.1 Introduction

Biomarkers ('biological markers') are substances, structures or processes that are able to be measured objectively, accurately and reproducibly influencing or predicting the incidence of outcomes or diseases (Strimbu and Tavel, 2010). Biomarkers have a particularly important role in IBD, where disease heterogeneity and complexity renders clinical markers limited in the ability to predict intestinal inflammation and long-term outcomes (Lichtenstein and McGovern, 2016).

IBD clinicians currently rely on a few clinically useful biomarkers including ESR, CRP, albumin and faecal calprotectin (Section 1.1.4). However, definitive assessment of mucosal healing (the best predictor of long-term outcome) generally requires invasive and expensive investigations (including endoscopy, radiology and histology) to classify, assess disease activity, monitor response to therapy and provide prognostic information.

Currently available IBD biomarkers suffer from many limitations. Blood-based biomarkers such as ESR, CRP and albumin are acute phase reactants which are raised in many inflammatory states; in contrast, faecal calprotectin is not affected by extra-intestinal inflammation and thus is more specific (Gisbert and McNicholl, 2009). However, faecal calprotectin is reflective of gut inflammation, is not disease-specific and is raised in several non-IBD gut inflammatory states such as nonsteroidal anti-inflammatory drug (NSAID) enteropathy and infectious gastroenteritis. Furthermore, there are issues with faecal

calprotectin around sampling difficulty and high intraday variability in some patients (Lasson *et al.*, 2015). Therefore, there is a clear need for novel non-invasive biomarkers in IBD to help in clinical decision making.

Beyond this, IBD clinicians yearn for a time when biomarkers will allow for treatment decisions in a ‘personalised’ or ‘precision’ medicine approach (Boyapati, Kalla, *et al.*, 2016). This will involve large scale prospective cohorts and patient inputted data leveraging big-data driven approaches. However, discovery-based approaches are currently burdened by significant challenges, and hypothesis/biology-based biomarkers have had the most success so far in IBD. Thus, there remains an important role in biomarker discovery for organic scientific thinking.

After establishing that mtDNA was elevated in patients with IBD, we wanted to assess its potential as a biomarker. Specifically, we aimed to assess the value of mtDNA in an exploratory cohort with regards to disease activity and prognosis.

3.2 Methods

3.2.1 Prospective study & mtDNA quantification

Methods are as described in Chapter 2 (Section 2.2) for the prospective study and mtDNA quantification.

3.2.2 Endoscopic disease activity stratification

Endoscopic disease severity was obtained from endoscopic reports generated by the gastroenterologist in charge of patients at time of sample collection. Endoscopy assessment was considered appropriate for inclusion if it was performed within 72 hours of a sample.

Only patients with UC were considered for inclusion in this analysis. CD can affect any part of the gastrointestinal tract from mouth to anus, which means that areas of inflammation may not be reachable via colonoscope (e.g. in the proximal small bowel). Furthermore, a normal colonoscopy does not necessary imply the absence of gut inflammation in CD. In contrast, UC affects the colon in a distal-to-proximal distribution, leading to a more robust understanding of the current level of gut inflammation from colonoscopy.

Disease activity was classified as ‘mild’, ‘moderate’ and ‘severe’ disease as per the endoscopic reports. In UC, ‘mild’, ‘moderate’ and ‘severe’ relate to the most widely used endoscopic scoring system in clinical practice (Mayo score,

Appendix F). Representative pictures of mild, moderate and severe endoscopic appearance are presented in Section 1.1.3.5 (Figure 1.2). If categorisation was unclear from the endoscopy report, the gastroenterologist who performed the procedure was consulted.

3.2.3 Colectomy data

Patients with ASUC were followed to determine whether they had undergone colectomy. If it was unclear from clinical notes whether a patient had undergone colectomy by the end of the study period, attempts were made to contact the patient for this information.

For patients who underwent colectomy, attempts were made to repeat sampling to compare pre- and post-colectomy mtDNA levels. If multiple samples were taken during admission for these patients, the first sample was considered as the 'pre-colectomy' sample. Patients who were unable or declined to be resampled were considered lost to follow-up.

3.2.4 Serum Calprotectin

ELISA for quantification of calprotectin in the serum was performed by Dr Rahul Kalla using a protocol as previously outlined (Kalla *et al.*, 2016). The methods are reproduced here with permission.

Samples were analysed in duplicate using the Calpro AS™ calprotectin ELISA. Prior to each 96 well run, 100µL of serum was first diluted (1:5) using a dilution buffer. 100µL of the diluted sample was then transferred onto 96 well plate coated with 3% bovine serum albumin (BSA).

A total of 100µL of standards, positive and negative controls and samples were plated in duplicates and all plates contained wells with blanks (no template control). This layout was then transferred onto the ELISA plate using a repetitive multichannel pipette and incubated for 45 minutes on a horizontal plate shaker. At the end of incubation, the liquid was discarded and 3 wash cycles (300µL per well) were performed using an automated plate washer. 100µL of enzyme conjugate was then added to each well using a multichannel pipette and the samples were incubated for 45 minutes on a horizontal plate shaker. A further 3-cycle wash step was performed followed by an enzyme substrate step (100µL per well) and incubation at room temperature for 30 minutes, protected from light. After incubation, 1M NaOH was added to standardise the incubation period and the plate was read at an optical density (OD) 405nm using an ELISA reader.

A 4-parameter standard curve was generated for each run and all standards and control OD were matched to the manufacturer recommended range. Any plate with standards and/or controls that were out of range were repeated. Samples with a calprotectin result of >2500 ng/mL were diluted and retested. Coefficients of variation of <10% were included in the analysis.

3.3 Results

3.3.1 Steroid use during ASUC

All patients with ASUC were on intravenous steroids at some point during their admission. There were 81 samples taken from patients with ASUC in the study. 3 samples were taken from patients who had been changed to oral prednisolone (all 40mg daily) at the time of sampling. Table 3.1 lists the number of samples taken categorised by the number of doses of intravenous steroid they had received prior to sampling. Patients with ASUC received twice daily dosing of 30mg of intravenous methylprednisolone.

Doses of IV steroids received prior to sampling	Number of samples
0	7
1	5
2	10
3	3
4	8
5	4
6	6
7	4
8	6
9	3
10	19
11	3

Table 3.1: Number of samples from patients with ASUC categorised into the number of doses of intravenous steroids prior to the sample being taken

3.3.2 mtDNA over the course of 5 days in ASUC

I sought to investigate the change in mtDNA level over the course of an admission for ASUC. I identified five patients with ASUC for mtDNA measurement over five consecutive days post admission. Daily mtDNA, CRP and albumin levels for each patient (1-5) is presented below.

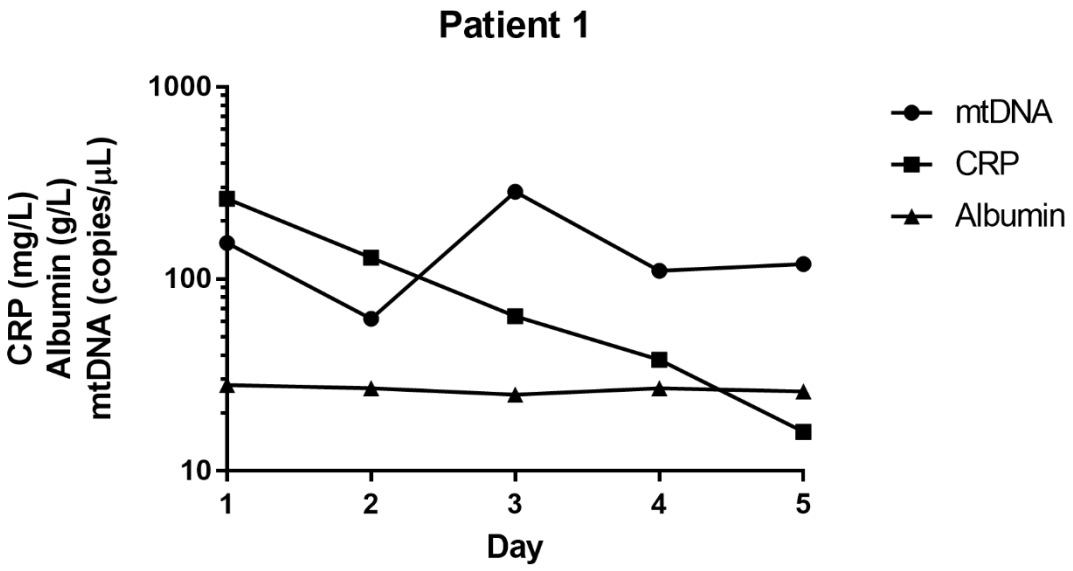

Figure 3.1: Patient 1 mtDNA, CRP and albumin over 5 days patient admission with ASUC. 39-year-old female who made initial good clinical response to IV steroids but required colectomy on day 7.

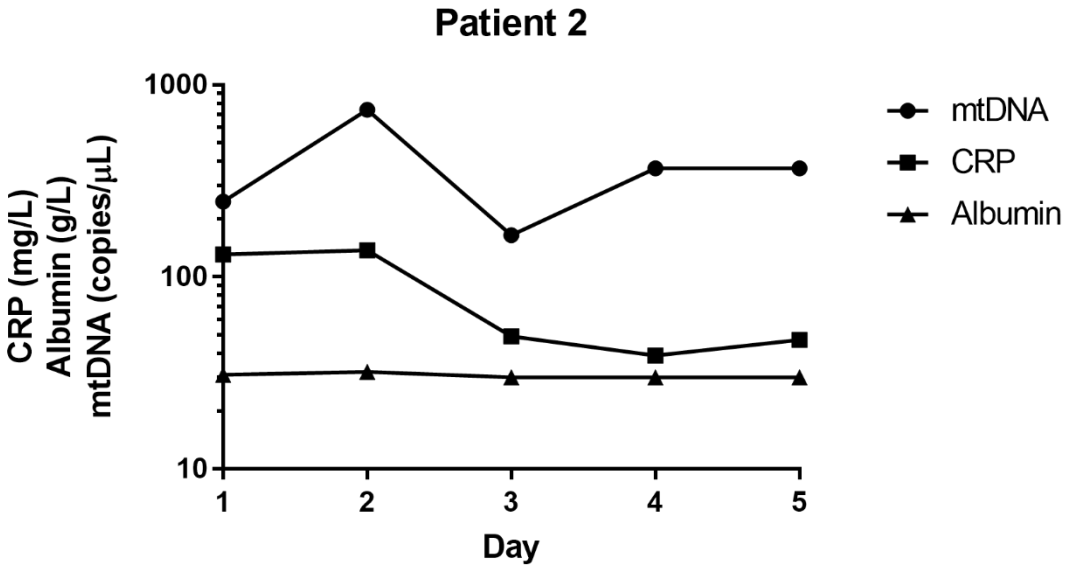

Figure 3.2: Patient 2 mtDNA, CRP and albumin over 5 days patient admission with ASUC. 29-year-old male, responded to IV steroids.

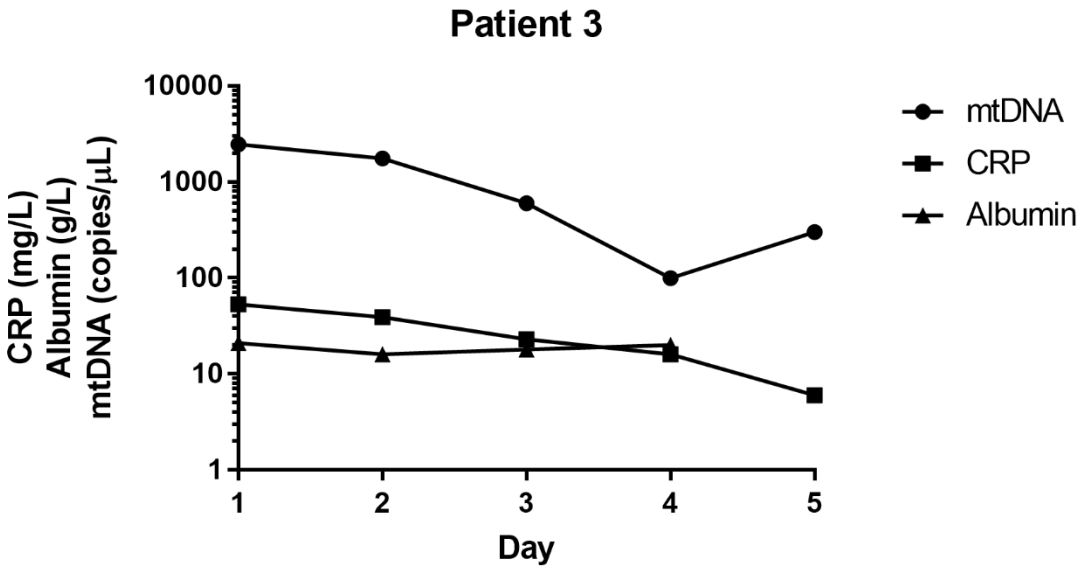

Figure 3.3: Patient 3 mtDNA, CRP and albumin over 5 days patient admission with ASUC. 29-year-old male, responded to IV steroids initially but required colectomy day 70 after subsequent relapse.

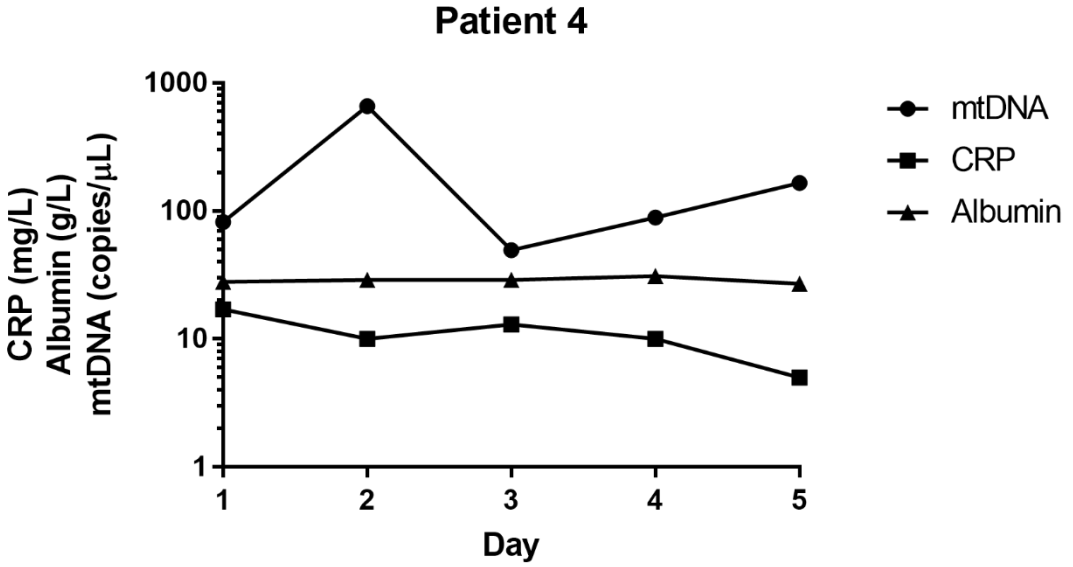

Figure 3.4: Patient 4 mtDNA, CRP and albumin over 5 days patient admission with ASUC. 35-year-old female, responded to IV steroids.

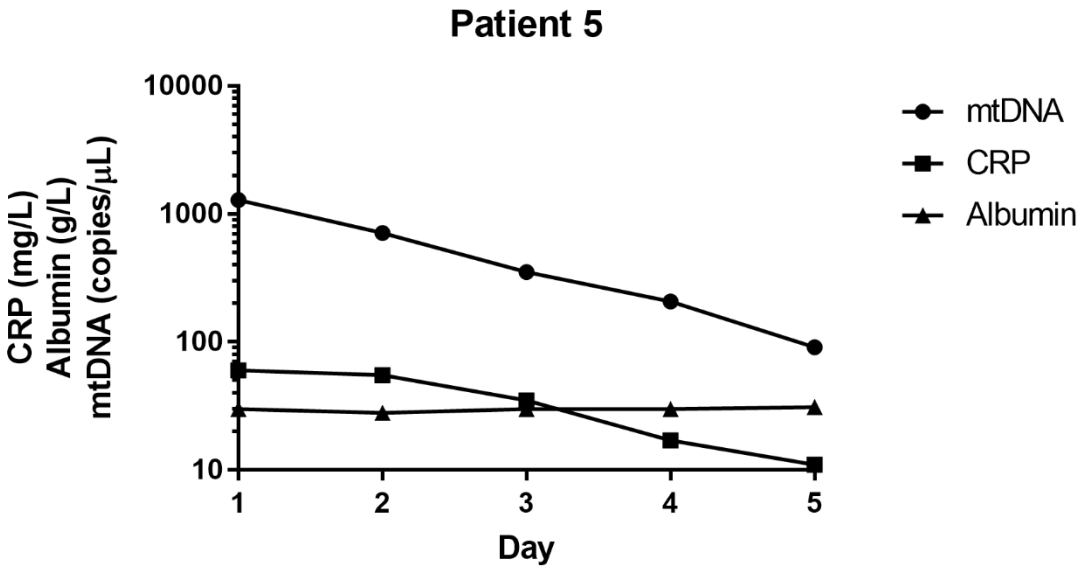

Figure 3.5: Patient 5 mtDNA, CRP and albumin over 5 days patient admission with ASUC. 81-year-old female, CRP peak 59, responded to IV steroids

3.3.3 MtDNA as a marker of disease activity

3.3.3.1 MtDNA levels stratified by clinical activity

Ulcerative colitis

In UC, patients were stratified using the SCCAI (Appendix A) as being in 'clinical remission' or with active disease and the latter group further classified into 'mild-moderate' or 'severe' activity based on whether they met Truelove and Witts' criteria for severe disease (Appendix A).

Those in with severe disease had significantly higher circulating plasma mtDNA levels (234.7 copies/µL [115.3 – 723.4]) compared to those in clinical remission (53.77 copies/µL [IQR 30.56 – 86.8]; $p<0.0001$) and mild-moderate disease (98.52 copies/µL [IQR 62.23 – 205.2]; $p=0.002$) (Figure 3.6).

Patients with clinically mild-moderate disease had significantly higher circulating plasma mtDNA levels compared to those in clinical remission (98.52 copies/µL [IQR 62.23 – 205.2] vs. 53.77 copies/µL [IQR 30.56 – 86.8]; $p=0.002$).

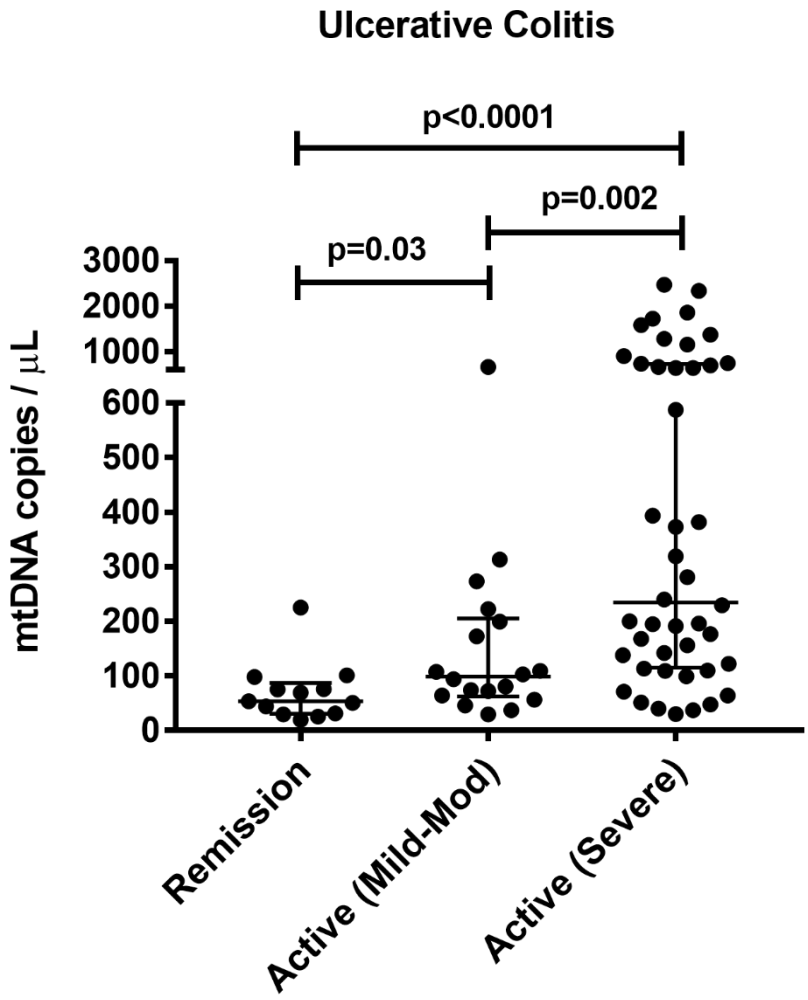

Figure 3.6: Plasma mtDNA (copies/µL) in UC in clinical remission, active and severe active (n= 13, 18 and 44 samples respectively; 8 UC individuals had samples taken more than 1 time point during active disease and in remission). Median ± IQR.

Crohn's disease

In the smaller cohort of CD, patients were stratified using the HBI (Appendix A) as being in 'clinical remission' or 'active' and the latter group further classified into severely active if they required hospitalisation and intravenous steroids.

Higher mtDNA levels were observed in those with severely active CD (159.1 copies/µL [IQR 90.17-421]) compared to those in remission (79.92 copies/µL [IQR 30.94 – 145.9] (p=0.04) (Figure 3.7).

There was no difference in circulating mtDNA between those categorised as mild-moderately active CD compared to those in clinical remission (179.9 copies/µL [IQR 110.8 – 551.1] vs. 79.92 copies/µL [IQR 30.94 – 145.9]; p=0.06). There was no difference in circulating mtDNA between those categorised as severely active compared to the mild-moderately active group (159.1 copies/µL [IQR 90.17-421] vs. 179.9 copies/µL [IQR 110.8 – 551.1] (p=0.72) (Figure 3.7).

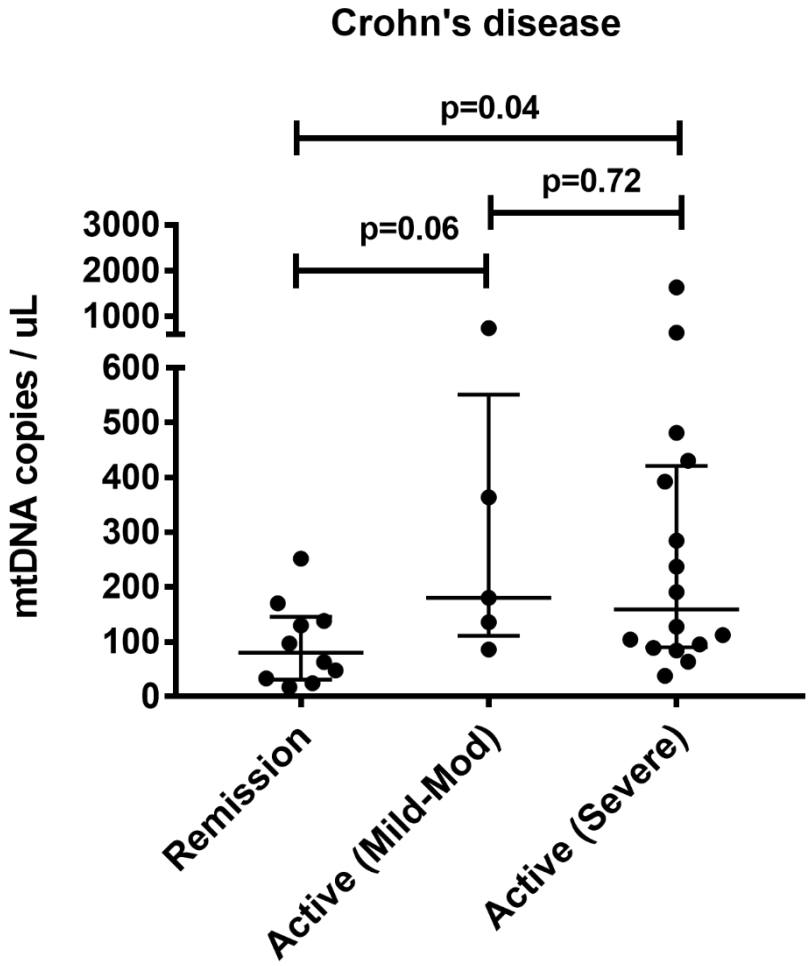

Figure 3.7: Plasma mtDNA (copies/µL) in CD, clinical remission, active and severe disease (n= 10, 5 and 16 samples respectively. One CD individual had samples taken more than 1 time point during active disease and in remission). Median ± IQR.

3.3.3.2 MtDNA levels stratified by endoscopic activity

A total of 68 samples had endoscopy reports suitable for inclusion in this analysis. Patients with endoscopically moderate (199.5 copies/µL [IQR 109 – 427.4]) and severe disease (255.4 copies/µL [96.71 – 641.4]) had significantly higher circulating mtDNA plasma levels in comparison to those with endoscopically mild disease (33.11 copies/µL [IQR 28.71 – 44.9] ($p<0.0001$ and $p=0.0002$ respectively) (Figure 3.8). Patients with moderate and severe disease on endoscopy did not have different levels of mtDNA detected in their plasma.

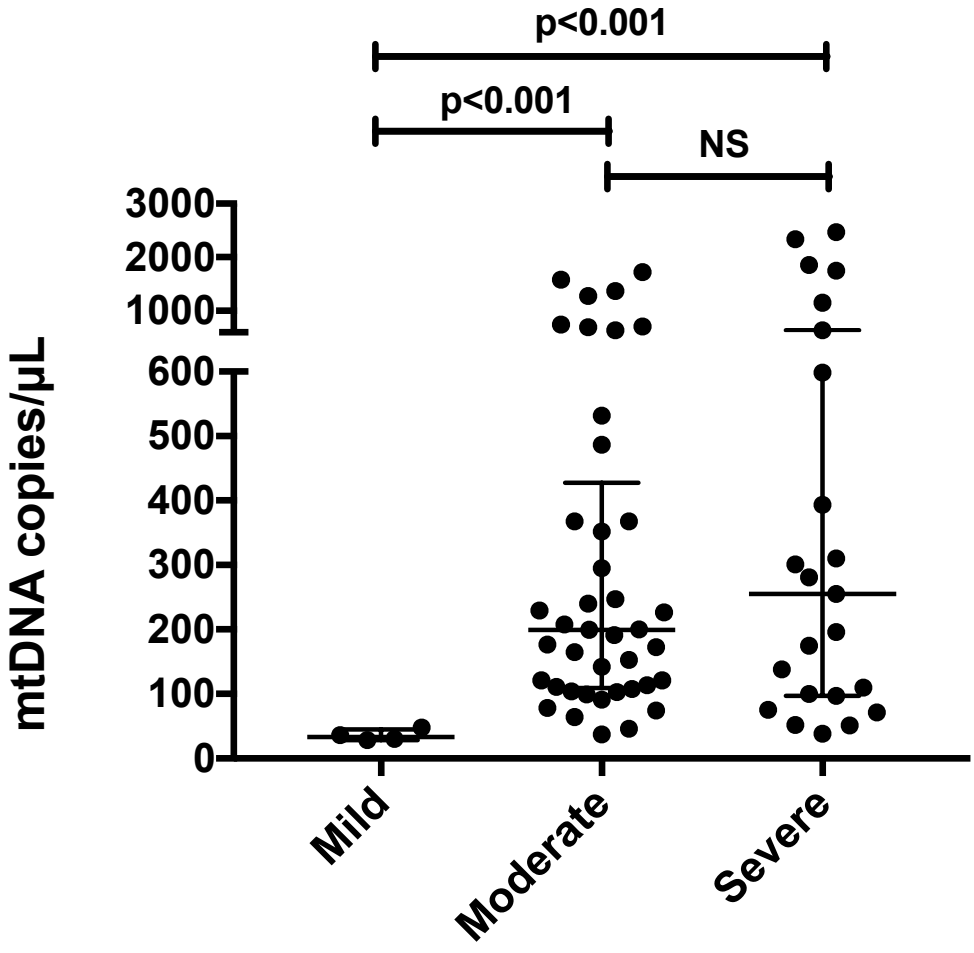

Figure 3.8: Circulating plasma mtDNA by endoscopic appearances in UC (mild, moderate, severe; n= 4, 41 and 23 samples respectively). Median ± IQR.

3.3.4 Correlation with existing biomarkers in IBD

3.3.4.1 CRP

For our IBD cohort samples, mtDNA levels was positively correlated with CRP when mtDNA was quantified using COXIII (r=0.35, p<0.0001; Figure 3.9) and ND2 genes (r=0.28, p=0.0003) (Figure 3.10)

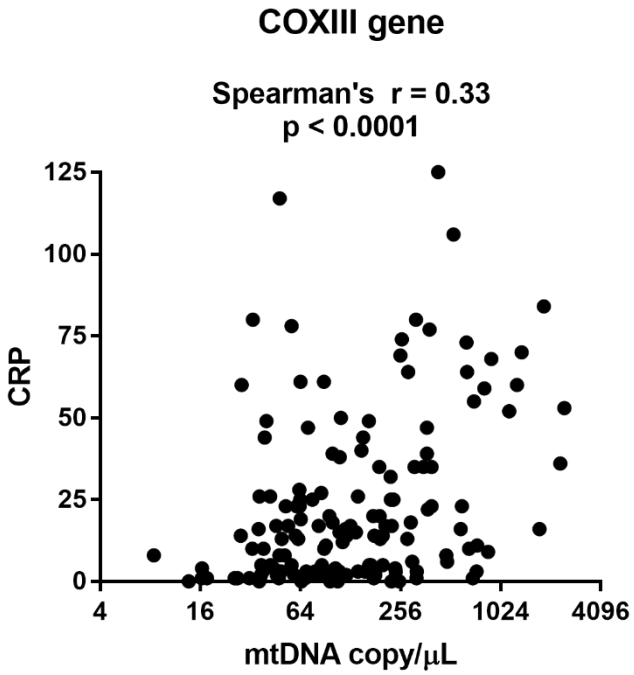

Figure 3.9: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by COXIII gene primers and CRP.

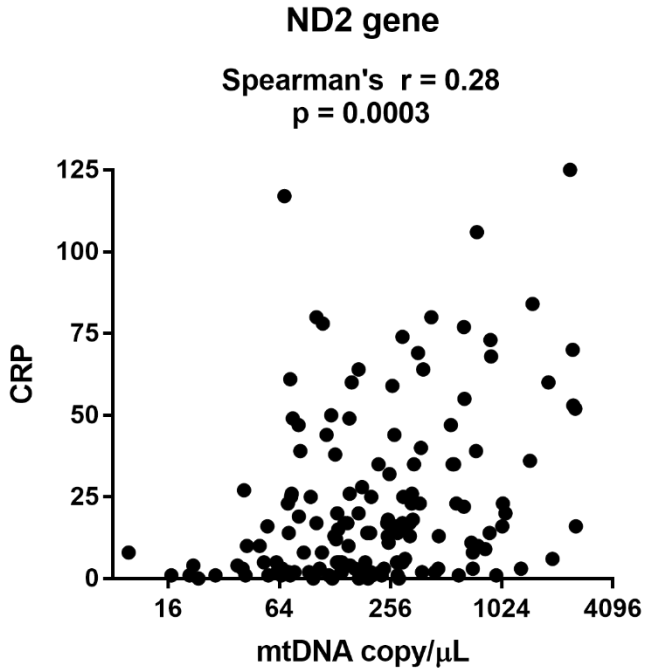

Figure 3.10: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by ND2 gene primers and CRP.

3.3.4.2 Albumin

For our IBD cohort samples, mtDNA levels was negatively correlated with albumin when mtDNA was quantified using COXIII (r=-0. 38, p<0.0001) (Figure 3.11) and ND2 genes (r=-0. 26, p=0.0009) (Figure 3.12).

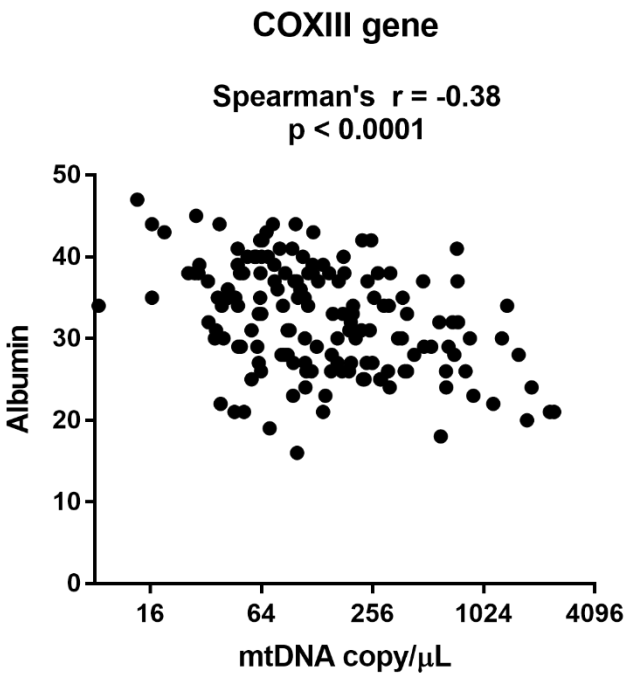

Figure 3.11: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by COXIII gene primers and albumin.

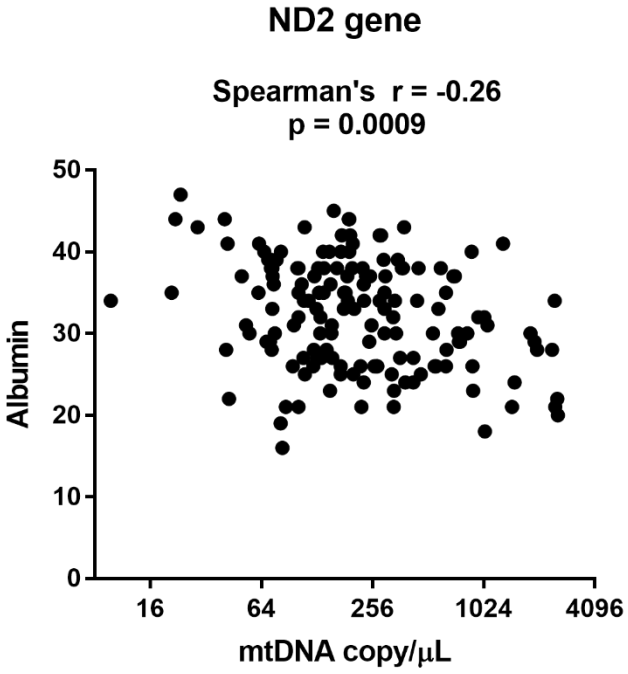

Figure 3.12: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by ND2 gene primers and albumin.

3.3.4.3 White cell count

For our IBD cohort samples, mtDNA levels was positively correlated with CRP when mtDNA was quantified using COXIII (r=0.36, p<0.0001; Figure 3.13) and ND2 genes (r=0.39, p<0.0001) (Figure 3.14).

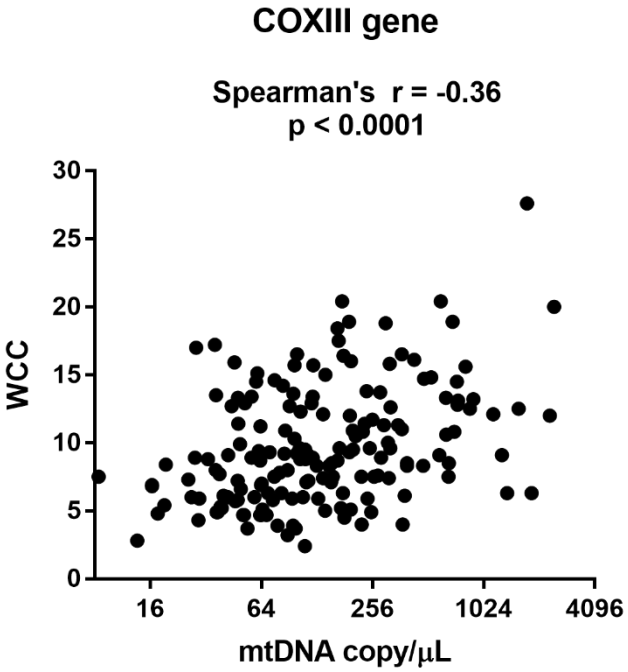

Figure 3.13: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by COXIII gene primers and WCC.

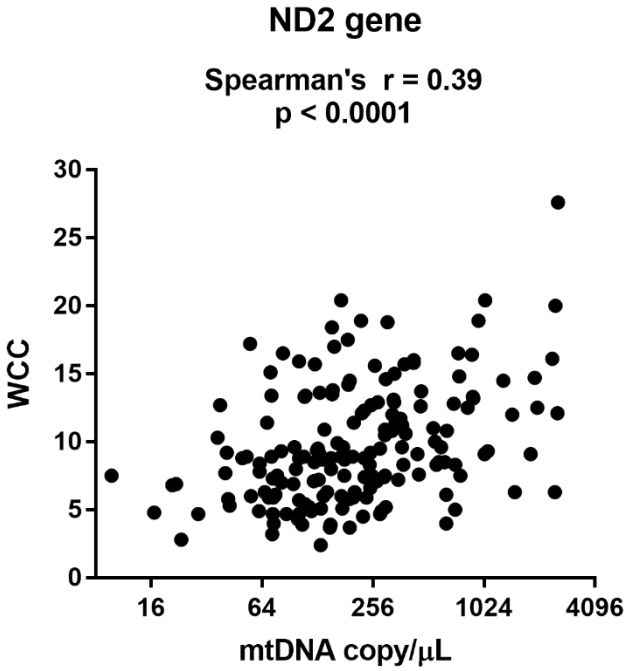

Figure 3.14: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by ND2 gene primers and WCC.

3.3.4.4 Serum calprotectin

Serum calprotectin data was available for 29 ASUC plasma samples and 14 serum samples paired with mtDNA data.

There was no significant correlation between plasma mtDNA levels and serum calprotectin when mtDNA was quantified using COXIII (r=0.22, p=0.24; Figure 3.15) or ND2 genes (r=0.35, p=0.06) (Figure 3.16).

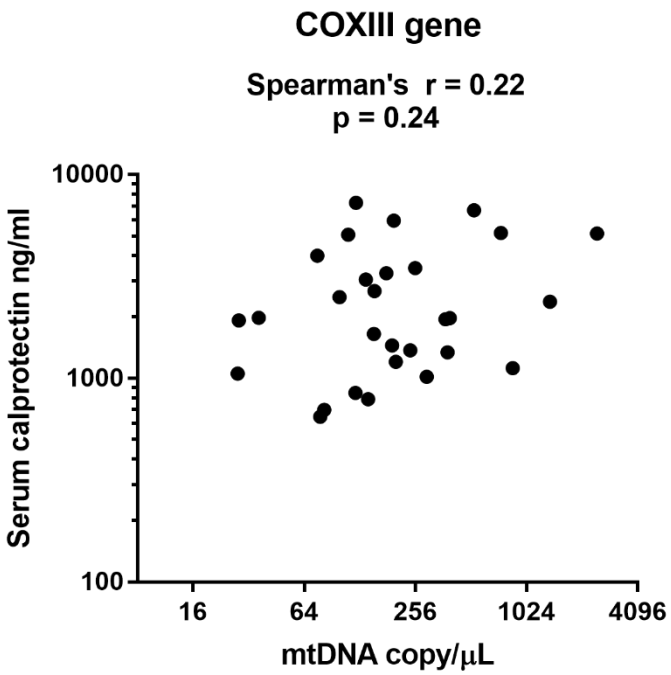

Figure 3.15: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by COXIII gene primers and serum calprotectin.

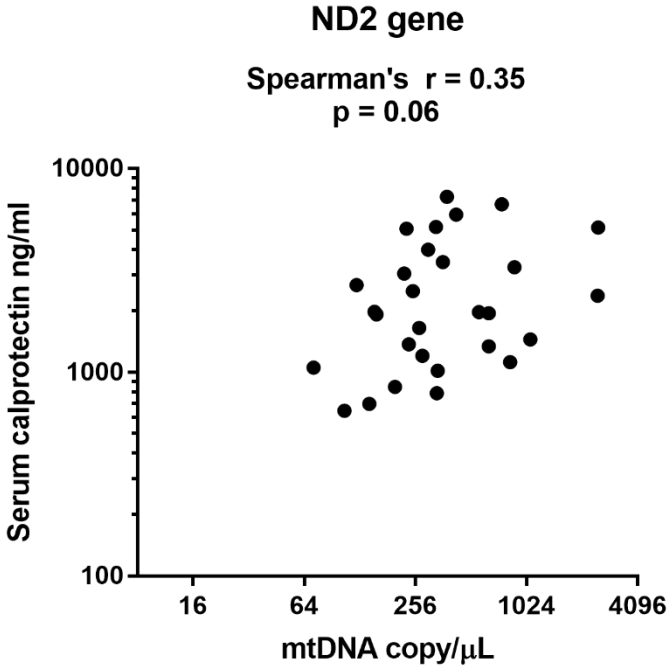

Figure 3.16: Spearman correlation between paired mtDNA plasma (copy/µL) as quantified by ND2 gene primers and serum calprotectin.

There was a significant positive correlation between serum mtDNA levels and serum calprotectin when mtDNA was quantified using COXIII (r=0.55, p=0.04; Figure 3.17) and ND2 genes (r=0.60, p=0.02) (Figure 3.18).

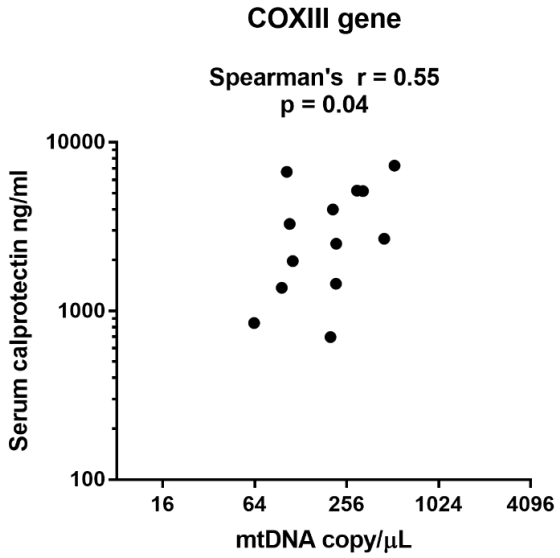

Figure 3.17: Spearman correlation between paired mtDNA serum (copy/µL) as quantified by COXIII gene primers and serum calprotectin.

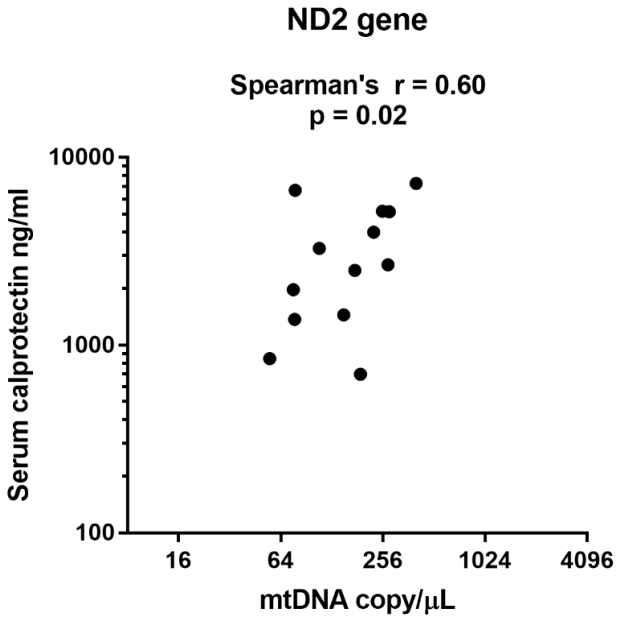

Figure 3.18: Spearman correlation between paired mtDNA serum (copy/µL) as quantified by ND2 gene primers and serum calprotectin.

3.3.5 mtDNA as a prognostic biomarker

The CD cohort had only one individual who underwent surgery during recruitment and follow up, precluding any further analysis. Of the 40 patients with ASUC, 12 (30%) underwent colectomy during follow-up after admission for ASUC. Follow up was for a median of 269 days (IQR 10-399).

mtDNA levels on admission for patients with ASUC who went on to require subsequent emergency colectomy were higher than those who responded to medical therapy (colectomy: 302.5 copies/µL [IQR 139 – 1553] vs no colectomy group 165 copies/µL [66.75 – 253]; p=0.04) (Figure 3.19).

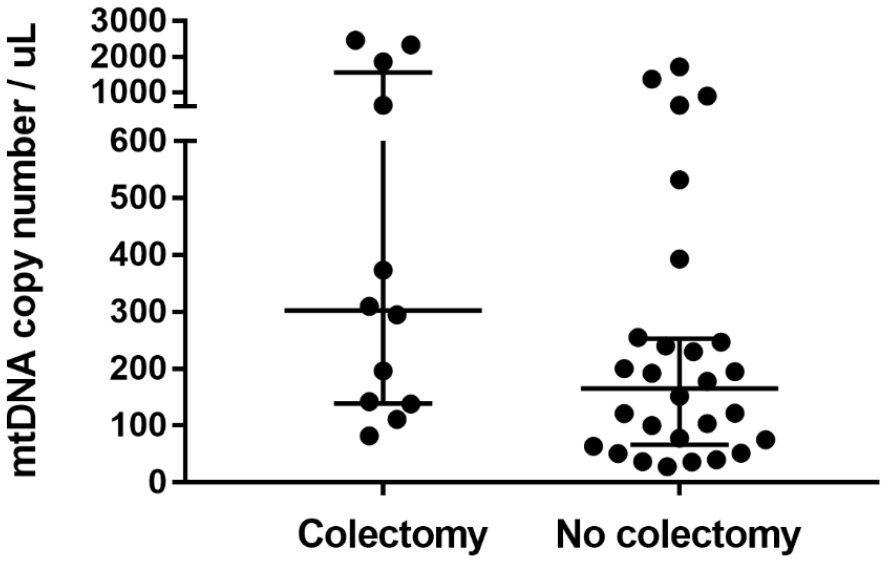

Figure 3.19: Plasma mtDNA (copy/µL) on initial sample for patients with ASUC who required colectomy (n=12) vs. no colectomy (n=28). Median ± IQR.

Comparative ROC curve analysis of mtDNA, CRP and albumin demonstrated AUROCs of 0.71, 0.76 and 0.82 in predicting colectomy (p=0.04, 0.01 and 0.002) respectively (Figure 3.20). There were no significant differences between AUROCs of mtDNA, CRP and albumin to predict colectomy.

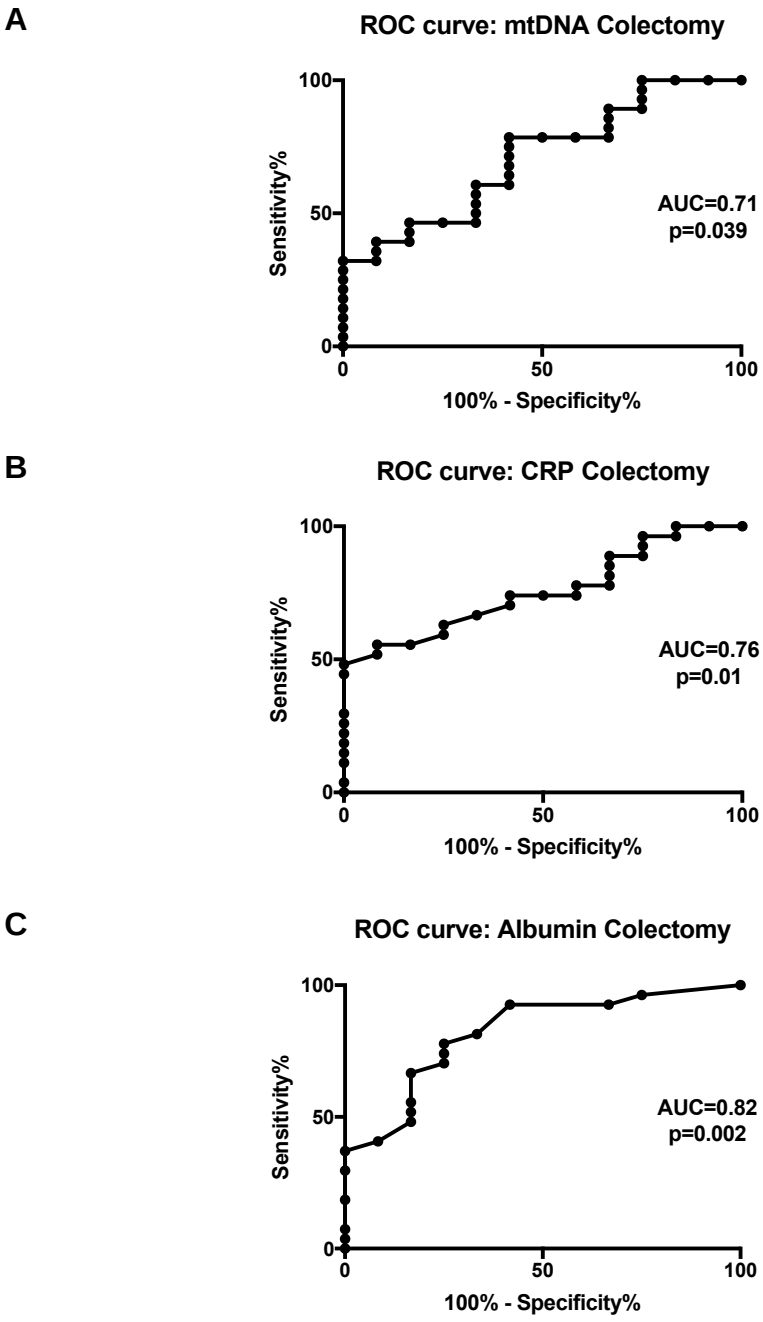

Figure 3.20: ROC curve analysis of **(A)** mtDNA, **(B)** CRP and **(C)** albumin in acute severe UC in-patients and colectomy.

To investigate the discriminant ability of mtDNA to predict subsequent colectomy, the cut off of maximum likelihood ratio was calculated from ROC curve data (1,545 copies/µL, likelihood ratio 7.0) and used to generate Kaplan-Meier survival curves with censored data for subsequent colectomy during follow up (Figure 3.21). There was a significant separation between the two survival curves (p=0.006).

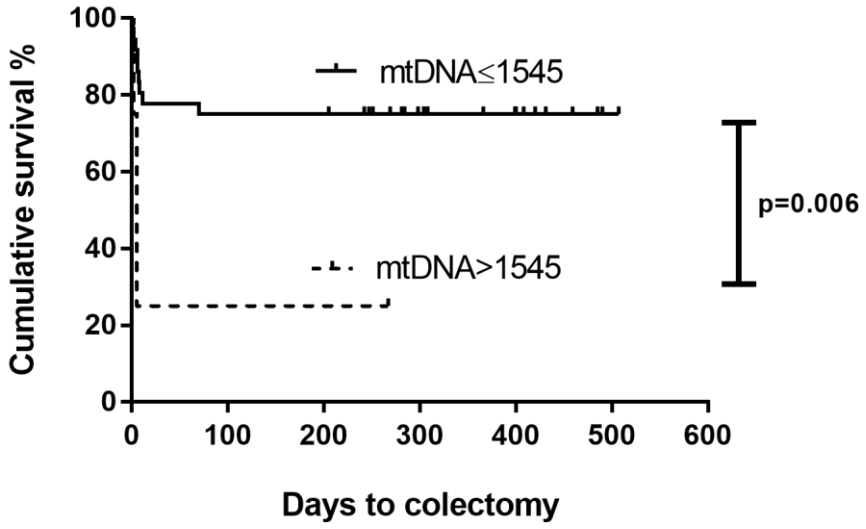

Figure 3.21: Kaplan-Meier survival curves for ASUC patients (mtDNA >1,545 vs. ≤ 1,545 copies/µL; log-rank Mantel-Cox; p = 0.006; ROC curve analysis of maximum likelihood ratio 7.0; n=40 (12 colectomy, 28 no colectomy)).

3.3.6 Characteristics of the 'high mtDNA' group

We identified a group of individuals with very high mtDNA levels (n=18, >600 copies/µL) and investigated if there were unique phenotypic characteristics that defined the group.

Multivariate analysis showed that CRP was independently associated with very high mtDNA levels (p=0.007, Table 3.2).

	p-value	**95% confidence interval**
Hb	0.621	0.973-1.046
WCC	0.083	0.984-1.310
Platelets	0.343	0.998-1.007
CRP	**0.007***	1.084-1.672
Albumin	0.861	0.360-2.346
Age	0.630	0.974-1.045
Smoking status	0.918	0.123-10.311
Sex	0.645	0.416-4.126

Table 3.2: Multivariate logistic regression to analyse parameters associated with high mtDNA levels in 160 samples with complete paired data with mtDNA levels (mtDNA >600 vs. ≤ 600 copies/µL).

3.3.7 Pre- and post-colectomy paired mtDNA levels

12 out of 40 patients (30%) with ASUC in the study underwent colectomy. Of these 12 patients, post-colectomy plasma samples were obtained from 8 patients (4 lost to follow up). This enabled pre- and post-colectomy matched mtDNA levels to be compared in these patients.

In a longitudinal series of acute severe UC with paired samples pre- and post-colectomy (median Δ107 days, IQR 89-189), plasma mtDNA fell following colectomy (n=8, p=0.008) (Figure 3.22).

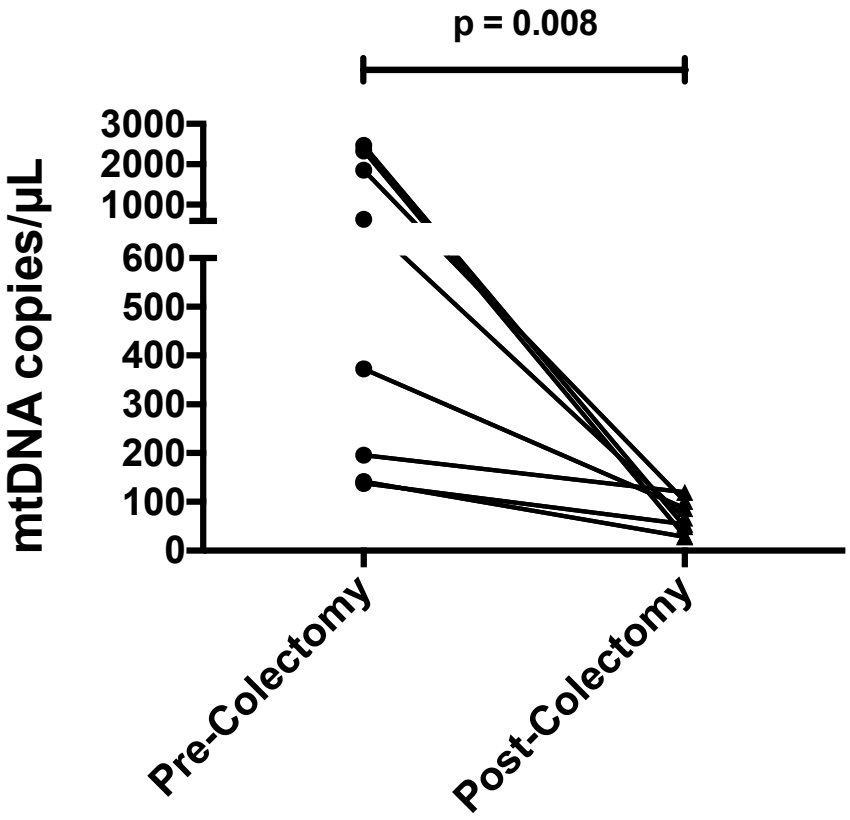

Figure 3.22: Longitudinal analysis of plasma mtDNA(copy/µL) in ASUC (n=8 patients) during active disease and the same patient post colectomy in clinical remission. All data represent median ± IQR.

3.3.8 Summary of results

- Higher circulating plasma mtDNA is associated with more severe disease in IBD as assessed by clinical indices. For ASUC, higher mtDNA levels are also associated with endoscopic severity.

- mtDNA is positively correlated with known markers of disease severity including CRP, albumin and WCC. There was a significant correlation found between serum calprotectin and serum mtDNA but not with plasma mtDNA.

- Patients with ASUC who did not respond to medical therapy and required colectomy had higher circulating mtDNA levels compared to those who responded to medical therapy. mtDNA performed similarly to CRP and albumin in predicting colectomy.

- Multivariate analysis showed CRP to be independently associated with high mtDNA levels.

- Sequential mtDNA samples on consecutive days during admission for ASUC demonstrated inter-day variability.

- mtDNA levels significantly dropped between pre- and post-colectomy plasma samples for patients with ASUC

3.4 Discussion

3.4.1 Precision medicine & the future role of biomarkers in IBD

3.4.1.1 Precision medicine

The completion of the human genome project in 2003 represented a major scientific landmark, ushering in a new era with hopes and expectations of fresh insights into disease mechanisms and treatments. In IBD, many important discoveries soon followed, notably the identification of more than 200 genetic susceptibility loci and characterisation of the gut microbiome (Huttenhower *et al.*, 2012). As 'big data', driven by advances in technology, becomes increasingly available and affordable, individuals with IBD and clinicians alike yearn for tangible outcomes from the promise of 'precision medicine' – precise diagnosis, monitoring and treatment.

Precision medicine is a major priority in health care, now recognised by all major stakeholders including governments, the pharmaceutical industry, clinicians and patients. In January 2015, United States President Barack Obama announced the Precision Medicine Initiative® (PMI): a concerted effort by multiple government agencies and backed by $215 million in federal funds to help facilitate a greater understanding of individual disease variability and its clinical translation (Collins and Varmus, 2015). A major component is the PMI Cohort Program, an ambitious plan to build a national research cohort of more than one million participants in a participant-centred, data-driven manner

with integrated multi-omic profiling. The PMI working group's report (Hudson, Lifton and Patrick-Lake, 2015) identified key scientific opportunities including a number relating to the importance of biomarkers (Table 3.3). Similarly, the Chinese government has plans to invest 20 billion yuan (around $3 billion) to support precision medicine research by 2030. In the UK, the 100,000 genomes project was launched in 2012 with the goal of large-scale integration of genetic information and health records from the National Health Service (http://www.genomicsengland.co.uk). In the same year, the National Phenome Centre was launched in UK, offering broad access to exploratory and targeted high-throughput metabolic phenotyping and computational biology facilities. These massive undertakings are potential game changers in the field of biomarker discovery and validation.

• Translating already identified environmental and genetic risk factors into conclusions on disease causes and population impact with population-based cohort studies as well as identifying new associations. • Interrogation of the wide variation in therapeutic response and adverse reactions • Discovery of biomarkers for identification of individuals with higher risks of developing disease to help more rational prevention efforts • Novel classification systems which transcend the existing grouping based on symptoms, signs, lab results by using molecular characterisation • Using biomarkers to assign patients into a variety of clinical trials targeting subsets based on these biomarkers to help with development of novel therapies

Table 3.3: Biomarker focussed scientific opportunities identified by the PMI

In IBD, the successful international partnerships in genetics and microbiome research already provide grounds for realistic optimism. A major concern remains the wide-ranging nature of the stochastic elements of IBD, which represent formidable hurdles with respect to study design and measurable outcomes. In our field, current creative research approaches are now beginning to integrate across molecular datasets (e.g. genetic + microbiome), override traditional boundaries of disease classifications (UC vs. CD) and most notably, increasingly rely on patient input using new technological applications to characterise the 'exposome' in IBD. Hence, a new theme of recombinant innovation is emerging with synergy arising from novel ideas within established and fresh datasets.

3.4.1.2 Progress towards precision medicine in IBD

Disease susceptibility, activity & behaviour

Although genetic data has provided invaluable insights into disease pathogenesis, the role for genetic data in predicting susceptibility, activity and disease behaviour however is less strong. Genetic information allied with other biological data (e.g. pheno-genomic status), maybe more informative. The strongest genetic signal, NOD2 status has been associated with ileal and fibrostenosing disease but the carriage of NOD2 mutant allele is uncommon. A combination of clinical, serological and genotypic data has been used to help predict the risks of surgery in CD (Dubinsky *et al.*, 2013). Recently, a study of 29,838 IBD patients tested for genetic-phenotype associations found that

predictive models based on generated genetic risk scores strongly distinguished colonic from ileal CD (Cleynen *et al.*, 2015).

Away from predicting susceptibility and behaviour in IBD, there is an unmet need for sensitive biomarkers to measure gut mucosal inflammation, which is necessary to provide objective data on disease activity and guide response to treatment. Presently, CRP and faecal calprotectin have better negative predictive values and are thus more useful in excluding significant inflammatory signals. Some progress can be expected in modalities to image inflammation (e.g. MRI) with or without the use of specific in-vivo labelling of inflammatory cells or targets. A recent study used confocal laser endomicroscopy to detect fluorescent antibody labelled membrane-bound TNF in intestinal immune cells of 25 CD patients and thus identify patients who are most likely to respond to anti-TNF therapy (Atreya *et al.*, 2014). Furthermore, a re-thinking of ways to measure established biomarkers such as a measuring serum (rather than faecal) calprotectin may improve the performance and applicability of these tests (Kalla *et al.*, 2016).

In search of better disease activity prediction, current approaches such as those exemplified by the EMBARK study (Faubion *et al.*, 2013) investigates panel of biomarkers by their correlations with endoscopy and radiological findings as the best reference measure. Such reference measure captures disease extent; location and burden well but are not specific enough to take in disease behaviour, complications and progression. As will be discussed later,

better biomarkers of activity may come from a refined approach measuring specific downstream effects of the biological pathway targeted.

Prediction of drug response and adverse effects

Exploiting the wealth of genetic data, the combination of phenotypic information with multiple susceptibility loci is shown to be predictive of primary non-response in anti-TNF therapy in paediatric IBD (Dubinsky *et al.*, 2010). There have been some notable successes in transcriptomics (gene expression) in IBD. Lee *et al.* showed that CD8+ T-cell immune signatures are better at predicting disease course than traditional clinical or serological markers (Lee *et al.*, 2011). Hence, this approach is useful to select individuals that might benefit from more aggressive medical treatment. At the mucosal level, one study of infliximab in UC used gene signatures to separate responders from non-responders with 95% sensitivity and 85% specificity (Arijs *et al.*, 2009).

In terms of predicting adverse effects, GWAS of azathioprine induced pancreatitis found increased susceptibility for HLA-DQA1-HLA-DRB1 variants with a 2.5 fold increased risk in heterozygotes and a 5 fold increased risk in homozygotes at rs2647087 (Heap *et al.*, 2014). Although an important finding, this potential biomarker highlights some of the difficulties encountered in translation to the clinic. For example, the low pre-test probability of pancreatitis means that even in the highest risk homozygotes, there is an 83% chance of taking thiopurines without developing pancreatitis. Would this justify the

exclusion of thiopurines in these patients? It will however improve risk counselling and awareness. In a future of greater therapeutic options, this may be more feasible. In addition, the number needed to test was 76, making it an expensive option for screening. However, cheaper point of care testing in the future, and the possible combination with other biomarkers could change the economics of such a test.

3.4.1.3 Biological basis of biomarker discovery

IBD clinicians will have increasing number of drugs available with over 20 currently in the developmental pipeline (D'Haens *et al.*, 2014)(Danese, 2012). Rather than a sequential approach of trying one drug after another, one of the goals for Precision Medicine is to identify individuals or disease phenotypes that are better suited for a particular drug from the outset (e.g. anti-leukocyte migration vs. anti-TNF for example).

This direction is appealing and cogent where recent advances in oncology and virology have shown the way. In breast cancer, HER2 (human epithelial growth factor) positivity provides prognostic information (more aggressive phenotype with higher recurrence rates) as well as therapeutic choice (response to monoclonal antibodies targeting HER2 such as trastuzumab) (Arteaga *et al.*, 2011). In non-small cell lung cancer, mutations in multiple oncogenes including ALK and EGFR can help direct tyrosine kinase therapy (Lindeman *et al.*, 2013). In hepatitis C, prior to the direct acting antiviral revolution, IL28B genotype

helped predict the likelihood of sustained viral response to interferon and ribavirin therapy.

In metastatic colorectal cancer, KRAS gene mutations predict response to anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibody therapy (Allegra *et al.*, 2009). Notably, the early studies were performed based on a hypothesis developed from an understanding of EGFR biology (Lièvre *et al.*, 2006); subsequent retrospective subset analysis of randomised controlled trials provided strong evidence for clinical use.

3.4.1.4 Hypothesis free vs hypothesis-based biomarker discovery

The critical question is: are we on the cusp of a therapeutic revolution underpinned by the inexorable wave of 'hypothesis free' big data, or will we end up drowning in a sea of potential biomarkers that we cannot translate into clinical practice? A number of critical enablers allow for optimism (Figure 1.2) including developments of large-scale prospective cohorts, advances in high throughput technology, advances in computational power and increasing emphasis on patient inputted data.

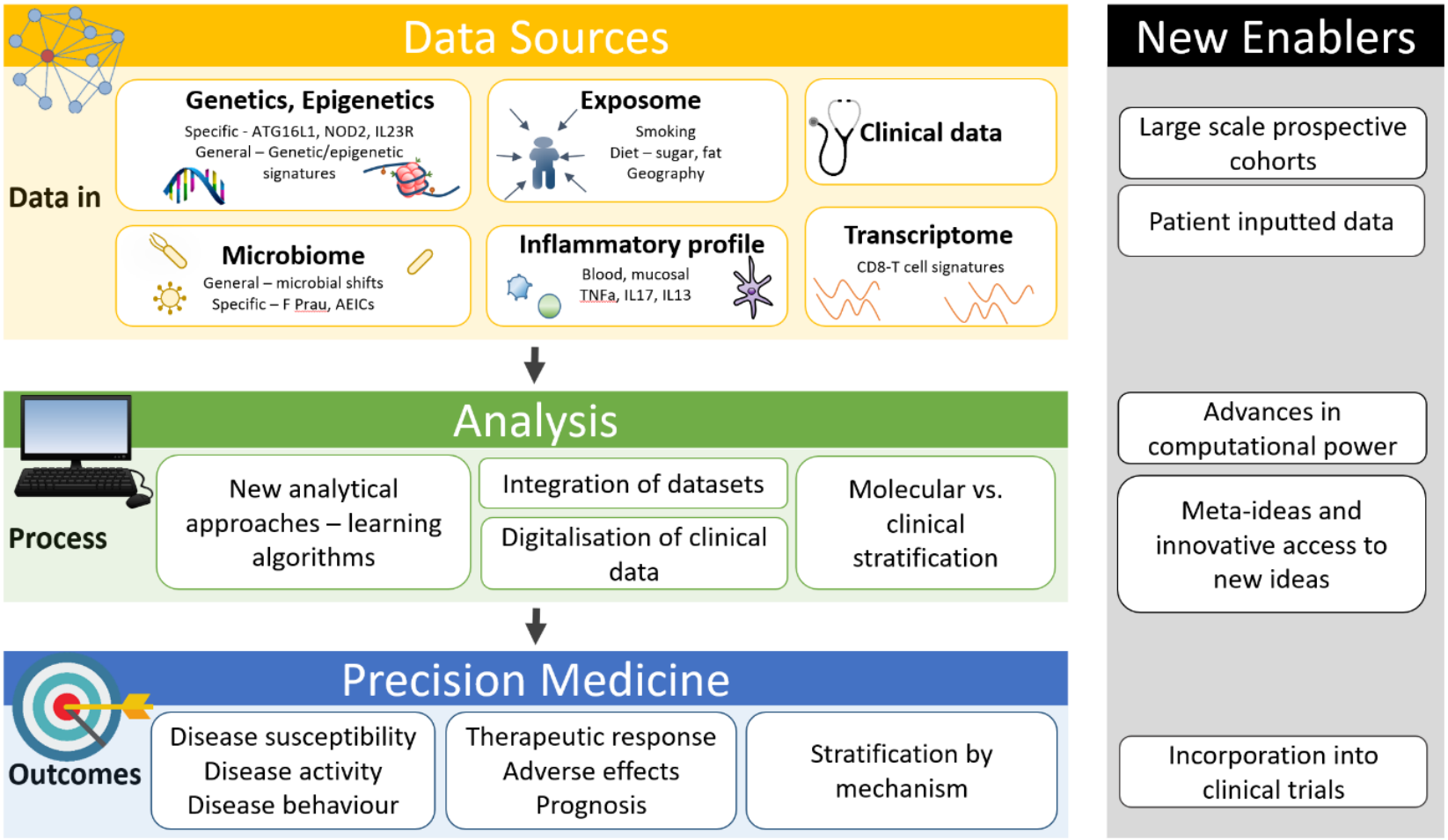

Figure 3.23: Critical enablers in the flow of precision medicine in inflammatory bowel disease (IBD) (Boyapati, Kalla, *et al.*, 2016)

Notwithstanding all these major interventions, there is a need for a dose of realism. In cancer research, where investment has been far greater, there has been a decrease in the number of Food and Drug Administration approved protein biomarkers over the last decade (Pavlou, Diamandis and Blasutig, 2013). In IBD, it is notable that biomarkers in existing use such as faecal calprotectin were found through hypothesis based investigation (Roseth *et al.*, 1992) rather than high throughput methods or *in silico* database analysis.

Discovery based approaches are currently burdened by several significant challenges (Table 3.4) although some potential solutions have been identified (Table 3.5). Selection bias from convenience sampling and data overfitting can result in over-interpretation of 'significant' *p*-values, potentially wasting valuable resources on random noise. For example, a host of studies have identified genetic polymorphisms as predictors of therapeutic response in IBD (Mosli *et al.*, 2014) but these have not been consistently replicated. Therefore, there remains room for advancements based on discoveries in related inflammatory conditions, serendipity and organic scientific thinking although big data now forms the ground for the generation of new hypotheses.

Methodological challenges
• Selection bias using convenience sampling • Increased flexibility and non-linearity in algorithms leading to overfitting • Potential confounders including interaction between the different 'omes' (e.g. microbiome studies with effect of host genome), disease activity, duration, location and effects of drug treatment, study design, heterogeneous cohorts • Standardisation of all steps in the process of biomarker discovery is optimal but in practice, difficult to achieve.
Cost challenges
• Lack of support from pharma companies not wanting to fragment markets • Large costs associated with biomarker validation for those biomarkers proposed by unbiased -omics testing
Integration & Adoption Challenges
• Electronic medical record integration – difficulties in standardisation, poor quality and granularity of inputted data. National approach easier in some countries (e.g. UK with NHS) than others e.g. USA • Adoption and acceptance by physicians and patients • Teamwork and large collaboration across institutions is particularly critical in relatively low incidence diseases such as IBD with creation of standard protocols and large cohort's obvious benefits but more complex, difficult and costly coordination an important challenge. • Privacy and data security

Table 3.4: Challenges with –omics research

There are many potential solutions to help resolve some of the earlier identified issues. For example, the importance of consistency in sample acquisition and processing and keeping patient cohorts as homogenous as possible cannot be overstated. We are approaching the stage where ‘big data’ will no longer be the rate-limiting step to progress; instead, it will be the clinician or researcher’s ingenuity in leveraging these assets into new knowledge which will be crucial. Increasingly, more targeted therapies will require more defined biomarkers to measure their effects on the respective biological pathways. This sets the scene for stratified clinical trials as seen in oncology for example.

- Same method of sample acquisition
- Homogenous patient subsets
- Studies of subjects with no prior medical therapy
- Data-driven approaches such as network interference
- Require prospective studies with multiple time points

Table 3.5: Potential solutions to help biomarker discovery

3.4.2 mtDNA as a biomarker in inflammatory diseases

In addition to higher levels of mtDNA found in various inflammatory diseases (Section 1.3.5), many studies have additionally suggested its role as a potential biomarker. In trauma, studies have demonstrated correlation with injury severity (Lam *et al.*, 2004; Nicole Y.L. Lam, Timothy H. Rainer, Rossa W.K. Chiu and Lo, 2004; Hsu *et al.*, 2013) and found higher mtDNA levels in non-survivors compared to survivors (Hsu *et al.*, 2013; Prikhodko *et al.*, 2015). Further studies have found elevated plasma mtDNA is an independent predictor of SIRS in trauma patients (Gu *et al.*, 2013) and correlates with SIRS score (Zhang *et al.*, 2017).

Di Caro *et al.* found higher mtDNA in the plasma of critically ill paediatric patients who were septic compared to similarly unwell but non-septic patients (Di Caro *et al.*, 2015). Another study in severe sepsis demonstrated mtDNA to be more powerful as a prognostic indicator than either sequential organ failure assessment or lactate concentration (Kung *et al.*, 2012). Similarly, some studies of patients in the intensive care setting have found that higher mtDNA levels are associated with poorer outcomes (Nakahira *et al.*, 2013; Krychtiuk *et al.*, 2015). In terms of chronic disease, a study found elevated anti-mtDNA antibodies in SLE, particularly in lupus nephritis where levels correlated with the lupus nephritis activity index better than did anti-dsDNA antibody levels (H. Wang *et al.*, 2015).

Collectively, these studies demonstrate the potential for mtDNA to be used as a biomarker in a variety of inflammatory diseases.

3.4.3 mtDNA as a potential novel biomarker in IBD

3.4.3.1 Biomarker landscape in IBD

Many dozens of potential genetic, blood-based, faecal, microbial and immunological biomarkers have been proposed in IBD (Dubinsky and Braun, 2015; Sands, 2015). However, apart from a few notable exceptions, biomarkers have not yet found widespread clinical application in clinical practice for a variety reasons (Table 3.6).

• Fails on the classic qualities of an ideal biomarker (simple, accurate, easy to perform, minimally invasive, cheap, rapid, reproducible) • Unclear or uncertain clinical utility: i.e. does not provide clinically useful information upon which to make decisions • low sensitivity/specificity • low prognostic/predictive values • Lack of validation: yet to be validated in independent cohorts or have had inconsistent results when validation has been attempted. • Some areas (such as microbiome-based biomarkers) are in their infancy

Table 3.6: Problems with biomarkers that have not found widespread clinical application in IBD

Most proposed biomarkers in IBD have failed to be implemented into clinical practice because they fail on the classic qualities of an ideal biomarker (simple, accurate, easy to perform, minimally invasive, cheap, rapid, reproducible) (Vermeire, Van Assche and Rutgeerts, 2006).

The use of DAMPs as biomarkers in IBD is established. The most relevant example is faecal calprotectin testing which has revolutionised IBD clinical practice with roles in differentiating IBD from functional gut disorders (Tibble *et al.*, 2000); as a marker of disease activity (Lin *et al.*, 2014) and to predict subsequent course of disease (Ho *et al.*, 2009). Calprotectin is a major cytosolic protein found in neutrophils and other inflammatory cells and is released by stressed cells during intestinal inflammation. Faecal calprotectin is now also a measurable outcome in current clinical IBD therapeutic trials.

The recent CALM study provides direct evidence for the benefit that biomarkers can play (Colombel *et al.*, 2018). In this prospective, open-label, multicentre, active-controlled study in moderate-severe CD, treatment using an escalating therapy strategy based on symptoms alone was compared to symptoms and biomarker targets (CRP < 5 mg/L and faecal calprotectin level < 250 µg/g). The investigators found a significantly higher number of patients reached the primary endpoint of endoscopic remission as well as steroid free remission with the 'treat-to-biomarker-target' approach compared to the approach based on symptom management alone.

As we move towards an era of 'big-data' (Section 3.4.1), hypothesis-free methods will propose many biomarkers going forward. However, a significant challenge remains to identify 'functional' biomarkers – i.e. those which have direct relevance to disease pathogenesis, which are more likely to help stratify patient populations that allow for yet further insights (Surinova *et al.*, 2011; Boyapati, Kalla, *et al.*, 2016).

3.4.3.2 Major findings

The findings in this chapter are consistent with the potential role of mtDNA as a novel biomarker in IBD.

mtDNA over a 5-day admission for ASUC

Data from five randomly selected patients with ASUC who agreed to consecutive day sample collection during admission revealed day to day variability in mtDNA levels. Notably, all patients were on intravenous steroids during their admission.

Some interesting observations may be made from this limited exploratory data. For example, mtDNA levels for patient 1 rose from day 2 to 3 whereas CRP consistently fell during the five days. Interestingly, this patient required a colectomy due to failure of medical therapy. Multiple samples from larger cohorts is required for both IBD patients and healthy controls to assess for inter-day and intra-day variability.

It is interesting to note that mtDNA did not fluctuate in tandem with CRP for all patients. It is possible that mtDNA has a completely different acute phase response time compared to other biomarkers of inflammation. This would not be unexpected, given existing acute phase markers such as CRP and ESR temporally differ after an inflammatory insult.

Correlation with disease activity

I found that mtDNA levels in UC correlated with disease severity as assessed by clinical indices. Patients with clinically mild-moderate UC had higher levels that those in clinical remission, and patients with severe disease had the highest levels. In CD, mtDNA levels in patients with clinical remission were significantly lower than those with severe disease but no different to those with mild-moderate disease.

CD is a more heterogenous condition compared to UC with inflammation affecting anywhere from the mouth to the anus (vs. colon alone) and being transmural in nature often leading to fistulas, fibrostenotic strictures and abscesses (vs. confluent superficial inflammation). This heterogeneity in CD means that clinical indices may not correspond accurately to extent of inflammation. A recent study of over 500 IBD patients found a poor correlation between the HBI clinical index and endoscopic scores in CD, whereas there was a good correlation between the SCCAI and endoscopic scores in UC (Taleban *et al.*, 2016). Given circulating mtDNA levels are likely a reflection of the extent of inflammation in IBD (and thus non-apoptotic cellular death and liberation of DAMPs), the more consistent finding between clinical indices and mtDNA in UC compared to CD is unsurprising.

As expected in UC, patients with endoscopically mild disease compared to moderate or severe disease had significantly lower mtDNA levels. There was no significant difference found between the moderate and severe groups. It is

important to note that there are limitations to the endoscopy data: it was retrospective; classification was not blinded; and there was no central scoring of endoscopic severity. Endoscopists were, however, blinded to the mtDNA levels, and all endoscopists were senior IBD specialists capable of accurately reporting endoscopic severity in UC.

A meta-analysis of the diagnostic accuracy of various biomarkers in IBD in predicting endoscopic activity found pooled area under the curve (AUC) for CRP and faecal calprotectin to be 0.49 (95% CI 0.34-0.64) and 0.88 (95% CI 0.84-0.90) (Mosli *et al.*, 2015). Based on the results presented here, prospective studies investigating the diagnostic utility of mtDNA in predicting endoscopic activity are warranted.

Correlation with existing biomarkers of activity

MtDNA correlated as expected with existing markers of severity including CRP (positive correlation), albumin (negative) and WCC (positive). This would be consistent with a recent publication of circulating DNA in elderly patients with venous thromboembolism which found strong correlations with CRP and WCC (Jiménez-Alcázar *et al.*, 2018). A potential confounding factor was that most patients with severe disease were on corticosteroids at the time of sampling leading to elevated WCC.

Serum calprotectin has recently been suggested as novel biomarker in IBD (Kalla *et al.*, 2016). Kalla *et al.* found serum calprotectin significantly correlated

with CRP (r=0.41), WCC (r=0.37) and albumin (r=-0.39) in IBD patients and predicted colectomy with a AUC of 0.69 (similar to CRP in this cohort).

Interestingly, I found that serum calprotectin had no significant correlation to either COXIII or ND2 mitochondrial genes in plasma, although there was a trend to significance for ND2 (r=0.35, p=0.06). This latter finding may be a result of multiple testing or a reflection of possible type II error. Notably, serum calprotectin did correlate positively and with COXIII (r=0.55, p=0.04) and ND2 (r=0.60, p=0.02) when mtDNA was tested in serum. Matched serum samples tested for calprotectin and mtDNA were aliquots from the same processed sample; the stronger correlation found in serum vs. plasma may be a reflection of this (compared to plasma which was separately processed as per the plasma protocol).

Prognostic indicator

Colectomy was chosen as the 'hard' endpoint most commonly used in ASUC research. Of the 40 patients with ASUC, 12 (30.0%) underwent colectomy over the follow up period following admission for ASUC, similar to colectomy rates reported in the infliximab rescue therapy era (Jarnerot *et al.*, 2005; Williams *et al.*, 2016).

I found that mtDNA performed similarly to CRP and albumin in predicting colectomy in ASUC (AUC 0.71, p=0.039). MtDNA compares favourably to other biomarkers reported in the literature including faecal calprotectin (AUC

0.65, p=0.04), serum calprotectin (AUC 0.69, 95% CI 0.53-0.81) and CRP (AUC 0.71. 95% CI 0.56-0.86) (Ho *et al.*, 2009; Hare *et al.*, 2014).

Further studies might seek to validate this finding in another cohort. A larger cohort may also enable multivariable models to build a prognostic 'score' based on multiple biomarkers including mtDNA. In addition, its use as a prognostic marker for other outcomes in IBD such as predicting disease relapse, abdominal surgery and hospitalisation could be assessed.

When a cutoff of 1,545 mtDNA copies/µL was used (guided by ROC curves for highest likelihood ratio), there was significant separation of the survival curves for colectomy on Kaplan-Meier analysis. This cut off requires validation other cohorts. However, in broad terms, this finding supports the idea that mtDNA levels may provide valuable prognostic information in some IBD patients.

Characteristics of patients with high mtDNA levels

The characteristics of patients with high mtDNA levels are of interest. An attempt was made to assess this through multivariable logistic regression analysis which found that only CRP was independently associated with high mtDNA levels. This is in keeping with the idea that a higher inflammatory burden is associated with higher mtDNA levels. A larger cohort may reveal further factors, which in turn could suggest further avenues of investigation.

Pre- and post-colectomy data

Patients who underwent colectomy had post-colectomy mtDNA levels that were significantly lower than pre-colectomy levels. All patients were resampled in clinic at a variable number of days post colectomy (median Δ107 days, IQR 89-189), and were in clinical remission with the diseased colon removed. This data is consistent with a possible role for mtDNA in monitoring disease activity over time. Furthermore, it is consistent with the hypothesis that mtDNA in the circulation arises from the inflamed gut in IBD (Chapter 4). Further studies with measurement of mtDNA at multiple time points would be useful to further define how mtDNA levels change post-colectomy.

3.4.4 Implications of findings

In this thesis, I have gone some way to helping identify mtDNA as a potential novel biomarker. It is important to emphasise the exploratory nature of this work. Further investigation is required, initially for validation of these findings, and subsequently to explore potential clinical or research applications. Nevertheless, I have shown that in some patients, plasma mtDNA levels correlate with disease activity and severity, and find mtDNA (and mitochondrial DAMPs more generally) to be a potential mechanistic biomarker in IBD.

Earlier, I highlighted the importance of such functional biomarkers to identify sub-mechanisms that drive the heterogenous clinical presentations and disease progression in IBD, where specific therapeutic interventions can be stratified accordingly (Section 3.4.1). However, several challenges exist to adoption of mtDNA as a biomarker. These include the variation in the methodology in which mtDNA is measured and reported in the literature (e.g. serum vs plasma, mtDNA specific PCR primers, plasma processing protocol). Standardisation of these protocols, including identification of ‘normal’ and ‘abnormal’ ranges, will be important prior to clinical use. Furthermore, further studies reporting clinically relevant predictive statistics in a variety of inflammatory conditions are required.

CHAPTER 4: SITE OF MITOCHONDRIAL DNA RELEASE IN IBD

Chapter 4 Contents

4.1 Introduction

Data presented in chapters 2 and 3 show elevated plasma levels of mtDNA in patients with IBD, with a potential role as a biomarker. A pertinent question is whether this observation is simply an epiphenomenon in IBD, or whether these mitochondrial DAMPs play a role in instigating and/or perpetuating inflammation. To investigate the precise contribution of mtDNA to IBD related inflammation would entail a substantial body of work in itself, beyond the time and funding constraints of this project.

As discussed earlier, a pro-inflammatory milieu of DAMPs along with mucosal oxidative stress and deregulation of homeostatic pathways after initial non-apoptotic cell death may promote further and ongoing intestinal damage (Boyapati, Rossi, *et al.*, 2016). I hypothesised that if mitochondrial DAMPs had a pathogenic role in the inflammation seen in IBD, they would be released at the level of the inflamed gut. In this chapter, I pursue multiple lines of evidence to investigate this hypothesis.

Firstly, mtDNA analysis of stool samples was performed to test the hypothesis that raised levels would be found in patients with IBD due to mitochondrial DAMP release into the faeces. This is conceptually similar to the DAMP S100A/9 (calprotectin) which is measured in the stool to test for gut inflammation. Secondly, transmission electron microscopy (TEM) of colonic epithelium from IBD patients was used to assess the presence of damaged mitochondria. Thirdly, expression of the downstream target of mtDNA (TLR9) was assessed in IBD vs non-IBD colonic specimens.

4.2 Methods

4.2.1 Stool mtDNA analysis

Stool collection

Stool was collected using a Sterilin Polystyrene 30mL container (ThermoFisher) and immediately stored at 4^{o}C. Within 4 hours, the sample was transferred to -80^{o}C until further use.

Isolation of DNA from stool

DNA was isolated using the QIAamp DNA Stool Mini Kit as per manufacturer's instructions. At Step 2, ASL buffer is used in the protocol; samples were processed in duplicate with one using the ASL buffer and one using phosphate buffered saline (PBS; to minimise processing-related liberation of DAMPs). PBS data is presented for mtDNA quantification.

200 mg of stool was placed in a 2mL microcentrifuge tube and placed on ice. 1.6mL of PBS or ASL buffer (see above) was added to each stool sample and then pulse-vortexed continuously until the stool sample was thoroughly homogenised (at least 1 minute). This was then centrifuged at 20,000g to pellet the stool particles. 1.4mL of the supernatant was then transferred to a new 2mL tube and the pellet was discarded. 1 InhibitEX Tablet (Qiagen) was added to the sample and vortexed immediately and continuously for 1 minute (until the tablet was completely suspended). The suspension was then incubated for 1 minute at room temperature (15-25^{o}C) to allow inhibitors to adsorb to the

InhibitEX matrix. The sample was then centrifuged at full speed for 3 minutes to pellet stool particles and inhibitors bound to InhibitEX matrix. Immediately post centrifugation, the supernatant was transferred to a new 1.5mL microcentrifuge tube and the pellet was discarded. The sample was centrifuged at 20,000g for 3 minutes.

25µL proteinase K was added to a new 2mL microcentrifuge tube and 600µL of supernatant from the sample was added. 600µL of Buffer AL was added and vortexed for 15 seconds. This was then incubated at 70^{o}C for 10 minutes and then 600µL of ethanol (96-100%) was added to the lysate, and then mixed by vortexing.

600µL of the lysate was then applied to the a QIAamp (Qiagen) spin column and centrifuged at 20,000g for 1 minute. The spin column was placed in a new collection tube and the filtrate was discarded. A second aliquot of 600µL lysate was applied to the spin column and centrifuged at 20,000g for 1 minute. The spin column was placed in a new collection tube and the filtrate was discarded. A third aliquot of 600µL lysate was applied to the spin column and centrifuged at 20,000g for 1 minute. The spin column was placed in a new collection tube and the filtrate was discarded.

500µL of Buffer AW1 was then added to the spin column and centrifuged at 20,000g for 1 minute. The spin column was placed in a new collection tube and the filtrate was discarded. 500µL of Buffer AW2 was then added to the

spin column and centrifuged at 20,000g for 3 minutes. The spin column was placed in a new collection tube and the filtrate was discarded. This was then centrifuged at 20,000g for a further 1 minute to help eliminate the chance of possible Buffer AW2 carryover.

The spin column was transferred to a new 1.5mL microcentrifuge tube and 200µL of Buffer AE was applied directly onto the QIAamp membrane. This was incubated for 1 minute at room temperature and then centrifuged at full speed for 1 minute to elute the DNA, with the elute stored at -20°C until analysed.

qPCR for absolute quantification of mtDNA in stool

The method previously described (Section 2.2.7) was used to quantify mtDNA in stool samples.

4.2.2 Transmission Electron Microscopy

Colonic pinch biopsies from IBD and non-IBD controls were obtained from distal colon during colonoscopy, briefly washed with sterile PBS and immediately transferred into 3% electron microscopy grade glutaraldehyde solution in 0.1M Sodium Cacodylate buffer, pH 7.3, for 2 hours before further processing (details available on request). For mouse studies, colons were flushed with PBS before transfer into electron microscopy solution as above. All TEMs were carried out at Electron Microscopy Unit, King's Building, University of Edinburgh.

4.2.3 Immunohistochemistry

Parrafin-embedded gut sections of healthy individuals, and individuals with IBD were provided by the Western General pathology department (via Dr Joseph Loane) using the Scottish Tissue Bank via Scottish Academic Health Sciences Collaboration (SAHSC) SR493. All IBD samples were coded and matched (sex, age and tissue location) with a non-IBD control group.

I am grateful to Dr Arina Tambrowska who optimised the protocol and performed the immunohistochemistry described below. The IHC protocol was optimised by testing various antigen retrieval protocols and primary antibody concentrations.

The sections were deparaffinised in xylene, rehydrated and retrieved using Tris-EDTA buffer. The endogenous peroxidase activity was quenched with 3% hydrogen peroxide. The sections were immunostained with polyclonal anti-TLR9 antibody (ab52967, Abcam) using Vectastatin Elite ABC Kit (Vector Laboratories) and diaminobenzidine. The sections were counterstained, cover-slipped and imaged with Zeiss Axiovert200 microscope using AxioVision 4.6 acquisition software (Carl Zeiss).

4.3 Results

4.3.1 Higher faecal mtDNA levels in ASUC

4.3.1.1 Processing methodology

In step 2 of the QIAamp DNA stool mini kit protocol, ASL buffer is used for lysis of stool samples. 6 samples were processed in duplicate with a single difference: ASL was substituted with PBS. The samples processed with ASL had significantly higher levels of mtDNA detected compared to matched PBS samples (1027 copies/nL [IQR 11.75-374] vs 171 copies/nL [193.2-2631], p=0.03, Wilcoxon), likely due to processing related liberation of mtDNA with ASL (Figure 4.1).

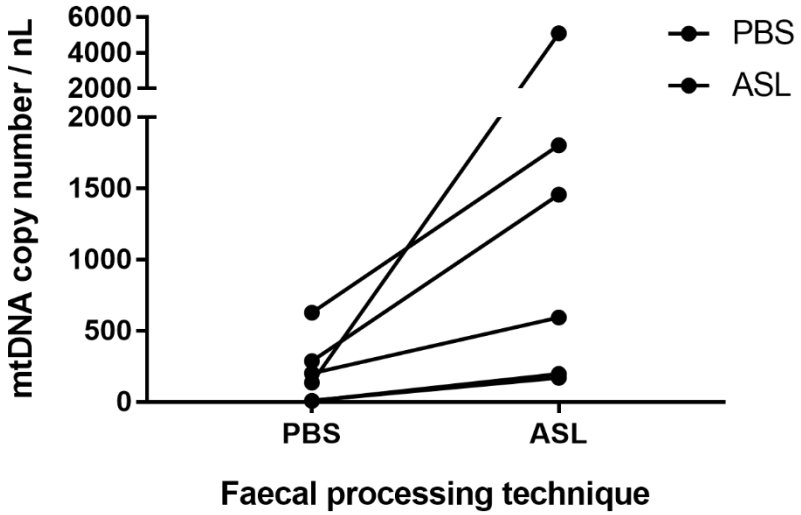

Figure 4.1: Comparison of matched samples with PBS and ASL used for step 2 of the QIAamp DNA stool mini protocol.

Herein, data from the PBS method is presented.

4.3.1.2 Faecal mtDNA is significantly elevated in ASUC

12 individuals with ASUC and 12 healthy controls were prospectively recruited to provide stool samples.

Significantly higher mtDNA levels ($p<0.0001$) were found in ASUC compared to non-IBD controls (222 copies/nL [IQR 19-313] vs. 1 copy/nL [IQR 0-7]) (Figure 4.2).

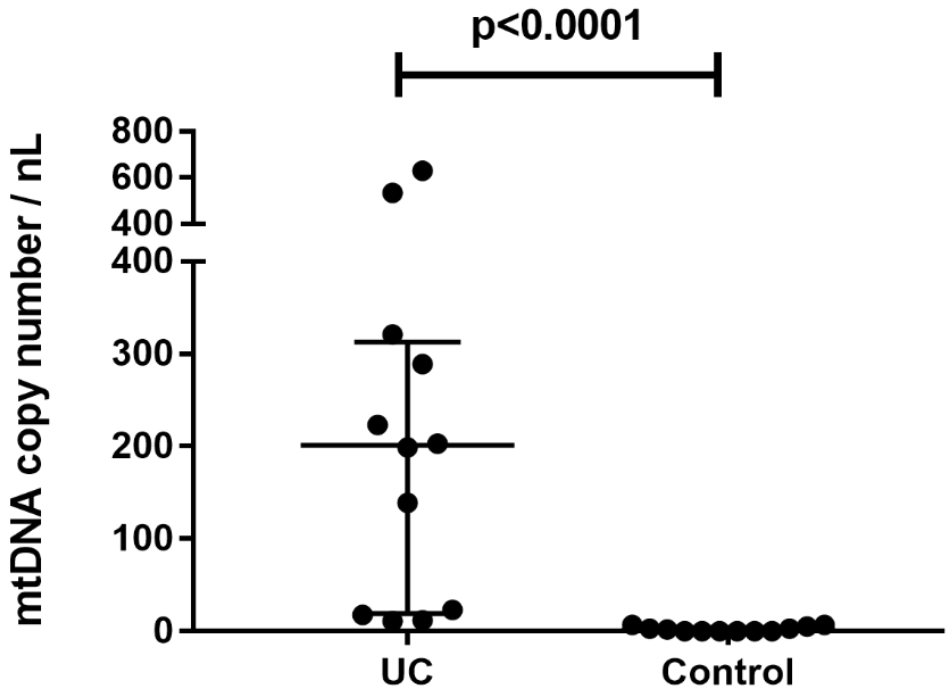

Figure 4.2: Faecal mtDNA (copy/nL) in active UC and non-IBD controls (n=12/group). Median ± IQR.

4.3.2 Matched stool vs plasma mtDNA

Of the 12 faecal samples analysed for mtDNA levels, 8 had matched (same day) plasma mtDNA data available for analysis (figure 4.3). There was no statistically significant correlation found (Spearman's r = 0.60, p=0.13).

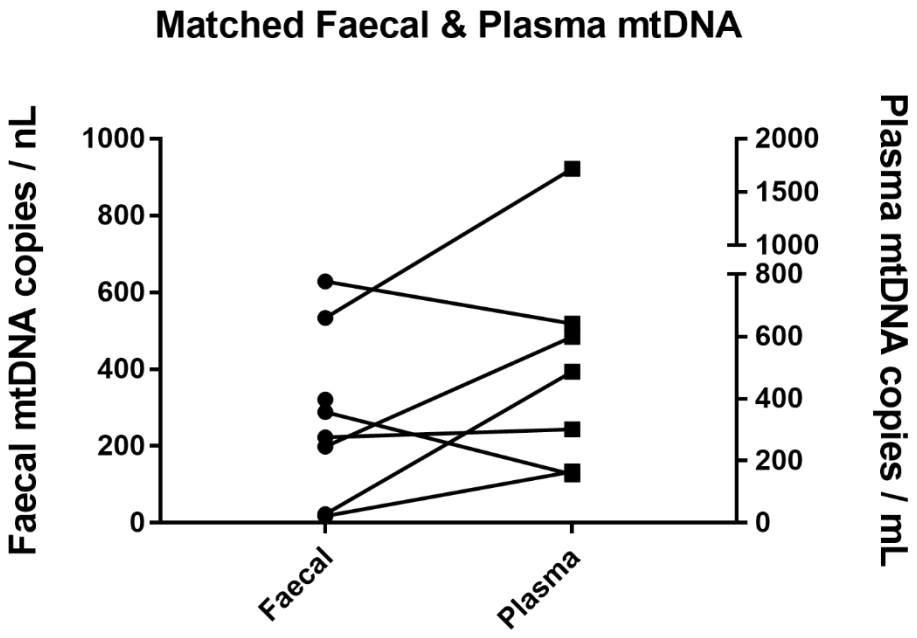

Figure 4.3: Faecal mtDNA (copy/nL) with matched plasma mtDNA (copy/mL) in active UC (n=8).

4.3.3 Active IBD is associated with mucosal mitochondrial damage

6 individuals with active UC and 6 non-IBD controls were prospectively recruited for pinch biopsies collected from the affected colon at time of colonoscopy. Patient characteristics are listed in Table 4.1.

Case	Age / Sex	Clinical Description
UC 1	23 years, Female	Active proctitis (UC), Mayo 2
UC 2	44 years; Male	Active left sided UC, Mayo 1
UC 3	41 years, Female	Active pan-UC, Mayo 2
UC 4	20 years, Male	Active left sided UC, Mayo 2
UC 5	33 years, Male	Active severe UC, to point of insertion (descending), Mayo 3
UC 6	49 years, Female	Active proctitis (UC), Mayo 1
HC 1	56 years; Male	Investigated for abdominal pain and weight loss; normal
HC 2	45 years; Female	Investigated for iron deficiency anaemia; internal haemorrhoids only
HC 3	62 years, Male	Investigated for PR bleeding; sigmoid diverticular disease found otherwise normal
HC 4	55 years, Male	Investigated for altered bowel habit and family history bowel cancer; normal
HC 5	39 years, Female	Investigated for iron deficiency anaemia; normal
HC 6	71 years, Male	Surveillance scope post polypectomy 2 years ago; left sided diverticular disease otherwise normal

Table 4.1: Clinical characteristics of individuals for transmission electron microscopy studies of distal colon. Mayo score refers to endoscopic classification (Appendix F)

Transmission electron microscopy (TEM) of the colon showed evidence of mitochondrial damage (with loss of inner cristae structure, increased lucency with swollen rounded appearances) in areas of cellular injury and were also extravasated within sub-epithelium in affected UC.

Representative images are shown in Figure 4.4 and images from participants are shown in Figure 4.5.

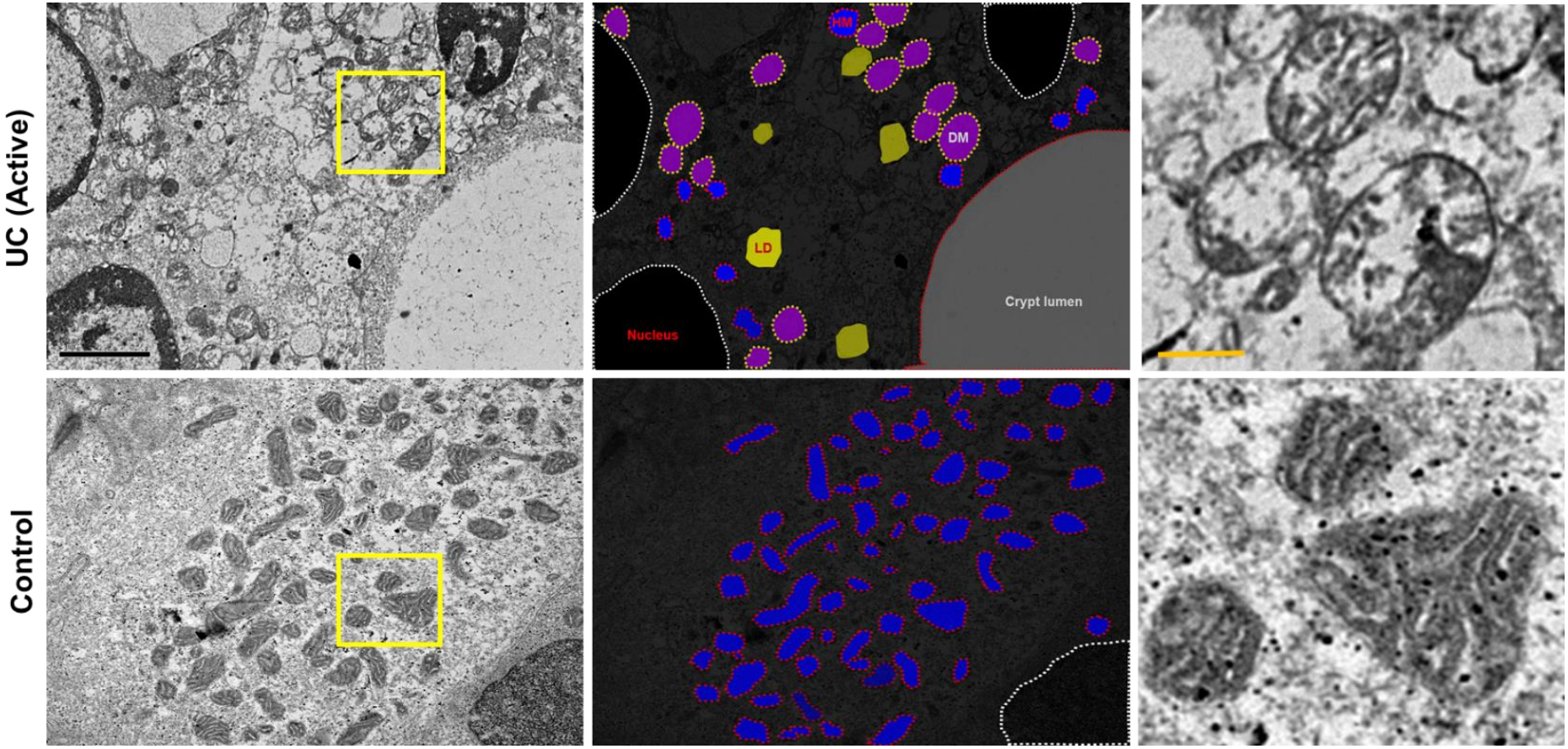

Figure 4.4: Representative transmission electron microscopy of distal colonic epithelium from active UC vs. non-IBD controls (n= 6/group; bar = 5µm). Annotated image: Purple – damage mitochondria (DM), Blue – healthy mitochondria (HM) and yellow – lipid droplets (LD). Black scale bar 2µm. Yellow insert – Damaged and healthy mitochondria from UC and controls respectively (Orange bar 0.5 µm).

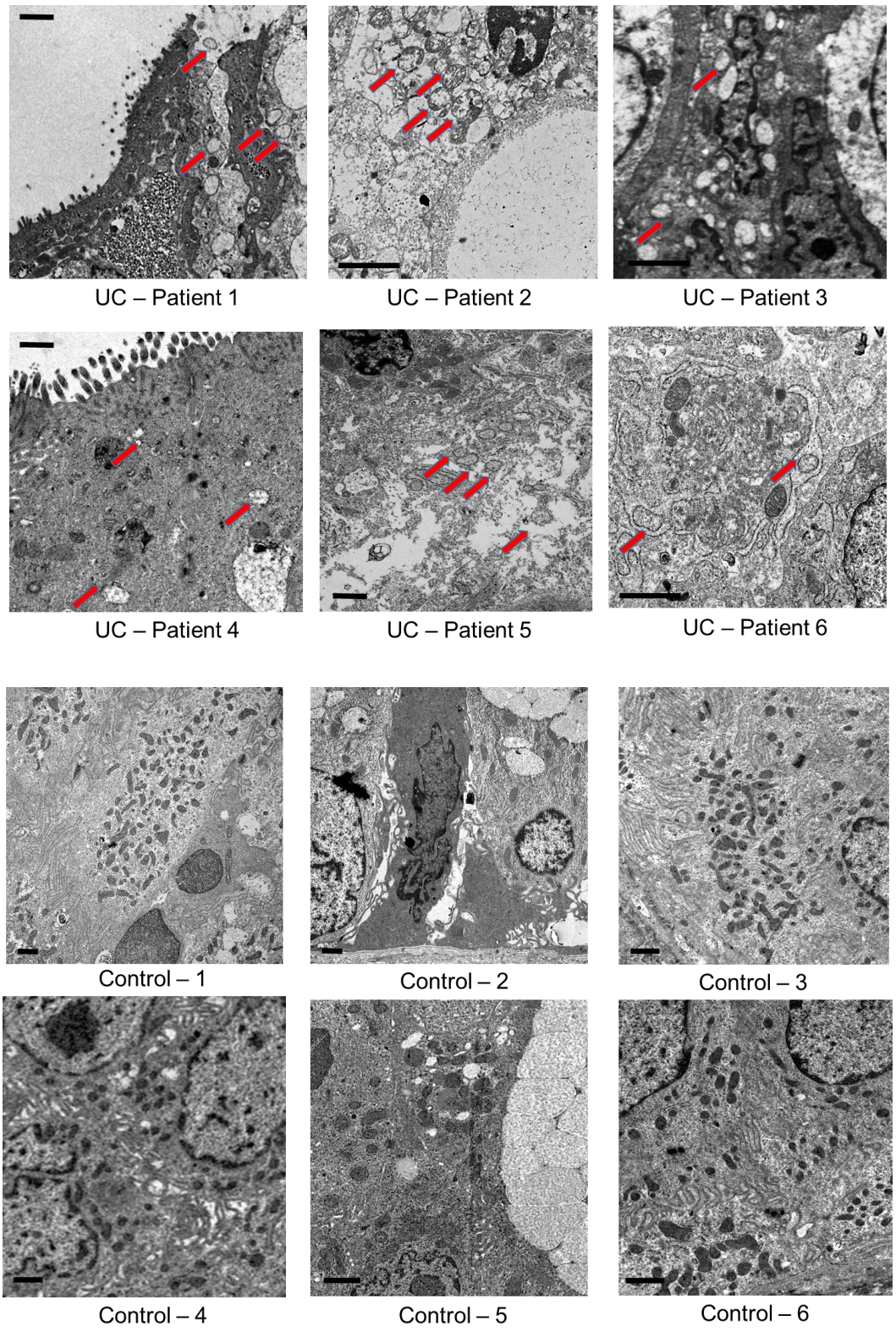

Figure 4.5: Representative colonic TEM images of 6 UC vs. 6 non-IBD individuals. Panel A – UC; Panel B – Non-IBD controls. Black bar - 2µm. Red arrows denote damaged mitochondria.

4.3.4 Immunohistochemistry

Colonic resection specimens from 14 IBD (7 UC and 7 CD) and 14 non-IBD controls were obtained for analysis.

We analysed TLR9 protein expression in human IBD colonic resection specimens and found increased frequency of lamina propria TLR9+ve cells in active UC and CD. Representative IHC is presented in Figure 4.6 and cell count data presented in Figure 4.7.

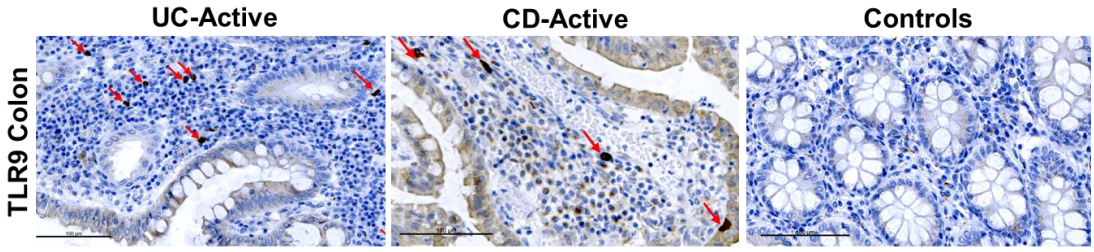

Figure 4.6: Immunohistochemistry for anti-TLR9 in human IBD colon (UC and CD; n=7/group vs. non-IBD control; n=14). Red arrows – TLR9 positive cells. Black scale bar 100 μm.

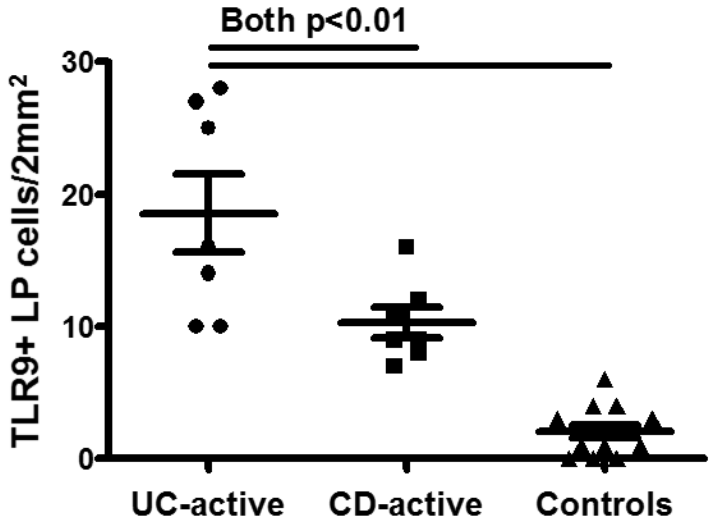

Figure 4.7: TLR9+ve cell counts in lamina propria (LP) of human IBD colon per 2mm^2 (UC and CD; n=7/group vs. non-IBD control; n=14). Mean ± SEM.

4.4 Discussion

4.4.1 Summary

The main finding of this thesis is that mitochondrial DAMPs can be found in the circulation of patients with active IBD (Chapter 2). Given that DAMPs are released during times of cellular stress and necrosis, this release is likely to occur at the gut mucosal level (Boyapati, Rossi, *et al.*, 2016). Local release of mtDNA at the site of inflammation would be in keeping with findings of higher mtDNA levels in the cerebrospinal fluid of patients with subarachnoid haemorrhage (Wang *et al.*, 2013), traumatic brain injury (Walko 3rd *et al.*, 2014) and multiple sclerosis (Varhaug *et al.*, 2016). More specifically to IBD, local release fits conceptually with the clinically useful finding of higher levels of another DAMP (calprotectin) in the stool in patients with gut inflammation.

The results in this chapter help support the hypothesis that mtDNA found in the circulation of IBD patients is released locally at the sites of inflammation in the gut. However, the functional consequence of mtDNA in IBD remains unclear. Significant further research, including *in vivo* studies, are required before this is demonstrated and any potential for therapeutic targets may be pursued (discussed further in Chapter 5). Time and resource restraints meant that a definitive exploration of mitochondrial DAMP release from inflamed mucosa and the functional consequence on this was thought to be unachievable.

I hypothesise that when present in overwhelming concentrations at the gut mucosal level, mtDNA may help instigate or propagate this inflammation. Prior to discussing the major findings in this chapter (Section 4.4.4), I present the current understanding of the cellular mechanisms of mtDNA as an inflammatory mediator including its release, clearance and manner in which mtDNA is thought to promote inflammation.

4.4.2 mtDNA release and clearance

4.4.2.1 Mechanisms of mtDNA release

Two levels of mtDNA release, cytosolic and then extracellular, are critically important steps. In the former, the mechanism of release of mtDNA from mitochondria relies on the opening of mitochondrial permeability transition pores in the inner mitochondrial membrane (Patrushev *et al.*, 2004). Inhibition of pore opening with cyclosporine A resulted in lower mtDNA in the cytosol after stimulation with LPS and ATP (Nakahira *et al.*, 2011). Ding *et al.* showed that the induction of ROS using ox-LDL increased mtDNA leakage into the cytosol in a dose dependent manner, and this effect was ameliorated with blockade of the ox-LDL receptor or a ROS inhibitor (Ding *et al.*, 2013).

In terms of extracellular release, cellular stress and necrosis are primary factors in the non-discriminant liberation of a host of mitochondrial components such as mtDNA, N-formyl peptides, ATP, TFAM and mitochondrial lipids. These mitochondrial constituents also exert their respective effects, which is

wide-ranging on key inflammatory pathways (extensively reviewed by Nakahira et al. (Nakahira, Hisata and Choi, 2015)). Aside from this non-selective release after uncontrolled cell death, several studies have suggested additional mechanisms such as necroptosis (or programmed necrosis) (Kaczmarek, Vandenabeele and Krysko, 2013). Blood transfusion induced endothelial necroptosis was recently found to increase extracellular mtDNA as a potential mechanism to explain transfusion related lung injury (Mangalmurti *et al.*, 2016). A recent study suggested that during necroptosis, mitochondria were released before plasma membrane rupture and they are then phagocytosed by monocyte-derived macrophages or dendritic cells triggering an inflammatory response as evidenced by cytokine production and cell maturation respectively (Maeda and Fadeel, 2014). Thus, ingestion of intact mitochondria may represent a distinct uptake mechanism following necroptosis.

In a separate study, platelets were also found to be a source for free extracellular mitochondria release and they then act as an endogenous substrate for bactericidal secreted phospholipase A_2IIA (sPLA$_2$-IIA) leading to mitochondrial membrane hydrolysis, loss of mitochondrial structural integrity and mtDNA release (Boudreau *et al.*, 2014). Intriguingly, Xin *et al.* found lower levels of mitochondria-derived ROS (mtROS) production when metformin was added to activated platelets and this was associated with decreased extracellular mtDNA release (Xin *et al.*, 2016). The authors found lower

complex I activity of the platelet mitochondrial respiratory chain, and suggested this as a mechanism for the observed suppressed mitochondrial dysfunction.

Whether there is an active element in mtDNA release is an interesting point of consideration. Active cellular transfer of mitochondria from stromal cells to rescue stricken lung alveoli cells in acute lung injury has been demonstrated (Islam *et al.*, 2012). Extracellular vesicles are important modes of intercellular communication and comprise of exosomes (endosomal) and microvesicles (plasma membrane derived), and is directed by exocytosis. Both chromosomal DNA (Balaj *et al.*, 2011; Waldenström *et al.*, 2012) and mtDNA have been observed in extracellular vesicles (Guescini, Genedani, *et al.*, 2010; Guescini, Guidolin, *et al.*, 2010; Ye *et al.*, 2017). In non-alcoholic steatohepatitis patients (NASH), a greater percentage of mitochondria were found inside extracellular microparticles and a higher percentage of microparticles contained mitochondria compared with lean subjects (Garcia-Martinez *et al.*, 2016). Additionally, a recent study found genomic DNA and mtDNA to be mainly detected in microvesicles, with only low levels found in exosomes (Cai *et al.*, 2017).

Further clarification is required on the concentration and significance of mtDNA in extracellular vesicles, and whether this has different immunostimulatory effects compared to cell-free or surface bound mtDNA. A recent study in chronic heart failure patients found plasma-derived exosomal-bound mtDNA

triggered an inflammatory response which could be blocked by chloroquine (a TLR9 inhibitor) (Ye *et al.*, 2017)..

As previously mentioned, the pro-inflammatory effects of mtDNA is dependent on its oxidisation (Shimada *et al.*, 2012; Pazmandi *et al.*, 2014). The highly oxidative extracellular milieu at sites of tissue inflammation in patients with chronic inflammatory disease may overwhelm anti-oxidant systems, further potentiating the inflammatory potential of DAMPs such as mtDNA (Boyapati, Rossi, *et al.*, 2016).

4.4.2.2 MtDNA degradation & clearance

Several well-described clearance mechanisms limit the pro-inflammatory nature of mtDNA. Autophagy, as discussed earlier, is important (Oka *et al.*, 2012) and defective autophagy is strongly linked to IBD. A proportion of circulating DNA in the bloodstream appears to cross the kidney barrier and be excreted in the urine (Botezatu *et al.*, 2000). Indeed, mtDNA has been detected in the urine at elevated levels in patients with progressive acute kidney injury (Whitaker *et al.*, 2015). This may be due to inflammatory state associated with this condition, the increased clearance with a disturbed kidney barrier or both.

Another possible mechanism of mtDNA clearance is phagocytosis by macrophages in a similar manner to the ingestion of the structurally similar bacterial DNA (Stacey, Sweet and Hume, 1996). As described earlier, the outcome of phagocytosis of intact mitochondria may be pro-, rather than anti-

inflammatory; these divergent effects may also be dependent on the phenotype of the phagocytosing cells (e.g. inflammatory vs. pro-resolution macrophages/monocytes, neutrophils and red blood cells) (Maeda and Fadeel, 2014; Mangalmurti *et al.*, 2016).

In general, non-host DNA in the circulation is digested in part by circulating nucleases, and mtDNA may be affected by a similar mechanism (Lo *et al.*, 1999). Intracellularly, DNases found in the autophagolysosome play a vital role to degrade mtDNA (Okabe *et al.*, 2005; Oka *et al.*, 2012). Oka *et al.* showed that cardiac-specific deletion of *DNAse II* resulted in mtDNA accumulation in cardiomyocytes and the development of heart failure (Oka *et al.*, 2012). In human umbilical vein endothelial cells (HUVECs), lysosomal DNases protect cells against inflammation from mtDNA damage induced by ox-LDL (Ding *et al.*, 2013). Here, siRNA knock down of DNAase II amplifies mtDNA-TLR9 mediated inflammatory response (Ding *et al.*, 2013).

It is unclear whether nucleases have a similar action on mtDNA in the extracellular space or is relevant in the physiological setting, especially when mtDNA are present in microvesicles or housed within intact mitochondria, which protect against DNase II. Intriguingly, DNase pre-treatment abolished renal mitochondrial injury that was observed after injection of mitochondrial debris (including mtDNA) in mice (Tsuji *et al.*, 2015). The precise role of DNase and its effect on the immunostimulatory effects of mtDNA is likely to be more

complex, however, as illustrated by a recent study which showed DNase II was required for TLR9 activation by bacterial genomic DNA (Chan *et al.*, 2015).

4.4.3 MtDNA inflammatory pathways

Current evidence shows that mtDNA-mediated inflammation is predominantly driven by the TLR9-, inflammasome- and more recently, STING pathways.

4.4.3.1 Toll-like receptor (TLR9)

TLR9 is located in the ER of various immune cells and translocates to the endosome upon sensing of hypomethylated DNA with CpG motifs, such as bacterial DNA (Latz *et al.*, 2004; Leifer *et al.*, 2004). Given its high frequency of unmethylated CpG dinucleotide repeats, it is postulated that mtDNA mediates inflammation dependent on the TLR9 pathway and potentially exerts a similar effect to bacterial CpG. TLR9 recognises a variety of types of oligodeoxynucleotides (ODNs) – for example, class A ODNs preferentially activate plasmacytoid dendritic cells whilst class B CpG ODNs activate B cells (Moseman *et al.*, 2004). Some of our understanding of how mtDNA may interact with TLR9 is extrapolated from work with class A ODNs, although they do not necessarily have the same effect. After activation of TLR9 by CpG DNA, inflammatory cytokine induction and Th1 immune responses occur (Hemmi *et al.*, 2000) and TLR9 is necessary in CpG DNA driven responses (Bauer *et al.*, 2001). TLR9 ligands can preferentially activate downstream pathways including pro-inflammatory NF_KB, and NLRP3 inflammasomes; and IRF-7

dependent type 1-IFN that can upregulate IL-1 receptor antagonist (Sasai, Linehan and Iwasaki, 2010; Petrasek *et al.*, 2011).

Most tissue injury models show better outcomes when *tlr9* gene is deleted. Wei *et al.* recently observed *tlr9*$^{-/-}$ mice have improved survival outcome in a necrotic lung model of cationic nanocarrier induced necrosis and mtDNA release *in vivo* (Wei *et al.*, 2015). Furthermore, the pulmonary inflammation seen post injection of mtDNA, was significantly reduced in *tlr9*$^{-/-}$ and *MyD88*$^{-/-}$ mice, underlining the importance of TLR9-MyD88 pathway (Wei *et al.*, 2015). Intravenous injection of mitochondrial debris with substantial amounts of mtDNA into mice induced a systemic inflammatory response in wild type mice which was significantly attenuated in *tlr9*$^{-/-}$ mice (Tsuji *et al.*, 2015). *Tlr9*$^{-/-}$ mice also have better survival compared to wild-type counterparts in severe renal ischaemia reperfusion injury with associated decreased circulating mtDNA (Bakker *et al.*, 2015). A similar protective effect is also seen in *tlr9*$^{-/-}$ mice with acute acetaminophen overdose with observed lower serum mtDNA and an absence of lung inflammation in contrast to the findings of wild type mice (Marques *et al.*, 2012). Nevertheless, the reduction in mtDNA in *tlr9*$^{-/-}$ mice is intriguing and could be explained by the reduced inflammation with lower resultant cellular necrosis.

Alternatively, it is possible that TLR9 is somehow involved in mtDNA release into the extracellular circulation. In a recent study using a murine model of non-alcoholic steatohepatitis (NASH), mtDNA from NASH hepatocytes resulted in

greater activation of TLR9 than mtDNA from control livers (Garcia-Martinez *et al.*, 2016). This suggests that mtDNA that is selectively modified during pathologic disease processes can augment the ensuing inflammatory response. Similarly, the level of TLR9 expression (due to various factors) appears to be important. In those with high mtDNA levels, higher TLR9 expression is associated with increased mortality in ICU as earlier discussed (Arnalich *et al.*, 2012).

Neutrophils have received the most attention in studies on mtDNA-TLR9 signalling in several different inflammatory settings. Zhang *et al.* found that mtDNA activates neutrophil p38 mitogen activated protein kinase (MAPK) through TLR9 with release of MMP8 (matrix metalloproteinase) and MMP9 (Zhang *et al.*, 2010; Zhang, Itagaki and Hauser, 2010), a finding confirmed in another study where phosphorylated p38 and MMP9 increased after mtDNA treatment of neutrophils (Sudakov *et al.*, 2015). A separate study reported similar findings where pre-treatment with TLR9 inhibitor ODN2088 inhibited activation of p38 MAPK and release of MMP-8 (Wei *et al.*, 2015). Gu *et al.* also found intratracheal administration of mtDNA provokes lung inflammation through TLR9-p38 MAPK (Gu *et al.*, 2015). Hip fracture in rats resulted in mtDNA release into circulation as well as higher TLR9 and NF-KB p65 activation and subsequent lung injury (Gan *et al.*, 2015). The role of other MAPKs such as extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK) remain unclear, and to our knowledge unexamined in this context. These data suggest a pathway where mtDNA activates

neutrophils through TLR9 binding and activation of the MAPK pathway with subsequent MMP8 and MMP9 release.

In considering mtDNA vis-à-vis the site and location of TLR9 receptor, mtDNA must be either displaced from whole mitochondria and moved into the cytosol or when extracellular, internalised by some mechanism(s) to act on endosomal TLR9. The endosomal location of TLR9 is most likely a mechanism to avoid unwanted activation (Barton and Kagan, 2009). It is unclear how extracellular mtDNA are internalised but possibilities include endocytosis, transmembrane diffusion, phagocytosis and receptor mediated endocytosis (Ziello, Huang and Jovin, 2010). Transmembrane diffusion is unlikely due to the highly (negatively) charged nature of DNA, which makes it difficult to pass through the cellular membrane. A recent study found that monocyte derived macrophages can take up whole mitochondria released from necroptosis suggesting that phagocytosis could be a relevant mechanism (Maeda and Fadeel, 2014). Given that the macrophage also has a clear role in resolving inflammation by clearing up cellular debris and apoptotic bodies, inadequate clearance of mitochondria following non-apoptotic cell death may lead to cellular corpses with mtDNA still abundantly present, being internalised by phagocytosis.

Typically, apoptotic corpses can suppress the transcription of pro-inflammatory cytokine genes, promote the secretion anti-inflammatory cytokines by phagocytes, and cause antigen-presenting cells to present dead cell antigen

in a manner that promotes immunological tolerance (reviewed by Zitvogel *et al.* (Zitvogel, Kepp and Kroemer, 2010)). It will be of interest to consider the fate of mtDNA when macrophages or dendritic cells phagocytose cellular corpses with mtDNA. Does this clear the mtDNA or does it regulate subsequent functions (e.g. immune responsiveness) in these cell types? This has yet to be studied in detail. It is also possible that binding to additional cofactors may facilitate the internalisation into immune cells and in this instance, HMGB1 and RAGE have been implicated (Tian *et al.*, 2007). In this study, HMGB1-CpG (class A) complexes resulted in TLR9/RAGE association and recruitment of MyD88 in B cells (Tian *et al.*, 2007). Here, RAGE was visualised as associating with the DNA and was internalised with some sequestered in endosome-like structures. However, this possible mechanism requires further investigation. It has also been proposed that activation of autoreactive B cells by CpG DNA occurs after B cell receptor engagement leading to delivery of CpG DNA to endosomal TLR9 (Viglianti *et al.*, 2003).

Although nucleic acid-sensing TLRs on immune cells are mainly found within cells, cell surface expression has also been described. Using flow cytometry, TLR9 has been detected on surface of resting B lymphocytes (Dasari *et al.*, 2005; Baiyee *et al.*, 2006) and peripheral blood mononuclear cells (Eaton-Bassiri *et al.*, 2004; Saikh *et al.*, 2004). One functional *ex vivo* study found primary human and mouse TLR9 surface expression in neutrophils which are upregulated by a variety of stimuli including TLR9 agonists (Lindau *et al.*, 2013). However, it remains unclear whether TLR9 can signal from the cell

surface. In other cell types, TLR9 is also expressed on the cell surface. For example, TLR9 is expressed on both the apical and basolateral membrane of intestinal epithelial cells although NF_KB is activated only via basolateral stimulation of CpG ligands (Ewaschuk *et al.*, 2007; Lee *et al.*, 2007). This is relevant at the gut mucosal interphase as this limits the extent of TLR9 activation at the apical surface, which is in contact with a luminal milieu rich with bacterial DNA. Hence, compromised intestinal barrier integrity and translocation of bacterial CpG from the lumen during gut pathology will lead to basolateral stimulation in this context. Whether mtDNA has a different propensity compared to bacterial CpG to trigger TLR9 depending on epithelial site has not been studied.

4.4.3.2 The inflammasome

The inflammasomes are targets of mtDNA leading to cleavage and activation of caspase-1 and downstream maturation of interleukin-1β (IL-1β) and IL-18 (Gurung, Lukens and Kanneganti, 2015). Here, it is cytosolic release of mtDNA that exerts the dominant effect on inflammasome activation. Of the several inflammasomes described, the NLRP3 inflammasome is the best characterised in this regard. Nakahira and colleagues showed that depletion of mtDNA reduced IL-1β secretion in macrophages following treatment with known inflammasome triggers, LPS and ATP (Nakahira *et al.*, 2011). Of interest, mtROS is a further key mediator in this process. Pharmacologic induction of mtROS correlates with higher secretion of active IL-1β in a NLRP3 and caspase-1-dependent manner and treatment with mtROS scavengers

suppresses this effect (Zhou *et al.*, 2011). The requirement for mtROS in NLRP3 activation has also been confirmed by other studies (Nakahira *et al.*, 2011; Shimada *et al.*, 2012; Zhang *et al.*, 2013; Won *et al.*, 2015) and may be explained by its oxidising effects on mtDNA. mtROS not only enhances the oxidative process, but also the cytosolic translocation of oxidised mtDNA that then binds directly to NLRP3 (Shimada *et al.*, 2012). Non-oxidised mtDNA is insufficient to activate the NLRP3 inflammasome, although it may stimulate IL-1β production via other inflammasomes such as AIM2 (Dombrowski *et al.*, 2012). Interestingly, genetic deletion of NLRP3 and caspase-1 results in less mtDNA release (Nakahira *et al.*, 2011; Won *et al.*, 2015). This suggests a positive-feedback loop, in which activation of the NLRP3 inflammasome by oxidised mtDNA further promotes mtDNA release. The overwhelming and/or persisting ROS production by inflammatory cells, for example, is known to damage macromolecules (DNA, as well as RNA, lipids, carbohydrates and proteins) of the surrounding cells. Activated neutrophils produce large amounts of ROS as part of their essential role in host defence (Holmstrom and Finkel, 2014). Hence this is a likely major contributory factor to mtDNA damage once the inflammatory process is triggered.

Other factors controlling mitochondria-mediated NLRP3 activation are also relevant. For example, defective autophagy increases caspase-1 activation, IL-1β and IL-18 production and cytosolic mtDNA translocation in LPS and ATP primed macrophages (Zhang *et al.*, 2013). Pharmacological inhibition of mitophagy/autophagy in human macrophages results in accumulation of

damaged mitochondria, ROS generation and IL-1β secretion (Zhou *et al.*, 2011) and increased NLRP3 expression in the presence of LPS (Ding *et al.*, 2014). Hence, defective autophagy leads to inadequate clearance of damaged mitochondria, priming the internal cellular environment for NLRP3 activation. It is noteworthy that given the diversity of NLRP3 activators, current literature suggest that the precise mechanism of NLRP3 activation is still under debate (Nakahira, Hisata and Choi, 2015). Although the role of the inflammasome is considered separate to TLR9 here, there is evidence that TLR/NFKB activation is a necessary priming step leading to NLRP3 upgregulation and subsequent downstream signalling. NF-κB-activating stimulus is required for cells to express pro-IL-1β and NLRP3 (Bauernfeind *et al.*, 2009). Imeada et al. showed that stimulation of TLR9 by DNA fragments during early acetaminophen-induced cell death can lead to the transcriptional activation of the IL-1β gene resulting in the formation of pro-IL-1β (Imaeda *et al.*, 2009). Using the acetaminophen hepatotoxicity model, they showed that NLRP3-deletion (and related inflammasome components ASC and Caspase-1) were protective against induced liver failure (Imaeda *et al.*, 2009). A further study however, did not show any effect of NLRP3-deletion on the outcomes of acetaminophen-induced liver failure (Williams *et al.*, 2011). Hence in the context of liver necrosis, the role for NLRP3 inflammasome remains controversial.

4.4.3.3 STING pathway

The role of mtDNA in innate immunity through the stimulator of interferon genes (STING) pathway has also been a focus of recent studies. STING is a

cytosolic protein anchored to the ER (Ishikawa and Barber, 2008). STING can be activated either by direct association with double-stranded (dsDNA) or by cyclic dinucleotides, which can be derived from intracellular bacteria or viruses, or produced by a DNA sensor, cyclic GMP-AMP (cGAMP) synthetase (cGAS) (Barber, 2014). This in turn, activates interferon regulatory factor 3 (IRF3) which ultimately translocates to the nucleus and transcribes type I interferon (IFN) genes and also NF_KB pathway (Ishikawa and Barber, 2008).

Two independent groups recently discovered that STING-mediated IFN response can also be activated by mtDNA (Rongvaux *et al.*, 2014; White *et al.*, 2014). They first observed that deficiency of apoptotic caspases (3, 7 and 9) resulted in upregulation of type I IFN genes. This response was dependent on Bak/Bax, pro-apoptotic proteins responsible for mitochondrial outer membrane permeabilisation leading to mtDNA release; and the release of cytochrome C that activates the intrinsic apoptotic pathway. Typically, apoptosis is considered immunologic silent e.g. it does not trigger an inflammatory response. However, these studies demonstrated that when caspases (9, 3/7) responsible for the completion of apoptotic process are inhibited or deleted, cytosolic mtDNA go on to activate cGAS/STING-mediated type I IFN signalling (Rongvaux *et al.*, 2014; White *et al.*, 2014). Hence, these caspases serve as a ‘brake’ on mtDNA-inflammatory effect during cell death. MtDNA released during cell death has been previously reported to provide a second signal that cooperates with an additional inflammatory signal (e.g., LPS) to activate the NLRP3 inflammasome and induce IL-1β production in murine macrophages (Shimada *et al.*, 2012). Further evidence of mtDNA role in STING-mediated

IFN responses comes from West *et al.* (West *et al.*, 2015). Here, partial deficiency of mtDNA binding protein TFAM was associated with increased concentrations of cytosolic mtDNA and enhanced type I IFN response, which was attenuated by knockdown of components of the STING pathway.

Aberrant mtDNA-STING signalling has been also implicated in human inflammatory diseases, such as SLE. As earlier discussed, Lood *et al.* showed that treatment of human neutrophils with SLE-abundant ribonucleoprotein immune complexes induces mtROS, mtDNA oxidisation and translocation of the mitochondria to the plasma membrane (Lood *et al.*, 2016). Oxidised mtDNA is then released extracellularly as a component of neutrophil extracellular traps (NETs). Transfection of NETs-derived mtDNA results in expression of IFN-β in human peripheral mononuclear cells. Systemic injection of oxidised mtDNA increases interferon-stimulated genes expression in spleen of wild type, but not STING deficient mice. Similar to inflammasomes, uncontrolled mtROS production promoting cytosolic mtDNA release is important in the STING activation and potentially in the case of autoimmunity. These studies highlight the importance of the innate cellular functions to handle the mtDNA release during the initiation of cell death, which ultimately will decide if the ensuing fate will be that of a silent or inflammatory outcome.

4.4.4 Local release of mtDNA in IBD

4.4.4.1 Abnormal mitochondria seen at the intestinal epithelial level

Our group recently found loss of mitochondrial protective mechanisms at the intestinal mucosal level rendered the mitochondria susceptible to damage, and triggered the onset of colitis in *multidrug resistant-1* (*mdr1*) deficient mice (Ho *et al.*, 2018). Other relevant IBD mice models with primary autophagy (*Irgm* and *Atg16l1*)(Adolph *et al.*, 2013; Liu *et al.*, 2013); including those with secondary autophagy impairments due to defective ER-stress (Kaser *et al.*, 2008) and NLRP6 inflammasome activity (Elinav *et al.*, 2011) all exhibited similar accumulations of damaged mitochondria within the gut epithelium as seen in *mdr1*-deficient mice. In addition, genome-wide association data sets show around 5% of IBD susceptibility genes have direct roles in regulating mitochondrial homeostasis (Ho *et al.*, 2018). These findings implicate defective mitochondria as a novel pathological mechanism in IBD.

In this thesis, TEM of the colon showed evidence of mitochondrial damage (with loss of inner cristae structure, increased lucency with swollen rounded appearances) in areas of cellular injury. These damaged mitochondria were also extravasated within sub-epithelium in affected UC. The nature of TEM did not allow more detailed discrimination of whether specific enterocyte or inflammatory cell types displayed a predilection towards mitochondrial damage. Further limitations of the TEM data described in this chapter include

the lack of blinding, and the data was observational only with no quantification of mitochondrial damage performed to compare UC and healthy controls.

Our findings of damaged mitochondria in areas of cellular injury on TEM are in keeping with studies in CD which found increased mitochondrial size and disrupted cristae (Soderholm *et al.*, 2002; Nazli *et al.*, 2004). In UC, mitochondrial abnormalities have been found on TEM even prior to any light microscopic changes (Delpre *et al.*, 1989; Hsieh *et al.*, 2006). Reduction in the mitochondrial electron transport complex activity has also been found in UC, and mitochondrial dysfunction has been found early in the development of DSS colitis in mice (Santhanam *et al.*, 2012).

4.4.4.2 Higher levels of mtDNA in the faeces

Stool testing provides a non-invasive method of intestinal assessment and has been used to test for inflammation (stool calprotectin) and gut microbiota (16S ribosomal DNA sequencing). Although not as accurate as assessment at the mucosal level (e.g. with endoscopy and histology), it is far more practical and less invasive.

I hypothesised that the widespread cellular disruption at the intestinal epithelial level would lead to local release of DAMPs (including mitochondrial DAMPs) into the faeces, and that these would be detectable. I find for the first time that mtDNA levels are significantly elevated in the stool of patients with UC compared to healthy controls. Using prospectively sampled stool, significantly

higher mtDNA levels were found in individuals with ASUC compared to non-IBD controls. Faecal mtDNA was significantly higher than plasma levels (~1000-fold) which may be explained by inherent nature of the different biological material.

These findings are in keeping with other DAMPs that have been detected as raised in the faeces of patients with IBD. In addition to calprotectin, lactoferrin (a marker of neutrophil degranulation that acts as an alarmin) is also detectable in the stool and can be used to differentiate IBD from functional disorders (Lewis, 2011). High levels of faecal S100A12 is found in active IBD, although existing studies are limited by size and most relate to the paediatric cohort (De Jong, Leach and Day, 2006; Kaiser *et al.*, 2007; Sidler, Leach and Day, 2008). Similarly, faecal HMGB1 is raised in intestinal inflammation associated with IBD (Vitali *et al.*, 2011; Palone *et al.*, 2014).

Samples of stool and plasma from the same patient on the same day did not show a statistically significant correlation. This may be due to multiple factors. This was a small dataset (n=8), so the analysis may have suffered from type II error. Furthermore, the stool mtDNA method is novel and requires validation (see below).

This is the first time mtDNA has been measured in stool. A widely used commercial kit was used to extract DNA from the stool samples (QIAamp DNA Stool Mini, Qiagen). A change to the protocol was made in order to reduce the

amount of cellular lysis (and thus minimise post processing liberation of DAMPs). This theory was supported with data presented showing when the manufacturer's lysis buffer (ASL) was used in matched specimens, mtDNA levels were dramatically higher than when PBS was used. The protocol includes an absorption resin to help remove PCR inhibitors. Despite this, there is a risk that the substitution of ASL to PBS led to incomplete removal of PCR inhibitors from the samples. Further work needs to be performed to optimise mtDNA detection in the stool.

4.4.4.3 Higher levels of TLR9 expression in active IBD

mtDNA has many similarities to bacterial DNA, particularly the high frequency of unmethylated CpG dinucleotide repeats. Once liberated from mitochondrial membranes, mtDNA can induce inflammation through three main pathways: TLR9, inflammasome and STING (Boyapati *et al.*, 2017) of which mtDNA-TLR9 is best characterised. TLR9 is expressed in many immune cells including dendritic cells, B-cells as well as in the cytoplasm and on the apical and basolateral surfaces of intestinal epithelial cells (Pedersen *et al.*, 2005). We hypothesised that if there was local release of mtDNA, TLR9 would be expressed in epithelial cells and in lamina propria immune cells.

The findings presented in this chapter confirms TLR9 expression in human intestinal epithelial cells and in lamina propria immune cells. TLR9 was expressed in the cytoplasm of the epithelial cells (similarly in the surface and in the crypt bases). We also noted cytoplasmic TLR9 expression in the immune

cells in the lamina propria. Moreover, we found that TLR9+ lamina propria cells were significantly more abundant in gut resection specimens of active IBD compared to non-IBD controls.

These data are in keeping with findings of strong TLR9 expression in the cytoplasm of epithelial cells from patients with UC compared to those from normal controls (Fan and Liu, 2015). This study also found TLR9 mRNA expression to be significantly higher in UC. However, our findings are in contrast to another study in which TLR9 mRNA expression was found to be reduced in inflamed IBD gut sections (Pedersen *et al.*, 2005).

In this thesis, we have showed that TLR9 expression is increased in IBD patients and that mtDNA is elevated in these patients. The data is associative and does not definitively show mtDNA dependent downstream function. It is possible that other ligands for TLR9 are raised in active IBD, leading to the higher expression seen. Further work should focus on demonstrating that mtDNA directly activates TLR9, and that this contributes to the inflammation associated with IBD.

CHAPTER 5:

CONCLUSIONS & FUTURE RESEARCH

Chapter 5 Contents

5.1 Preamble

A detailed discussion of the results are presented within each chapter (Sections 2.4, 3.4 and 4.4). This final chapter will discuss the overall implications of the thesis and focus on how future research could extend this exploratory work.

5.2 Mitochondrial DNA as a biomarker

DAMPs offer great potential as biomarkers in disease diagnosis, prediction of outcome, monitoring of progression and response to treatment. The role of calprotectin as an established IBD biomarker has been discussed, as have the numerous other DAMPs found in high levels in serum, faeces or at the mucosal level in IBD (Section 2.4.6.4, Table 2.3). At a broader level, investigating whether respective IBD sub-phenotypes have specific DAMP-signatures offers an opportunity to stratify patients for therapy and clinical trials.

MtDNA is becoming increasingly appreciated as a highly potent DAMP and relevant in the inflammatory cascade of human inflammatory diseases. In this thesis, I show for the first time that mtDNA is present at high levels in IBD, levels correlate with disease activity in some patient groups, and that mtDNA has the potential to be a novel biomarker.

MtDNA's potential as a biomarker offers a unique opportunity to stratify and identify individuals that may benefit from specific therapeutic targeting of downstream inflammation pathways (e.g. TLR9, NLRP3 or STING pathways). As discussed earlier, there are numerous studies in sepsis, trauma and acute single organ injury which have already demonstrated individuals with high mtDNA levels and TLR9 expression have worse prognosis. Therefore, there are clear groups in which stratification is useful.

However, several challenges exist to its implementation as a clinically useful biomarker as evidenced by numerous potentially promising biomarkers failing to be incorporated into IBD clinical practice. Further work should initially focus on validating mtDNA as a biomarker in different and larger cohorts, with prospective correlation to endoscopic and/or histologic activity as a surrogate for longer term outcomes. Other more fundamental issues exist such as variations in the method of quantification. Standardisation of these protocols, including identification of 'normal' and 'abnormal' ranges will be necessary.

5.3 Defining the effect of mtDNA in IBD

The main findings presented in this thesis of raised mtDNA in the circulation of patients with active IBD raises numerous questions. Foremost among these is whether the observation of elevated mtDNA (and mitochondrial DAMPs more generally) contribute to, or are simply a consequence of, inflammation in IBD. Indeed, the finding of raised mtDNA levels is not isolated to IBD, suggesting that its effect is unlikely to be IBD-specific (Section 1.3.5). Furthermore, numerous other DAMPs have been found to be elevated in IBD and experimental colitis including calprotectin, S100A12 and HMGB1 (Section 2.4.6.4) raising the question as to the relative significance of mtDNA compared to other DAMPs in IBD.

Although extensive further research is required, there are many reasons to believe that mtDNA may be a highly relevant functional DAMP in IBD. There is an increasing understanding of the functional role of mtDNA in other inflammatory diseases as well as IBD-specific factors which support its proposed status as an active player. In this section, I discuss the evidence implicating mtDNA as a functional mediator in inflammatory disease in general and IBD specifically, and further avenues of investigation to help define its possible functional role in IBD.

mtDNA contributes to the inflammatory response

Collins *et al.*, first reported the inflammatory potential of mtDNA in 2004, when they found that mtDNA (and not nuclear DNA) induced TNFα and caused

inflammatory arthritis when injected into the joints of the mice (Collins *et al.*, 2003). There are now numerous studies utilising *in vivo* injection of mtDNA to provoke local and/or systemic inflammation (Zhang, Itagaki and Hauser, 2010; Gan *et al.*, 2015; Hu *et al.*, 2015; Tsuji *et al.*, 2015; Xie *et al.*, 2017).

Moreover, there are now several *in vivo* studies to show that genetic deletion or pharmacologic interference of these pathways reduce the inflammatory effect of mtDNA. MtDNA's role as a TLR9 agonist has received most attention where many inflammatory models show better outcomes when TLR9-signalling is abolished. *Tlr9*–deletion is protective against SIRS following systemic administration of mitochondrial DAMPs (Tsuji *et al.*, 2015); and in lung (Wei *et al.*, 2015), liver (Marques *et al.*, 2012) and kidney (Bakker *et al.*, 2015) injury models characterised by high mtDNA release. Blocking TLR9 using inhibitory ligands has been shown to improve mtDNA-driven mouse models of cardiac failure (Oka *et al.*, 2012) and NASH (Garcia-Martinez *et al.*, 2016).

Collectively, these studies support the role of mtDNA as a direct contributor to inflammatory disease and not simply an epiphenomenon.

mtDNA-TLR9 in IBD

The role of mtDNA-TLR9 in IBD and intestinal inflammation however, is more complex. TLR9 is expressed both in the intestinal epithelium and, in resident and recruited lamina propria immune cells. Whereas NFKB-activation is

prototypically pro-inflammatory (Zhang, Lenardo and Baltimore, 2017), intestinal epithelial NFKB-activation is cytoprotective and important to maintain barrier integrity (Nenci *et al.*, 2007). Hence in *tlr9*-deficiency, the intestinal epithelium is postulated to have lower protective NFKB-activation and is more susceptible to injury. Along these lines, CpG oligonucleotide treatment in mouse studies using low dose DSS colitis showed either no difference or a protective role with the beneficial effects seen before the induction of colitis (Rachmilewitz *et al.*, 2004; Lee *et al.*, 2006; Rose, Sakamoto and Leifer, 2012). In contrast, CpG oligonucleotide treatment given during colitis worsened inflammation (Obermeier *et al.*, 2005). Similarly, our group has showed that *tlr9*-deletion was protective in 2% DSS colitis (a higher concentration than previously studied) with less weight loss, milder histology score and preserved colon length (unpublished).

It is likely, therefore, that the effect of TLR9 activation is dependent on the stage of inflammation, severity of colitis (and thus extent of mtDNA release) and the cellular context of where TLR9 is blocked. In health, TLR-NFKB activation from PAMPs (ligands from commensal microflora) promotes cytoprotective factors. However, if epithelial barrier integrity is breached, or cytoprotective factors overwhelmed in active or severe disease, TLR9 activation may augment colitis, potentially driven by extracellular mtDNA in IBD. This may help explain why a recent clinical study of a TLR9-agonist in moderate-to-severe UC failed to show overall clinical improvement (Atreya *et al.*, 2016).

This area is ripe for further investigation. In active intestinal inflammation, the local environment is enriched with various DAMPs and microbial ligands for TLR9 – a key avenue of investigation will be to clarify the relative effect of mtDNA in this context. There should be a focus on how this varies with disease severity, duration and extent, and the ultimate cellular target of mtDNA (e.g. intestinal epithelial cells vs. lamina propria immune cells).

Oxidised mtDNA and relevance to IBD

MtDNA in disease may have higher inflammatory potential than from healthy controls. Two recent studies in human disease (NASH and SLE) demonstrate that equivalent respective mtDNA concentrations have higher inflammatory potential compared to mtDNA from healthy controls likely due to differences in oxidisation (Caielli *et al.*, 2016; Garcia-Martinez *et al.*, 2016).

It is pertinent that the inflamed gut environment is associated with mitochondrial damage, which leads to increased mtROS production (Brookes, 2004; Ho *et al.*, 2018). MtROS potentiates the oxidisation of mtDNA, critical to its role as an inflammasome agonist (Shimada *et al.*, 2012; Novak and Mollen, 2015). Excessive mtROS also inhibits autophagy, necessary for the clearance of damaged mitochondria. Hence, defective autophagy (Oka *et al.*, 2012; Caielli *et al.*, 2016) as seen in IBD further influences mtDNA's inflammatory capacity prior to its release.

Beyond simply a secondary phenomenon following uncontrolled cell death, recent studies in SLE show that neutrophils with impaired mitophagy can actively extrude mtDNA which upon oxidisation becomes a potent activator of plasmacytoid dendritic cells (Caielli *et al.*, 2016). Furthermore, oxidised mtDNA can become bound to neutrophil extracellular traps (NET) following NETosis, a cell death pathway characterised by extrusion of chromatin bound to cytosolic and granular content, which can further enhance and maintain its inflammatory potential (Lood *et al.*, 2016).

Further investigation should aim to clarify several areas including a) how oxidisation of mtDNA differs in IBD compared to health and other inflammatory conditions; b) whether mtDNA associated with IBD has a higher proinflammatory potential; and c) the extent of NET-bound oxidised mtDNA and its precise effects in IBD.

mtDNA release in IBD

Uncontrolled cell death and increased necrosis of inflammatory and intestinal epithelial cells are necessary preceding events for mtDNA (and DAMP) release into the extracellular milieu, and these are hallmark features of active IBD (Günther *et al.*, 2011). In this thesis, indirect evidence is presented to support local mtDNA release at the level of the inflamed gut in IBD. Damaged mitochondria were present in the colonic epithelium of active IBD compared to non-IBD patients on TEM. It is known that damaged and dysfunctional mitochondria produce increased levels of ROS, and excessive mtROS has

multiple effects including oxidisation of mtDNA (Brookes, 2004). Furthermore, faecal levels of mtDNA were significantly raised in active IBD compared to non-IBD samples, as was TLR9 expression in colonic resection specimens. Further work is required to extend these associative data and interrogate the mechanisms behind local mtDNA release in IBD. A number of areas require exploration, including direct evidence of TLR9 activation by mtDNA locally; how the manner of cell death (e.g. necroptosis vs. necrosis) in IBD affects mtDNA release; the role of cytosolic mtDNA in IBD; and the contribution of DNases on mtDNA in the circulation.

5.4 mtDNA as a therapeutic target in IBD

The role of DAMPs as functionally active mediators of inflammation makes this class a highly novel and exciting therapeutic target in IBD. This approach in related inflammatory diseases has already shown promise in animal models (Boyapati, Rossi, *et al.*, 2016). At present, most potential DAMP therapeutics have yet to be studied in human clinical trials. Many challenges exist, and these include: understanding complex disease-specific DAMP biology with their diverse often competing effects; how to localize therapeutic effects to the site of inflammation; deciphering DAMP-PRR and DAMP-DAMP interactions; understanding the triggers for DAMP release; and how DAMP mediated signalling varies depending on context.

The current evidence offers a rich ream of translational opportunities to target mtDNA-mediated inflammation. There are many plausible approaches which include targeting cytosolic mtDNA release (e.g. directly at MPT using cyclosporine or by specific mitochondrial anti-oxidant strategies e.g. $MitoQ_{10}$ to reduce mtROS), augmenting clearance (e.g. using autophagy activators or correcting factors leading to impaired autophagy), diverting the cellular response following mitochondrial damage (e.g. induction of pro-apoptotic caspases) and reducing the inflammatory potential of mtDNA (e.g. DNAses to digest NET-bound mtDNA and reducing oxidisation of mtDNA), augmenting damaged mitochondrial clearance mechanisms (e.g. mitophagy activation), interfering with mtDNA-TLR9 activation (using inhibitory CpG ligands) and

targeting downstream NFKB- and MAPK-pathways in the relevant inflammatory cell groups (e.g. neutrophils).

Similarly, much is known about the biological effects of *N*-formylated peptides and its cognate receptors (FPR1, 2 and 3). Activation of FPR1 drives neutrophil chemotaxis and stimulates a variety of antimicrobial responses, including degranulation, ROS production and cytokine release. Both FPR1 gene-deletion and pharmacologic inhibition are protective in inflammatory lung disease (Dorward *et al.*, 2015, 2017). There are other DAMPs such as calprotectin (s100a8/9), HMGB1 and IL-33 with known pro-inflammatory mechanisms found in active IBD (Boyapati, Rossi, *et al.*, 2016). The relative importance of these DAMPs is not yet clarified but offers multiple avenues of investigation.

The aim of this thesis was not to determine whether mtDNA was a therapeutic target, although this question is naturally raised in the investigation of mechanistic biomarkers. Significant work in precisely defining mtDNA's role in IBD in different conditions and on different cell types is required prior to work in this regard. However, once a framework is established, there is significant translational potential to investigate how existing novel therapies can modulate these pathways. If direct evidence is found suggesting blockade of mitochondrial DAMPs can reduce inflammation in the gut, novel therapies can be envisaged. These projects would require significant investment and partnerships from research councils or industry.

5.5 Model for mitochondrial dysfunction in IBD

In this thesis, I have shown that mitochondrial DAMPs are released in IBD and correlate with disease activity. Supportive evidence suggests that release is at the level of cellular damage in the gut and that they may be measured as a novel biomarker in the circulation and stool for some patients with IBD. Given that many lines of evidence implicate mtDNA as a functional pro-inflammatory mediator, these findings have direct translational importance.

The colonic epithelial mitochondria, uniquely juxtaposed with the austere luminal environment are particularly susceptible to damage. Overall, I propose a model whereby epithelial mitochondrial dysfunction in IBD leads to the accumulation of damaged mitochondria, increased mtROS which can either directly activate inflammatory signalling (e.g. through the inflammasome) or lead to a vicious cycle of further mitochondrial dysfunction (Figure 5.1). This accumulation is potentiated by defective homeostatic mechanisms such as autophagy. Non-apoptotic cell death can lead to release of these mitochondrial DAMPs into the extracellular milieu which can then activate innate inflammatory pathways.

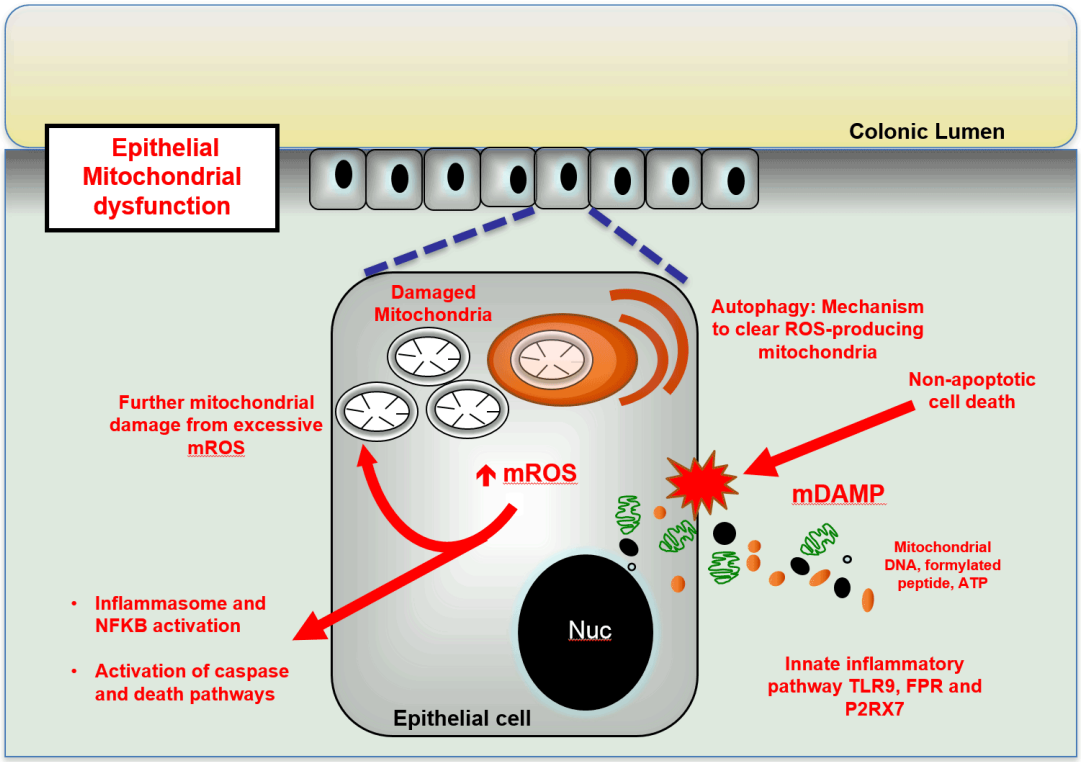

Figure 5.1: Proposed model for contribution of mitochondria as a proinflammatory source in IBD

More broadly, our findings open avenues to explore a new mechanistic layer which may further expand the current model of IBD pathogenesis to incorporate the 'danger' model. Indeed, one of the strengths of this work is the novelty associated with addressing this relatively unexplored area in IBD. Hence DAMPs as 'enemies within', may represent a potential major player in addition to established data implicating genetic susceptibility, and exogenous microbial and environmental factors in the pathogenesis of IBD.

REFERENCES

Adams, A. T. *et al.* (2014) ‘Two-stage genome-wide methylation profiling in childhood-onset Crohn’s Disease implicates epigenetic alterations at the VMP1/MIR21 and HLA loci.’, *Inflammatory bowel diseases*, 20(10), pp. 1784–93. doi: 10.1097/MIB.0000000000000179.

Adolph, T. E. T. E. *et al.* (2013) ‘Paneth cells as a site of origin for intestinal inflammation.’, *Nature*, 503(7475), pp. 272–6. doi: 10.1038/nature12599.

Akira, S., Uematsu, S. and Takeuchi, O. (2006) ‘Pathogen recognition and innate immunity’, *Cell*, pp. 783–801. doi: 10.1016/j.cell.2006.02.015.

Allegra, C. J. *et al.* (2009) ‘American Society of Clinical Oncology Provisional Clinical Opinion: Testing for KRAS Gene Mutations in Patients With Metastatic Colorectal Carcinoma to Predict Response to Anti–Epidermal Growth Factor Receptor Monoclonal Antibody Therapy’, *Journal of Clinical Oncology* , 27(12), pp. 2091–2096. doi: 10.1200/JCO.2009.21.9170.

Arijs, I. *et al.* (2009) ‘Mucosal gene signatures to predict response to infliximab in patients with ulcerative colitis.’, *Gut*, 58(12), pp. 1612–1619. doi: 10.1136/gut.2009.178665.

Arnalich, F. *et al.* (2012) ‘Circulating cell-free mitochondrial DNA: a better early prognostic marker in patients with out-of-hospital cardiac arrest.’, *Resuscitation*. European Resuscitation Council, American Heart Association, Inc., and International Liaison Committee on Resuscitation.~Published by Elsevier Ireland Ltd, 83(7), pp. e162-3. doi: 10.1016/j.resuscitation.2012.03.032.

Arnalich, F. *et al.* (2013) ‘Development of a new multiplex quantitative real-time PCR assay for the detection of the mtDNA(4977) deletion in coronary artery disease patients: a link with telomere shortening’, *Crit Care.*, 17(3), p. R90. doi: 10.1186/cc12735.

Arteaga, C. L. *et al.* (2011) ‘Treatment of HER2-positive breast cancer: current status and future perspectives’, *Nature Reviews Clinical Oncology*, 9(1), pp. 16–32. doi: 10.1038/nrclinonc.2011.177.

Atreya, R. *et al.* (2014) ‘In vivo imaging using fluorescent antibodies to tumor necrosis factor predicts therapeutic response in Crohn’s disease.’, *Nature medicine*, 20(3), pp. 313–8. doi: 10.1038/nm.3462.

Atreya, R. *et al.* (2016) ‘Clinical effects of a topically applied toll-like receptor 9 agonist in active moderate-to-severe ulcerative colitis’, *Journal of Crohn’s and Colitis*, 10(11), pp. 1294–1302. doi: 10.1093/ecco-jcc/jjw103.

Baiyee, E. E. *et al.* (2006) ‘Expression and function of Toll-like receptor 9 in severely injured patients prone to sepsis’, *Clinical and Experimental Immunology*. Blackwell Science Inc, 145(3), pp. 456–462. doi: 10.1111/j.1365-2249.2006.03160.x.

Bakker, P. J. *et al.* (2015) 'TLR9 Mediates Remote Liver Injury following Severe Renal Ischemia Reperfusion', *Plos One*, 10(9), p. e0137511. doi: 10.1371/journal.pone.0137511.

Balaj, L. *et al.* (2011) 'Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences', *Nature*, 2, p. 180. doi: 10.1038/ncomms1180.Tumour.

Banas, B., Kost, B. P. and Goebel, F. D. (2004) 'Platelets, a typical source of error in real-time PCR quantification of mitochondrial DNA content in human peripheral blood cells', *Mol Biol Evol.*, 21(12), p. 2310–8. Epub 2004 Sep 1.

Barber, G. N. (2014) 'STING-dependent cytosolic DNA sensing pathways', *Trends Immunol*, 35(2), pp. 88–93. doi: 10.1016/j.it.2013.10.010.

Barton, G. M. and Kagan, J. C. (2009) 'A cell biological view of Toll-like receptor function: regulation through compartmentalization.', *Nature reviews. Immunology*, 9(8), pp. 535–42. doi: 10.1038/nri2587.

Bauer, S. *et al.* (2001) 'Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition', *Proc Natl Acad Sci U S A*, 98(16), pp. 9237–9242. doi: 10.1073/pnas.161293498.

Bauernfeind, F. G. *et al.* (2009) 'Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression.', *Journal of immunology (Baltimore, Md. : 1950)*. United States, 183(2), pp. 787–791. doi: 10.4049/jimmunol.0901363.

Baumgart, D. C. and Sandborn, W. J. (2012) 'Crohn's disease.', *Lancet*. Elsevier Ltd, 380(9853), pp. 1590–605. doi: 10.1016/S0140-6736(12)60026-9.

Beaugerie, L. *et al.* (2006) 'Predictors of crohn's disease', *Gastroenterology*, 130(3), pp. 650–656. doi: 10.1053/j.gastro.2005.12.019.

Best, W. R. *et al.* (1976) 'Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study.', *Gastroenterology*, 70(3), pp. 439–44. doi: 10.1016/S0016-5085(76)80163-1.

Bhagirath, V. C., Dwivedi, D. J. and Liaw, P. C. (2015) 'Comparison of the Pro-Inflammatory and Pro-Coagulant Properties of Nuclear, Mitochondrial, and Bacterial DNA', *Shock*, 4, p. 4.

Bhat H. K *et al*. (2004) ' Quantitative analysis of total mitochondrial DNA: competitive polymerase chain reaction versus real-time polymerase chain reaction', *Journal of Biochemistry and Molecular Toxicology*, 18(4), pp. 180-6.

Bland, J. M and Altman, D. G (1986) 'Statistical methods for assessing agreement between two methods of clinical measurement.', Lancet, 1(8476) pp. 307-10.

Blander, J. M. and Sander, L. E. (2012) ‘Beyond pattern recognition: Five immune checkpoints for scaling the microbial threat’, *Nature Reviews Immunology*, pp. 215–225. doi: 10.1038/nri3167.

Bliksøen, M. *et al.* (2012) ‘Increased circulating mitochondrial DNA after myocardial infarction.’, *International journal of cardiology*, 158(1), pp. 132–4. doi: 10.1016/j.ijcard.2012.04.047.

Boddy, J. L. *et al.* (2005) ‘Prospective Study of Quantitation of Plasma DNA Levels in the Diagnosis of Malignant versus Benign Prostate Disease Prospective Study of Quantitation of Plasma DNA Levels in the Diagnosis of Malignant versus Benign Prostate Disease’, pp. 1394–1399.

Boirivant, M. *et al.* (1988) ‘The clinical significance of serum C reactive protein levels in Crohn’s disease. Results of a prospective longitudinal study.’, *Journal of clinical gastroenterology*. United States, 10(4), pp. 401–405.

Borghini, A. *et al.* (2015) ‘Increased circulating cell-free DNA levels and mtDNA fragments in interventional cardiologists occupationally exposed to low levels of ionizing radiation’, *Environ Mol Mutagen.*, 56(3), p. 293–300. doi: 10.1002/em.21917. Epub 2014 Oct 18.

Botezatu, I. *et al.* (2000) ‘Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism.’, *Clinical chemistry*. United States, 46(8 Pt 1), pp. 1078–1084.

Boudreau, L. H. *et al.* (2014) ‘Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation’, *Blood.*, 124(14), p. 2173–83. doi: 10.1182/blood–2014–05–573543. Epub 2. doi: 10.1182/blood-2014-05-573543.

Boyapati, R. K., Kalla, R., *et al.* (2016) *Biomarkers in Search of Precision Medicine in IBD*, *Am J Gastroenterol*. American College of Gastroenterology. doi: 10.1038/ajg.2016.441.

Boyapati, R. K., Rossi, A. G., *et al.* (2016) ‘Gut mucosal DAMPs in IBD: From mechanisms to therapeutic implications’, *Mucosal Immunology*. Society for Mucosal Immunology, pp. 567–582. doi: 10.1038/mi.2016.14.

Boyapati, R. K. *et al.* (2017) ‘Advances in the understanding of mitochondrial DNA as a pathogenic factor in inflammatory diseases’, *F1000Research*, 6. doi: 10.12688/f1000research.10397.1.

Boyapati, R. K., Satsangi, J. and Ho, G.-T. (2015) ‘Pathogenesis of Crohn’s disease’, *F1000Prime Reports*, 7(44). doi: 10.12703/P7-44.

Brookes, P. S. (2004) ‘Calcium, ATP, and ROS: a mitochondrial love-hate triangle’, *AJP: Cell Physiology*, 287(4), pp. C817–C833. doi: 10.1152/ajpcell.00139.2004.

Budnik, L. T. *et al.* (2013) 'Circulating mitochondrial DNA as biomarker linking environmental chemical exposure to early preclinical lesions elevation of mtDNA in human serum after exposure to carcinogenic halo-alkane-based pesticides.', *PloS one*, 8(5), p. e64413. doi: 10.1371/journal.pone.0064413.

Cai, Y. *et al.* (2017) 'Mitochondrial DNA–enriched microparticles promote acute-on-chronic alcoholic neutrophilia and hepatotoxicity', *JCI Insight*, 2(14). doi: 10.1172/jci.insight.92634.

Caielli, S. *et al.* (2016) 'Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus', *The Journal of Experimental Medicine*. The Rockefeller University Press, 213(5), pp. 697–713. doi: 10.1084/jem.20151876.

Cao, H. *et al.* (2014) 'Circulatory mitochondrial DNA is a pro-inflammatory agent in maintenance hemodialysis patients', *PLoS One.*, 9(12), p. e113179. doi: 10.1371/journal.pone.0113179. eColle.

Di Caro, V. *et al.* (2015) 'Plasma Mitochondrial DNA - A Novel Damp in Pediatric Sepsis.', *Shock*, Publish Ah. Available at: http://journals.lww.com/shockjournal/Fulltext/publishahead/Plasma_Mitochondrial_DNA___A_Novel_Damp_in.98410.aspx.

Carp, H. (1982) 'Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils.', *The Journal of experimental medicine*. United States, 155(1), pp. 264–275.

Chan, M. P. *et al.* (2015) 'DNase II-dependent DNA digestion is required for DNA sensing by TLR9', *Nature Communications*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 6, p. 5853.

Le Chatelier, E. *et al.* (2013) 'Richness of human gut microbiome correlates with metabolic markers.', *Nature*, 500(7464), pp. 541–6. doi: 10.1038/nature12506.

Chiu, R. W. *et al.* (2001) 'Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma.', *Clinical chemistry*, 47(9), pp. 1607–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11514393.

Chiu, R. W. K. *et al.* (2003) 'Quantitative analysis of circulating mitochondrial DNA in plasma.', *Clinical chemistry*, 49(5), pp. 719–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12709361.

Choi, S. H. *et al.* (2009) 'Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake', *Circulation Research*, 104(12), pp. 1355–1363. doi: 10.1161/CIRCRESAHA.108.192880.

Chou, C.-C. C. *et al.* (2008) 'Plasma nuclear DNA and mitochondrial DNA as prognostic markers in corrosive injury patients.', *Digestive surgery*, 25(4), pp. 300–4. doi: 10.1159/000152846.

Cleynen, I. *et al.* (2015) 'Inherited determinants of Crohn ' s disease and ulcerative colitis phenotypes : a genetic association study', *Lancet*, 6736(15), pp. 1–12. doi: 10.1016/S0140-6736(15)00465-1.

Collins, F. S. and Varmus, H. (2015) 'A New Initiative on Precision Medicine.', *The New England journal of medicine*, 372(9), pp. 793–5. doi: 10.1056/NEJMp1500523.

Collins, L. V *et al.* (2003) 'Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses', *Cell Mol Biol (Noisy-le-grand).*, 49(8), p. 1205–11.

Colombel, J.-F. *et al.* (2018) 'Effect of tight control management on Crohn's disease (CALM): a multicentre, randomised, controlled phase 3 trial.', *Lancet (London, England)*. England, 390(10114), pp. 2779–2789. doi: 10.1016/S0140-6736(17)32641-7.

Colombel, J. F. and Mahadevan, U. (2017) 'Inflammatory Bowel Disease 2017: Innovations and Changing Paradigms', *Gastroenterology*, 152(2), pp. 309–312. doi: 10.1053/j.gastro.2016.12.004.

Cossarizza, A. *et al.* (2011) 'Increased plasma levels of extracellular mitochondrial DNA during HIV infection: A new role for mitochondrial damage-associated molecular patterns during inflammation', *Mitochondrion*. Elsevier B.V. and Mitochondria Research Society, 11(5), pp. 750–755. doi: 10.1016/j.mito.2011.06.005.

Crouser, E. D. *et al.* (2009) 'Monocyte activation by necrotic cells is promoted by mitochondrial proteins and formyl peptide receptors', *Critical Care Medicine*, 37(6), pp. 2000–2009. doi: 10.1097/CCM.0b013e3181a001ae.

D'Haens, G. R. *et al.* (2014) 'Future directions in inflammatory bowel disease management.', *Journal of Crohn's & colitis*. European Crohn's and Colitis Organisation, 8(8), pp. 726–734. doi: 10.1016/j.crohns.2014.02.025.

Dai, Z. *et al.* (2015) 'Plasma Mitochondrial DNA Levels as a Biomarker of Lipodystrophy Among HIV-infected Patients Treated with Highly Active Antiretroviral Therapy (HAART).', *Current molecular medicine*. Netherlands, 15(10), pp. 975–979.

Danese, S. (2012) 'New therapies for inflammatory bowel disease: from the bench to the bedside.', *Gut*, 61(6), pp. 918–32. doi: 10.1136/gutjnl-2011-300904.

Dantham, S. *et al.* (2016) 'Plasma circulating cell-free mitochondrial DNA in the assessment of Friedreich's ataxia.', *Journal of the neurological sciences*. Netherlands, 365, pp. 82–88. doi: 10.1016/j.jns.2016.04.016.

Dasari, P. *et al.* (2005) 'Expression of toll-like receptors on B lymphocytes', *Cellular Immunology*, 236(1–2), pp. 140–145. doi: http://dx.doi.org/10.1016/j.cellimm.2005.08.020.

Dechartres, A. *et al.* (2011) 'Single-center trials show larger treatment effects than multicenter trials: Evidence from a meta-epidemiologic study', *Annals of Internal Medicine*, 155(1), pp. 39–52. doi: 10.7326/0003-4819-155-1-201107050-00006.

Delpre, G. *et al.* (1989) 'Ultrastructural abnormalities in endoscopically and histologically normal and involved colon in ulcerative colitis.', *The American journal of gastroenterology*. United States, 84(9), pp. 1038–1046.

Dhondup, Y. *et al.* (no date) 'Low Circulating Levels of Mitochondrial and High Levels of Nuclear DNA Predict Mortality in Chronic Heart Failure', *Journal of Cardiac Failure*. doi: http://dx.doi.org/10.1016/j.cardfail.2016.06.013.

Dick, K. J. *et al.* (2014) 'DNA methylation and body-mass index: a genome-wide analysis.', *Lancet (London, England)*, 383(9933), pp. 1990–8. doi: 10.1016/S0140-6736(13)62674-4.

Ding, Z. *et al.* (2013) 'Oxidant stress in mitochondrial DNA damage, autophagy and inflammation in atherosclerosis', *Scientific Reports*. Nature Publishing Group, 3, p. 1077. doi: 10.1038/srep01077.

Ding, Z. *et al.* (2014) 'LOX-1, mtDNA damage, and NLRP3 inflammasome activation inmacrophages: Implications in atherogenesis', *Cardiovascular Research*, 103(4), pp. 619–628. doi: 10.1093/cvr/cvu114.

Dombrowski, Y. *et al.* (2012) 'Honey bee (Apis mellifera) venom induces AIM2 inflammasome activation in human keratinocytes', *Allergy*, 67(11), pp. 1400–1407.

Dorward, D. A. (2014) *Determining the contribution of formylated peptides and formyl peptide receptor 1 to the pathogenesis of acute lung injury*. University of Edinburgh. Available at: http://hdl.handle.net/1842/17966.

Dorward, D. A. *et al.* (2015) 'The Role of Formylated Peptides and Formyl Peptide Receptor 1 in Governing Neutrophil Function during Acute Inflammation', *The American Journal of Pathology*, 185(5), pp. 1172–1184. doi: 10.1016/j.ajpath.2015.01.020.

Dorward, D. A. *et al.* (2017) 'Novel role for endogenous mitochondrial formylated peptide-driven formyl peptide receptor 1 signalling in acute respiratory distress syndrome.', *Thorax*. England, 72(10), pp. 928–936. doi: 10.1136/thoraxjnl-2017-210030.

Dubinsky, M. and Braun, J. (2015) 'Diagnostic and Prognostic Microbial Biomarkers in Inflammatory Bowel Diseases', *Gastroenterology*. Elsevier, Inc, 149(5), p. 1265–1274.e3. doi: 10.1053/j.gastro.2015.08.006.

Dubinsky, M. C. *et al.* (2010) 'Genome Wide Association (GWA) Predictors Of Anti-TNFα Therapeutic Responsiveness In Pediatric Inflammatory Bowel Disease (IBD)', *Inflammatory bowel diseases*, 16(8), pp. 1357–66. doi: 10.1002/ibd.21174.

Dubinsky, M. C. *et al.* (2013) 'Multidimensional prognostic risk assessment identifies association between IL12B variation and surgery in Crohn's disease.', *Inflammatory bowel diseases*, 19(8), pp. 1662–70. doi: 10.1097/MIB.0b013e318281f275.

Dyall, S. D., Brown, M. T. and Johnson, P. J. (2004) 'Ancient invasions: from endosymbionts to organelles.', *Science (New York, N.Y.)*. United States, 304(5668), pp. 253–257. doi: 10.1126/science.1094884.

Eaton-Bassiri, A. *et al.* (2004) 'Toll-Like Receptor 9 Can Be Expressed at the Cell Surface of Distinct Populations of Tonsils and Human Peripheral Blood Mononuclear Cells ', *Infection and Immunity*. American Society for Microbiology, 72(12), pp. 7202–7211. doi: 10.1128/IAI.72.12.7202-7211.2004.

Elinav, E. *et al.* (2011) 'NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis.', *Cell*. Elsevier Inc., 145(5), pp. 745–57. doi: 10.1016/j.cell.2011.04.022.

Ellinger, J. *et al.* (2009) 'Circulating mitochondrial DNA in the serum of patients with testicular germ cell cancer as a novel noninvasive diagnostic biomarker', *BJU International*, 104(1), pp. 48–52. doi: 10.1111/j.1464-410X.2008.08289.x.

Ellinger, J. *et al.* (2012) 'Circulating mitochondrial DNA in serum: A universal diagnostic biomarker for patients with urological malignancies', *Urologic Oncology: Seminars and Original Investigations*. Elsevier Inc., 30(4), pp. 509–515. doi: 10.1016/j.urolonc.2010.03.004.

Ellinger, J. J. *et al.* (2008) 'Mitochondrial DNA in serum of patients with prostate cancer: a predictor of biochemical recurrence after prostatectomy.', *BJU international*, 102(5), pp. 628–32. doi: 10.1111/j.1464-410X.2008.07613.x.

Ewaschuk, J. B. *et al.* (2007) 'Surface expression of Toll-like receptor 9 is upregulated on intestinal epithelial cells in response to pathogenic bacterial DNA.', *Infection and immunity*, 75(5), pp. 2572–9. doi: 10.1128/IAI.01662-06.

Fan, Y. and Liu, B. (2015) 'Expression of Toll-like receptors in the mucosa of patients with ulcerative colitis', *Experimental and Therapeutic Medicine*. D.A. Spandidos, 9(4), pp. 1455–1459. doi: 10.3892/etm.2015.2258.

Faubion, W. A. J. *et al.* (2013) 'EMerging BiomARKers in Inflammatory Bowel Disease (EMBARK) study identifies fecal calprotectin, serum MMP9, and serum IL-22 as a novel combination of biomarkers for Crohn's disease activity: role of cross-sectional imaging.', *The American journal of gastroenterology*. United States, 108(12), pp. 1891–1900. doi: 10.1038/ajg.2013.354.

Galluzzi, L. *et al.* (2012) ‘Mitochondrial Control of Cellular Life, Stress, and Death’, *Circulation Research*, 111(9), p. 1198 LP-1207. Available at: http://circres.ahajournals.org/content/111/9/1198.abstract.

Gan, L. *et al.* (2015) ‘Significance of Serum mtDNA Concentration in Lung Injury Induced by Hip Fracture’, *Shock.*, 44(1), p. 52–7. doi: 10.1097/SHK.0000000000000366.

Garcia-Martinez, I. *et al.* (2016) ‘Hepatocyte mitochondrial DNA drives nonalcoholic steatohepatitis by activation of TLR9’, *The Journal of Clinical Investigation*. The American Society for Clinical Investigation, 126(3). doi: 10.1172/JCI83885.

Garlanda, C., Dinarello, C. A. and Mantovani, A. (2013) ‘The Interleukin-1 Family: Back to the Future’, *Immunity*, pp. 1003–1018. doi: 10.1016/j.immuni.2013.11.010.

Garrabou, G. *et al.* (2012) ‘The effects of sepsis on mitochondria.’, *The Journal of infectious diseases*, 205(3), pp. 392–400. doi: 10.1093/infdis/jir764.

Gisbert, J. P. and McNicholl, A. G. (2009) ‘Questions and answers on the role of faecal calprotectin as a biological marker in inflammatory bowel disease’, *Dig Liver Dis*, 41(1), pp. 56–66. doi: S1590-8658(08)00191-6 [pii] 10.1016/j.dld.2008.05.008.

Gourlain, K. *et al*. (2003) 'Quantitative analysis of human mitochondrial DNA using a real-time PCR assay', *HIV Medicine*, 4(3), pp 287-92.

Gu, X. *et al.* (2013) ‘The plasma mitochondrial DNA is an independent predictor for post-traumatic systemic inflammatory response syndrome.’, *PloS one*. Edited by C. M. Stover. San Francisco, USA: Public Library of Science, 8(8), p. e72834. doi: 10.1371/journal.pone.0072834.

Gu, X. *et al.* (2015) ‘Intratracheal administration of mitochondrial DNA directly provokes lung inflammation through the TLR9–p38 MAPK pathway’, *Free Radical Biology and Medicine*. Elsevier, 83, pp. 149–158. doi: 10.1016/j.freeradbiomed.2015.02.034.

Guescini, M., Genedani, S., *et al.* (2010) ‘Astrocytes and Glioblastoma cells release exosomes carrying mtDNA.’, *Journal of neural transmission (Vienna, Austria : 1996)*. Austria, 117(1), pp. 1–4. doi: 10.1007/s00702-009-0288-8.

Guescini, M., Guidolin, D., *et al.* (2010) ‘C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction’, *Experimental Cell Research*, 316(12), pp. 1977–1984. doi: 10.1016/j.yexcr.2010.04.006.

Gunther, C. *et al.* (2012) 'Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium', *Gut*, 62(7), pp. 1062–1071. doi: 10.1136/gutjnl-2011-301364.

Günther, C. *et al.* (2011) 'Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis.', *Nature*, 477(7364), pp. 335–9. doi: 10.1038/nature10400.

Gurung, P., Lukens, J. R. and Kanneganti, T.-D. (2015) 'Mitochondria: diversity in the regulation of the NLRP3 inflammasome', *Trends in molecular medicine*, 21(3), pp. 193–201.

Hajizadeh, S. *et al.* (2003) 'Extracellular mitochondrial DNA and oxidatively damaged DNA in synovial fluid of patients with rheumatoid arthritis.', *J Biol Chem.*, 5(5), p. 43893–6. Epub 2003 Sep 17. doi: 10.1186/ar787.

Hare, N. C. *et al.* (2014) 'Serum calprotectin: A novel biomarker to predict outcome in acute severe ulcerative colitis?', *Journal of Crohn's and Colitis*, 8, p. S210.

Harvey, R. F. and Bradshaw, J. M. (1980) 'A Simple Index of Crohn's disease Activity', *The Lancet*, 315(8167), p. 514. doi: 10.1016/S0140-6736(80)92767-1.

Hauser, C. J. *et al.* (2010) 'Mitochondrial damage associated molecular patterns from femoral reamings activate neutrophils through formyl peptide receptors and P44/42 MAP kinase.', *Journal of orthopaedic trauma*. United States, 110(4 Suppl 2), p. 3–11. doi: 10.1097/BOT.0b013e3181ec4991.

Heap, G. a *et al.* (2014) 'HLA-DQA1-HLA-DRB1 variants confer susceptibility to pancreatitis induced by thiopurine immunosuppressants.', *Nature genetics*, 46(10), pp. 1131–4. doi: 10.1038/ng.3093.

Hemmi, H. *et al.* (2000) 'A Toll-like receptor recognizes bacterial DNA', *Nature*, 408(6813), pp. 740–745. doi: 10.1038/35047123.

Ho, G.-T. *et al.* (2018) 'MDR1 deficiency impairs mitochondrial homeostasis and promotes intestinal inflammation', *Mucosal Immunology*, 11(1). doi: 10.1038/mi.2017.31.

Ho, G.-T., Boyapati, R. K. and Satsangi, J. (2015) 'Ulcerative colitis', *Medicine (United Kingdom)*. Elsevier, 43(5), pp. 276–281. doi: 10.1016/j.mpmed.2015.02.004.

Ho, G. T. *et al.* (2009) 'Fecal Calprotectin Predicts the Clinical Course of Acute Severe Ulcerative Colitis', *The American Journal of Gastroenterology*, 104(3), pp. 673–678. doi: 10.1038/ajg.2008.119.

Holmstrom, K. M. and Finkel, T. (2014) ‘Cellular mechanisms and physiological consequences of redox-dependent signalling.’, *Nature reviews. Molecular cell biology*. England, 15(6), pp. 411–421. doi: 10.1038/nrm3801.

Hou, Y.-L. *et al.* (2013) ‘Clinical significance of serum mitochondrial DNA in lung cancer.’, *Clinical biochemistry*. The Canadian Society of Clinical Chemists, 46(15), pp. 1474–7. doi: 10.1016/j.clinbiochem.2013.04.009.

Hsieh, S.-Y. *et al.* (2006) ‘Comparative proteomic studies on the pathogenesis of human ulcerative colitis’, *Proteomics*, 6(19). doi: 10.1002/pmic.200500541.

Hsu, C. C. *et al.* (2013) ‘Plasma mitochondrial DNA levels in patients with trauma and severe sepsis: time course and the association with clinical status.’, *Journal of critical care*. Elsevier Inc., 28(6), pp. 1027–31. doi: 10.1016/j.jcrc.2013.05.006.

Hu, Q. *et al.* (2015) ‘Mitochondrial Damage-Associated Molecular Patterns (MTDs) Are Released during Hepatic Ischemia Reperfusion and Induce Inflammatory Responses’, *Plos One*, 10(10), p. e0140105. doi: 10.1371/journal.pone.0140105.

Huang, C.-Y. Y. *et al.* (2014) ‘Circulating free mitochondrial DNA concentration and its association with erlotinib treatment in patients with adenocarcinoma of the lung.’, *Oncology letters*, 7(6), pp. 2180–2184. doi: 10.3892/ol.2014.2006.

Hudson, K., Lifton, R. R. and Patrick-Lake, B. (2015) ‘The Precision Medicine Initiative Cohort Program – Building a Research Foundation for 21st Century Medicine’, *Precision Medicine Initiative (PMI) Working Group Report to the Advisory Committee to the Director, NIH*, p. 103. Available at: http://www.nih.gov/precisionmedicine/09172015-pmi-working-group-report.pdf (Accessed: 13 March 2016).

Huttenhower, C. *et al.* (2012) ‘Structure, function and diversity of the healthy human microbiome’, *Nature*. England, 486(7402), pp. 207–214. doi: 10.1038/nature11234.

Imaeda, A. B. *et al.* (2009) ‘Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome.’, *The Journal of clinical investigation*. United States, 119(2), pp. 305–314. doi: 10.1172/JCI35958.

Imai, Y. *et al.* (2008) ‘Identification of Oxidative Stress and Toll-like Receptor 4 Signaling as a Key Pathway of Acute Lung Injury’, *Cell*, 133(2), pp. 235–249. doi: 10.1016/j.cell.2008.02.043.

Irvine, E. J. (1995) ‘Usual therapy improves perianal Crohn’s disease as measured by a new disease activity index. McMaster IBD Study Group.’, *Journal of clinical gastroenterology*, 20(1), pp. 27–32. doi: 10.1007/BF02053756.

Ishikawa, H. and Barber, G. N. (2008) 'STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling', *Nature*, 455(7213), pp. 674–678. doi: 10.1038/nature07317.

Islam, M. N. *et al.* (2012) 'Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury', *Nat Med*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 18(5), pp. 759–765. Available at: http://dx.doi.org/10.1038/nm.2736.

Iyer, S. S. *et al.* (2009) 'Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome.', *Proceedings of the National Academy of Sciences of the United States of America*, 106, pp. 20388–20393. doi: 10.1073/pnas.0908698106.

Jarnerot, G. *et al.* (2005) 'Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study.', *Gastroenterology*. United States, 128(7), pp. 1805–1811.

Jiménez-Alcázar, M. *et al.* (2018) 'Circulating extracellular DNA is an independent predictor of mortality in elderly patients with venous thromboembolism', *PLOS ONE*. Public Library of Science, 13(2), p. e0191150. Available at: https://doi.org/10.1371/journal.pone.0191150.

Johnson, G. B., Brunn, G. J. and Platt, J. L. (2004) 'Cutting Edge: An Endogenous Pathway to Systemic Inflammatory Response Syndrome (SIRS)-Like Reactions through Toll-Like Receptor 4', *The Journal of Immunology*, 172(1), pp. 20–24. doi: 10.4049/jimmunol.172.1.20.

De Jong, N. S. H., Leach, S. T. and Day, A. S. (2006) 'Fecal S100A12: A novel noninvasive marker in children with Crohn's disease', *Inflammatory Bowel Diseases*, 12(7), pp. 566–572. doi: 10.1097/01.ibd.0000227626.72271.91.

Jostins, L. *et al.* (2012) 'Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease.', *Nature*. Nature Publishing Group, 491(7422), pp. 119–24. doi: 10.1038/nature11582.

Jung, M. and Klotzek, S. (2003) 'Letters Changes in Concentration of DNA in Serum and Plasma during Storage of Blood Samples', *Clinical Chemistry*, pp. 1028–1029.

Jylhävä, J. *et al.* (2013) 'Characterization of the role of distinct plasma cell-free DNA species in age-associated inflammation and frailty', *Aging cell*, 12(3), pp. 388–97. doi: 10.1111/acel.12058.

Kaczmarek, A., Vandenabeele, P. and Krysko, D. V (2013) 'Necroptosis: the release of damage-associated molecular patterns and its physiological relevance.', *Immunity*. Elsevier Inc., 38(2), pp. 209–23. doi: 10.1016/j.immuni.2013.02.003.

Kageyama, Y. *et al.* (2018) 'The relationship between circulating mitochondrial DNA and inflammatory cytokines in patients with major depression', *Journal of Affective Disorders*. Elsevier. doi: 10.1016/j.jad.2017.06.001.

Kaiser, T. *et al.* (2007) 'Faecal S100A12 as a non-invasive marker distinguishing inflammatory bowel disease from irritable bowel syndrome', *Gut*, 56(12), pp. 1706–1713. doi: 10.1136/gut.2006.113431.

Kalla, R. *et al.* (2016) 'Serum Calprotectin: A Novel Diagnostic and Prognostic Marker in Inflammatory Bowel Diseases', *American Journal of Gastroenterology*, 111(12). doi: 10.1038/ajg.2016.342.

Kaplan, G. G. (2015) 'The global burden of IBD: From 2015 to 2025', *Nature Reviews Gastroenterology and Hepatology*, pp. 720–727. doi: 10.1038/nrgastro.2015.150.

Kaser, A. *et al.* (2008) 'XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease.', *Cell*, 134(5), pp. 743–56. doi: 10.1016/j.cell.2008.07.021.

Kavlick, M.F *et al*. (2011) 'Quantification of human mitochondrial DNA using synthesized DNA standards', *Journal of Forensic Science*, pp. 1457-63. doi: 10.1111/j.1556-4029.2011.01871

King, M. and Attardi, G. (1989) 'Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation', *Science*, 246(4929), pp. 500–503. doi: 10.1126/science.2814477.

Kohler, C. *et al.* (2009) 'Levels of plasma circulating cell free nuclear and mitochondrial DNA as potential biomarkers for breast tumors.', *Molecular cancer*, 8(11), p. 105. doi: 10.1186/1476-4598-8-105.

Kono, H. and Rock, K. L. (2008) 'How dying cells alert the immune system to danger.', *Nature reviews. Immunology*, 8(4), pp. 279–89. doi: 10.1038/nri2215.

Krychtiuk, K. *et al.* (2017) 'Mitochondrial DNA predicts mortality in acute but not in chronic heart failure', *Intensive Care Medicine Experimental*, 5(2). doi: 10.1186/s40635-017-0151-4.

Krychtiuk, K. A. *et al.* (2015) 'Mitochondrial DNA and Toll-Like Receptor-9 Are Associated With Mortality in Critically Ill Patients', *Critical Care Medicine*, 43(12). Available at: http://journals.lww.com/ccmjournal/Fulltext/2015/12000/Mitochondrial_DNA_and_Toll_Like_Receptor_9_Are.14.aspx.

Kung, C.-T. *et al.* (2012) 'Plasma nuclear and mitochondrial DNA levels as predictors of outcome in severe sepsis patients in the emergency room.', *Journal of translational medicine*. Journal of Translational Medicine, 10(1), p. 130. doi: 10.1186/1479-5876-10-130.

Lakra, W. S. *et al.* (2011) 'The value of serial plasma nuclear and mitochondrial DNA levels in patients with acute ischemic stroke.', *Clinica chimica acta; international journal of clinical chemistry*. Elsevier B.V., 412(5–6), pp. 476–9. doi: 10.1016/j.cca.2010.11.036.

Lam, N. Y. *et al.* (2004) 'Plasma mitochondrial DNA concentrations after trauma', *Fertil Steril.*, 81(1), p. 73–9.

Lasson, A. *et al.* (2015) 'The intra-individual variability of faecal calprotectin: a prospective study in patients with active ulcerative colitis.', *Journal of Crohn's & colitis*, 9(1), pp. 26–32. doi: 10.1016/j.crohns.2014.06.002.

Latz, E. *et al.* (2004) 'TLR9 signals after translocating from the ER to CpG DNA in the lysosome.', *Nature immunology*. United States, 5(2), pp. 190–198. doi: 10.1038/ni1028.

Lauring, A. S. *et al.* (2012) 'Lack of Evidence for mtDNA as a Biomarker of Innate Immune Activation in HIV Infection', *PLoS ONE*, 7(11), p. e50486. doi: 10.1371/journal.pone.0050486.

Lee, H. C. *et al.* (2004) 'Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma', *Mol Biol Evol.*, 21(6), p. 1081–4. Epub 2004 Mar 10.

Lee, J. *et al.* (2006) 'Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells', *Nature Cell Biology*, 8(12), pp. 1327–1336. doi: 10.1038/ncb1500.

Lee, J. *et al.* (2007) 'Toll-like receptor signaling in intestinal epithelial cells contributes to colonic homoeostasis.', *Current opinion in gastroenterology*, 23, pp. 27–31. doi: 10.1097/MOG.0b013e3280118272.

Lee, J. C. *et al.* (2011) 'Gene expression profiling of CD8 + T cells predicts prognosis in patients with Crohn disease and ulcerative colitis', *Journal of Clinical Investigation*, 121(10), pp. 4170–4179. doi: 10.1172/JCI59255DS1.

Lee, T. H. *et al.* (2001) 'Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma', *Transfusion*, 41(2), pp. 276–282. doi: DOI 10.1046/j.1537-2995.2001.41020276.x.

Leifer, C. A. *et al.* (2004) 'TLR9 is localized in the endoplasmic reticulum prior to stimulation.', *Journal of immunology (Baltimore, Md. : 1950)*. United States, 173(2), pp. 1179–1183.

Lennard-Jones, J. E. (1989) 'Classification of inflammatory bowel disease.', *Scandinavian journal of gastroenterology. Supplement*, 170, pp. 2-6-9. doi: 10.3109/00365528909091339.

Lennard, L. (2002) ‘TPMT in the treatment of Crohn’s disease with azathioprine.’, *Gut*, 51, pp. 143–146. doi: 10.1136/gut.51.2.143.

Lewis, J. D. (2011) ‘The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease’, *Gastroenterology*, 140(6), p. 1817–1826.e2. doi: 10.1053/j.gastro.2010.11.058.

Lichtenstein, G. R. and McGovern, D. P. B. (2016) ‘Using Markers in IBD to Predict Disease and Treatment Outcomes: Rationale and a Review of Current Status’, *The American Journal of Gastroenterology Supplements*, 3(3), pp. 17–26. doi: 10.1038/ajgs.2016.17.

Lièvre, A. *et al.* (2006) ‘KRAS Mutation Status Is Predictive of Response to Cetuximab Therapy in Colorectal Cancer’, *Cancer Research* , 66(8), pp. 3992–3995. doi: 10.1158/0008-5472.CAN-06-0191.

Lin, J.-F. *et al.* (2014) ‘Meta-analysis: fecal calprotectin for assessment of inflammatory bowel disease activity.’, *Inflammatory bowel diseases*, 20(8), pp. 1407–15. doi: 10.1097/MIB.0000000000000057.

Lindau, D. *et al.* (2013) ‘Primary blood neutrophils express a functional cell surface Toll-like receptor 9’, *European Journal of Immunology*, 43, pp. 2101–2113. doi: 10.1002/eji.201142143.

Lindberg, J. *et al.* (2013) ‘Plasma bacterial and mitochondrial DNA distinguish bacterial sepsis from sterile systemic inflammatory response syndrome and quantify inflammatory tissue injury in nonhuman primates’, *Eur Urol.*, 63(1), pp. 55–62. doi: 10.1097/SHK.0b013e318276f4ca.

Lindeman, N. I. *et al.* (2013) ‘Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors’, *Journal of Thoracic Oncology*, 8(7), pp. 823–859. doi: 10.1021/ac901991x.

Lindqvist, D. *et al.* (2016) ‘Increased plasma levels of circulating cell-free mitochondrial DNA in suicide attempters: Associations with HPA-axis hyperactivity’, *Translational Psychiatry*, 6(12). doi: 10.1038/tp.2016.236.

Lindqvist, D. *et al.* (2018) ‘Circulating cell-free mitochondrial DNA, but not leukocyte mitochondrial DNA copy number, is elevated in major depressive disorder’, *Neuropsychopharmacology*. doi: 10.1038/s41386-017-0001-9.

Liu, B. *et al.* (2013) ‘Irgm1-deficient mice exhibit Paneth cell abnormalities and increased susceptibility to acute intestinal inflammation.’, *American journal of physiology. Gastrointestinal and liver physiology*, 305(8), pp. G573-84. doi: 10.1152/ajpgi.00071.2013.

Liu, J. *et al.* (2015) ‘Circulating cell-free mitochondrial deoxyribonucleic acid is increased in coronary heart disease patients with diabetes mellitus’, *Journal of Diabetes Investigation*. doi: 10.1111/jdi.12366.

Liu, J. Z. *et al.* (2015) 'Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations', *Nature Genetics*, 47(9), pp. 979–989. doi: 10.1038/ng.3359.

Lo, Y. M. *et al.* (1998) 'Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis.', *American journal of human genetics*, 62(4), pp. 768–75. doi: 10.1086/301800.

Lo, Y. M. *et al.* (1999) 'Rapid clearance of fetal DNA from maternal plasma.', *American journal of human genetics*, 64(1), pp. 218–24. doi: 10.1086/302205.

Lood, C. *et al.* (2016) 'Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease', *Nat Med*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 22(2), pp. 146–153. Available at: http://dx.doi.org/10.1038/nm.4027.

Lu, C.-H. *et al.* (2010) 'The value of serial plasma nuclear and mitochondrial DNA levels in adult community-acquired bacterial meningitis.', *QJM : monthly journal of the Association of Physicians*, 103(3), pp. 169–75. doi: 10.1093/qjmed/hcp201.

Lu, H. *et al.* (2016) 'Diagnostic and prognostic potential of circulating cell-free genomic and mitochondrial DNA fragments in clear cell renal cell carcinoma patients.', *Clinica chimica acta; international journal of clinical chemistry*. Netherlands, 452, pp. 109–119. doi: 10.1016/j.cca.2015.11.009.

Lui, Y. Y. N. *et al.* (2002) 'Predominant Hematopoietic Origin of Cell-free DNA in Plasma and Serum after Sex-mismatched Bone Marrow Transplantation', 427, pp. 421–427.

Lüthi, A. U. *et al.* (2009) 'Suppression of Interleukin-33 Bioactivity through Proteolysis by Apoptotic Caspases', *Immunity*, 31(1), pp. 84–98. doi: 10.1016/j.immuni.2009.05.007.

Macy, E. M. *et al*. (1997) ' Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications', *Clinical Chemistry*, 43(1).

Maeda, a and Fadeel, B. (2014) 'Mitochondria released by cells undergoing TNF-α-induced necroptosis act as danger signals', *Cell Death and Disease*. Nature Publishing Group, 5(7), p. e1312. doi: 10.1038/cddis.2014.277.

Mangalmurti, N. *et al.* (2016) 'Mitochondrial DNA Released Following Necroptosis Accumulates on RBCs', *Am J Respir Crit Care Med.* American Thoracic Society (American Thoracic Society International Conference Abstracts), 193, pp. A4309–A4309. doi: doi:10.1164/ajrccm-conference.2016.193.1_MeetingAbstracts.A4309.

Marques, P. E. *et al.* (2012) 'Chemokines and mitochondrial products activate neutrophils to amplify organ injury during mouse acute liver failure', *Hepatology*, 56(5), pp. 1971–1982. doi: 10.1002/hep.25801.

Martin, S. J., Henry, C. M. and Cullen, S. P. (2012) 'A Perspective on Mammalian Caspases as Positive and Negative Regulators of Inflammation', *Molecular Cell*, pp. 387–397. doi: 10.1016/j.molcel.2012.04.026.

Martinon, F. *et al.* (2006) 'Gout-associated uric acid crystals activate the NALP3 inflammasome', *Nature*, 440(7081), pp. 237–241. doi: nature04516 [pii]\r10.1038/nature04516.

Matzinger, P. (1994) 'Tolerance, Danger, and the Extended Family', *Annual Review of Immunology*, 12(1), pp. 991–1045. doi: 10.1146/annurev.iy.12.040194.005015.

Matzinger, P. (2002) 'The danger model: a renewed sense of self.', *Science (New York, N.Y.)*, 296(April), pp. 301–305. doi: 10.1126/science.1071059.

McDermott, E. *et al.* (2016) 'DNA Methylation Profiling in Inflammatory Bowel Disease Provides New Insights into Disease Pathogenesis.', *Journal of Crohn's & colitis*, 10(1), pp. 77–86. doi: 10.1093/ecco-jcc/jjv176.

McGill, M. R. *et al.* (2012) 'The mechanism underlying acetaminophen- induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation', *Journal of Clinical Investigation*, 122(4), pp. 1574–83. doi: 10.1172/JCI59755.of.

McGill, M. R. *et al.* (2014) 'Serum mitochondrial biomarkers and damage-associated molecular patterns are higher in acetaminophen overdose patients with poor outcome.', *Hepatology (Baltimore, Md.)*, 60(4), pp. 1336–45. doi: 10.1002/hep.27265.

Mehra, N. *et al.* (2007) 'Circulating mitochondrial nucleic acids have prognostic value for survival in patients with advanced prostate cancer.', *Clinical cancer research : an official journal of the American Association for Cancer Research*, 13(2 Pt 1), pp. 421–6. doi: 10.1158/1078-0432.CCR-06-1087.

Molodecky, N. a *et al.* (2012) 'Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review.', *Gastroenterology*. Elsevier Inc., 142(1), p. 46–54.e42; quiz e30. doi: 10.1053/j.gastro.2011.10.001.

Moseman, E. A. *et al.* (2004) 'Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells.', *Journal of immunology (Baltimore, Md. : 1950)*. United States, 173(7), pp. 4433–4442.

Mosli, M. H. *et al.* (2014) 'Toward a personalized medicine approach to the management of inflammatory bowel disease.', *The American journal of gastroenterology*. Nature Publishing Group, 109(7), pp. 994–1004. doi: 10.1038/ajg.2014.110.

Mosli, M. H. *et al.* (2015) 'C-reactive protein, fecal calprotectin, and stool lactoferrin for detection of endoscopic activity in symptomatic inflammatory bowel disease patients: A systematic review and meta-analysis', *American Journal of Gastroenterology*, pp. 802–819. doi: 10.1038/ajg.2015.120.

Nakahira, K. *et al.* (2011) 'Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome.', *Nature immunology*. Nature Publishing Group, 12(3), pp. 222–30. doi: 10.1038/ni.1980.

Nakahira, K. *et al.* (2013) 'Circulating Mitochondrial DNA in Patients in the ICU as a Marker of Mortality: Derivation and Validation', *PLoS Medicine*. Public Library of Science, 10(12), pp. 1–12. doi: 10.1371/journal.pmed.1001577.

Nakahira, K., Hisata, S. and Choi, A. M. (2015) 'The roles of Mitochondrial DAMPs in Diseases.', *Antioxidants & redox signaling*, 23(17), pp. 1–78. doi: 10.1089/ars.2015.6407.

Nanda, K. S., Cheifetz, A. S. and Moss, A. C. (2013) 'Impact of antibodies to infliximab on clinical outcomes and serum infliximab levels in patients with inflammatory bowel disease (IBD): a meta-analysis.', *The American journal of gastroenterology*, 108(1), pp. 40–47. doi: 10.1038/ajg.2012.363.

Nasi, M. *et al.* (2015) 'Decreased Circulating mtDNA Levels in Professional Male Volleyball Players', *Int J Sports Physiol Perform*, 10, p. 10.

Nazli, A. *et al.* (2004) 'Epithelia under metabolic stress perceive commensal bacteria as a threat.', *The American journal of pathology*. United States, 164(3), pp. 947–957. doi: 10.1016/S0002-9440(10)63182-3.

Nenci, A. *et al.* (2007) 'Epithelial NEMO links innate immunity to chronic intestinal inflammation', *Nature*, 446(7135), pp. 557–561. doi: 10.1038/nature05698.

Nicole Y.L. Lam, Timothy H. Rainer, Rossa W.K. Chiu, G. M. J. and Lo, and Y. M. D. (2004) 'Plasma Mitochondrial DNA Concentrations after Trauma', *Clinical chemistry*, 50(1), pp. 211–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14709653 (Accessed: 15 November 2014).

Nimmo, E. R. *et al.* (2012) 'Genome-wide methylation profiling in Crohn's disease identifies altered epigenetic regulation of key host defense mechanisms including the Th17 pathway', *Inflammatory Bowel Diseases*, 18(5), pp. 889–899. doi: 10.1002/ibd.21912.

Novak, E. A. and Mollen, K. P. (2015) 'Mitochondrial dysfunction in inflammatory bowel disease', *Frontiers in Cell and Developmental Biology*, 3. doi: 10.3389/fcell.2015.00062.

Nunnari, J. and Suomalainen, A. (2012) 'Mitochondria: In sickness and in health', *Cell*. Elsevier Inc., 148(6), pp. 1145–1159. doi: 10.1016/j.cell.2012.02.035.

Obermeier, F. *et al.* (2005) 'CpG motifs of bacterial DNA essentially contribute to the perpetuation of chronic intestinal inflammation', *Gastroenterology*, 129, pp. 913–927. doi: 10.1053/j.gastro.2005.06.061.

Oka, T. *et al.* (2012) 'Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure.', *Nature*. Nature Publishing Group, 485(7397), pp. 251–5. doi: 10.1038/nature10992.

Okabe, Y. *et al.* (2005) 'Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation', *J Exp Med*, 202(10), pp. 1333–1339. doi: 10.1084/jem.20051654.

Ordás, I. *et al.* (2012) 'Ulcerative colitis.', *Lancet*, 380(9853), pp. 1606–19. doi: 10.1016/S0140-6736(12)60150-0.

Orholm, M. *et al.* (1991) 'Familial occurence of inflammatory bowel disease', *N Eng J Med*, 324, pp. 84–8.

Palone, F. *et al.* (2014) 'Role of HMGB1 as a Suitable Biomarker of Subclinical Intestinal Inflammation and Mucosal Healing in Patients with Inflammatory Bowel Disease.', *Inflammatory bowel diseases*, 0(0), pp. 1–10. doi: 10.1097/MIB.0000000000000113.

Patrushev, M. *et al.* (2004) 'Mitochondrial permeability transition triggers the release of mtDNA fragments', *Cellular and Molecular Life Sciences*, 61(24), pp. 3100–3103. doi: 10.1007/s00018-004-4424-1.

Pavlou, M. P., Diamandis, E. P. and Blasutig, I. M. (2013) 'The long journey of cancer biomarkers from the bench to the clinic.', *Clinical chemistry*. United States, 59(1), pp. 147–157. doi: 10.1373/clinchem.2012.184614.

Pazmandi, K. *et al.* (2014) 'Oxidative modification enhances the immunostimulatory effects of extracellular mitochondrial DNA on plasmacytoid dendritic cells.', *Free Radic Biol Med.* Elsevier, 77:281-90.(doi), p. 10.1016/j.freeradbiomed.2014.09.028. Epub 2014 Oct. doi: 10.1016/j.freeradbiomed.2014.09.028.

Pedersen, G. *et al.* (2005) 'Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium', *Clin Exp Immunol*, 141(2), pp. 298–306. doi: 10.1111/j.1365-2249.2005.02848.x.

Petrasek, J. *et al.* (2011) 'Type I interferons protect from Toll-like receptor 9-associated liver injury and regulate IL-1 receptor antagonist in mice.', *Gastroenterology*. United States, 140(2), p. 697–708.e4. doi: 10.1053/j.gastro.2010.08.020.

Peyrin-Biroulet, L. *et al.* (2010) 'The natural history of adult Crohn's disease in population-based cohorts', *Am J Gastroenterol*, 105, pp. 289–297.

Pimentel, M. *et al.* (2000) 'Identification of a prodromal period in Crohn's disease but not ulcerative colitis', *Am J Gastroenterol*, 95, pp. 3458–62.

Pinti, M. *et al.* (2014) 'Circulating mitochondrial DNA increases with age and is a familiar trait: Implications for "inflamm-aging".', *European journal of immunology*, 44(5), pp. 1552–62. doi: 10.1002/eji.201343921.

Prikhodko, A. S. *et al.* (2015) 'Pure Mitochondrial DNA Does Not Activate Human Neutrophils in vitro', *Biochemistry (Mosc).*, 80(5), pp. 629–35. doi: 10.1134/S0006297915050168.

Puskarich, M. A. *et al.* (2012) 'Circulating cell-free mitochondrial DNA as a novel cancer biomarker: opportunities and challenges', *Mitochondrial DNA*, 23(5), pp. 329–332. doi: 10.3109/19401736.2012.696625.

Qin, C. *et al.* (2016) 'Release of mitochondrial DNA correlates with peak inflammatory cytokines in patients with acute myocardial infarction.', *Anatolian journal of cardiology*. Turkey. doi: 10.14744/AnatolJCardiol.2016.7209.

Qin, J. *et al.* (2012) 'A metagenome-wide association study of gut microbiota in type 2 diabetes.', *Nature*, 490(7418), pp. 55–60. doi: 10.1038/nature11450.

Rabiet, M.-J., Huet, E. and Boulay, F. (2005) 'Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR.', *European journal of immunology*. Germany, 35(8), pp. 2486–2495. doi: 10.1002/eji.200526338.

Rachmilewitz, D. *et al.* (2004) 'Toll-Like Receptor 9 Signaling Mediates the Anti-inflammatory Effects of Probiotics in Murine Experimental Colitis', *Gastroenterology*, 126(2), pp. 520–528. doi: 10.1053/j.gastro.2003.11.019.

van Rheenen, P. F., van de Vijver, E. and Fidler, V. (2010) 'Faecal calprotectin for screening of patients with suspected inflammatory bowel disease: diagnostic meta-analysis.', *BMJ (Clinical research ed.)*, 341, p. c3369. doi: 10.1136/bmj.c3369.

Rock, K. L. *et al.* (2008) 'The inflammatory response to cell death.', *Annual review of pathology*, 3, pp. 99–126. doi: 10.1146/annurev.pathmechdis.3.121806.151456.

Rongvaux, A. *et al.* (2014) ‘Apoptotic Caspases Prevent the Induction of Type I Interferons by Mitochondrial DNA’, *Cell*, 159(7), pp. 1563–1577. doi: 10.1016/j.cell.2014.11.037.

Rose, W. A. 2nd, Sakamoto, K. and Leifer, C. A. (2012) ‘TLR9 is important for protection against intestinal damage and for intestinal repair’, *Scientific Reports*, 2. doi: 10.1038/srep00574.

Roseth, A. G. *et al.* (1992) ‘Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study.’, *Scandinavian journal of gastroenterology*. NORWAY, 27(9), pp. 793–798.

van Rossum, I. A. *et al.* (2010) ‘Mitochondrial DNA and anti-mitochondrial antibodies in serum of autistic children.’, *Journal of neuroinflammation*. BioMed Central Ltd, 7(1), p. 80. doi: 10.1186/1742-2094-7-80.

Saikh, K. U. *et al.* (2004) ‘Human monocytes infected with Yersinia pestis express cell surface TLR9 and differentiate into dendritic cells.’, *Journal of immunology (Baltimore, Md. : 1950)*. United States, 173(12), pp. 7426–7434.

Saitoh, T. *et al.* (2008) ‘Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production.’, *Nature*, 456(7219), pp. 264–8. doi: 10.1038/nature07383.

Sandborn, W. J. *et al.* (2003) ‘Repifermin (keratinocyte growth factor-2) for the treatment of active ulcerative colitis: A randomized, double-blind, placebo-controlled, dose-escalation trial’, *Alimentary Pharmacology and Therapeutics*, 17(11), pp. 1355–1364. doi: 10.1046/j.1365-2036.2003.01589.x.

Sandler, N. *et al.* (2018) ‘Mitochondrial DAMPs Are Released During Cardiopulmonary Bypass Surgery and Are Associated With Postoperative Atrial Fibrillation.’, *Heart, lung & circulation*. Australia, 27(1), pp. 122–129. doi: 10.1016/j.hlc.2017.02.014.

Sands, B. E. (2015) ‘Biomarkers of Inflammation in Inflammatory Bowel Disease.’, *Gastroenterology*. Elsevier Ltd, pp. 1275–1285. doi: 10.1053/j.gastro.2015.07.003.

Santhanam, S. *et al.* (2012) ‘Mitochondrial electron transport chain complex dysfunction in the colonic mucosa in ulcerative colitis.’, *Inflammatory bowel diseases*. United States, 18(11), pp. 2158–2168. doi: 10.1002/ibd.22926.

Sappington, P. L. *et al.* (2002) ‘HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice’, *Gastroenterology*, 123(3), pp. 790–802. doi: 10.1053/gast.2002.35391.

Sasai, M., Linehan, M. M. and Iwasaki, A. (2010) ‘Bifurcation of Toll-like receptor 9 signaling by adaptor protein 3.’, *Science (New York, N.Y.)*. United States, 329(5998), pp. 1530–1534. doi: 10.1126/science.1187029.

Schaefer, L. (2014) ‘Complexity of Danger: The Diverse Nature of Damage-associated Molecular Patterns’, *Journal of Biological Chemistry*, 289(51), pp. 35237–35245. doi: 10.1074/jbc.R114.619304.

Schroeder, K. W., Tremaine, W. J. and Ilstrup, D. M. (1987) ‘Coated Oral 5-Aminosalicylic Acid Therapy for Mildly to Moderately Active Ulcerative Colitis’, *New England Journal of Medicine*, 317(26), pp. 1625–1629. doi: 10.1056/NEJM198712243172603.

Schwartz, D., Loftus, E. and Tremaine, W. et al (2002) ‘The natural history of fistulizing Crohn’s disease in Olmsted County, Minnesota’, *Gastroenterology*, 122, pp. 875–80.

Shimada, K. *et al.* (2012) ‘Oxidized Mitochondrial DNA Activates the NLRP3 Inflammasome during Apoptosis’, *Immunity*. Elsevier Inc., 36(3), pp. 401–414. doi: 10.1016/j.immuni.2012.01.009.

Shockett, P. E. *et al.* (2016) ‘Plasma cell-free mitochondrial DNA declines in response to prolonged moderate aerobic exercise.’, *Physiological reports*. United States, 4(1). doi: 10.14814/phy2.12672.

Sidler, M. A., Leach, S. T. and Day, A. S. (2008) ‘Fecal S100A12 and fecal calprotectin as noninvasive markers for inflammatory bowel disease in children’, *Inflammatory Bowel Diseases*, 14(3), pp. 359–366. doi: 10.1002/ibd.20336.

Simmons, J. D. *et al.* (2013) ‘Elevated levels of plasma mitochondrial DNA DAMPs are linked to clinical outcome in severely injured human subjects.’, *Annals of surgery*, 258(4), pp. 591-6-8. doi: 10.1097/SLA.0b013e3182a4ea46.

Soderholm, J. D. *et al.* (2002) ‘Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn’s disease.’, *Gut*. England, 50(3), pp. 307–313.

Sozzi, G. *et al.* (2005) ‘Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays.’, *Journal of the National Cancer Institute*, 97(24), pp. 1848–50. doi: 10.1093/jnci/dji432.

Stacey, K. J., Sweet, M. J. and Hume, D. A. (1996) ‘Macrophages ingest and are activated by bacterial DNA.’, *Journal of immunology (Baltimore, Md. : 1950)*, 157, pp. 2116–2122.

Steenholdt, C. *et al.* (2014) ‘Individualised therapy is more cost-effective than dose intensification in patients with Crohn’s disease who lose response to anti-TNF treatment: a randomised, controlled trial’, *Gut*, 63(6), pp. 919–927. doi: 10.1136/gutjnl-2013-305279.

Stertz, L. *et al.* (2015) ‘Damage-associated molecular patterns and immune activation in bipolar disorder’, *Acta Psychiatr Scand*, 16(10), p. 12417.

Strimbu, K. and Tavel, J. A. (2010) ‘What are Biomarkers?’, *Current opinion in HIV and AIDS*, 5(6), pp. 463–466. doi: 10.1097/COH.0b013e32833ed177.

Sudakov, N. P. *et al.* (2015) ‘Level of blood cell-free circulating mitochondrial DNA as a novel biomarker of acute myocardial ischemia’, *Biochemistry (Moscow)*. Pleiades Publishing, 80(10), pp. 1387–1392. doi: 10.1134/S000629791510020X.

Surinova, S. *et al.* (2011) ‘On the development of plasma protein biomarkers.’, *Journal of proteome research*. United States, 10(1), pp. 5–16. doi: 10.1021/pr1008515.

Surmiak, M. P. *et al.* (2015) ‘Circulating mitochondrial DNA in serum of patients with granulomatosis with polyangiitis’, *Clin Exp Immunol.*, 181(1), p. 150–5. doi: 10.1111/cei.12628. Epub 2015 May 24.

Taanman, J. W. (1999) ‘The mitochondrial genome: structure, transcription, translation and replication.’, *Biochimica et biophysica acta*. Netherlands, 1410(2), pp. 103–123.

Takeuchi, O. and Akira, S. (2010) ‘Pattern Recognition Receptors and Inflammation’, *Cell*, pp. 805–820. doi: 10.1016/j.cell.2010.01.022.

Taleban, S. *et al.* (2016) ‘Clinical Activity and Quality of Life Indices Are Valid Across Ulcerative Colitis But Not Crohn’s Disease Phenotypes’, *Digestive Diseases and Sciences*, 61(9), pp. 2627–2635. doi: 10.1007/s10620-016-4180-8.

Taylor, R. C., Cullen, S. P. and Martin, S. J. (2008) ‘Apoptosis: Controlled demolition at the cellular level’, *Nature Reviews Molecular Cell Biology*, pp. 231–241. doi: 10.1038/nrm2312.

Taylor, R. W. and Turnbull, D. M. (2005) ‘Mitochondrial DNA mutations in human disease’, *Nat Rev Genet*, 6(5), pp. 389–402. doi: 10.1038/nrg1606.MITOCHONDRIAL.

Thijssen, M. A. M. A. *et al.* (2002) ‘Difference between free circulating plasma and serum DNA in patients with colorectal liver metastases’, *Anticancer Research*, 22(1 A), pp. 421–425.

Tian, J. *et al.* (2007) ‘Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE.’, *Nature immunology*, 8(5), pp. 487–496. doi: 10.1038/ni1457.

Tibble, J. J. *et al.* (2000) ‘A simple method for assessing intestinal inflammation in Crohn’s disease.’, *Gut*, 47(4), pp. 506–513. doi: 10.1136/gut.47.4.506.

Timmermans, K. *et al.* (2015) 'Plasma Nuclear and Mitochondrial Dna Levels, and Markers of Inflammation, Shock, and Organ Damage in Patients with Septic Shock', *Shock (Augusta, Ga.)*. Available at: http://europepmc.org/abstract/MED/26717107.

Timmermans, K. *et al.* (2016) 'Plasma levels of danger-associated molecular patterns are associated with immune suppression in trauma patients', *Intensive Care Medicine*, 42(4), pp. 551–561. doi: 10.1007/s00134-015-4205-3.

Truelove, S. C. (1955) 'Cortisone in Ulcerative Colitis Final Report on a Therapeutic Trial', *British Medical Journal*, 2(4947), pp. 1041–1048. doi: 10.1136/bmj.2.4947.1041.

Tsuji, N. *et al.* (2015) 'Role of Mitochondrial DNA in Septic AKI via Toll-Like Receptor 9.', *Journal of the American Society of Nephrology : JASN*. doi: 10.1681/ASN.2015040376.

Turner, D. *et al.* (2007) 'Response to Corticosteroids in Severe Ulcerative Colitis: A Systematic Review of the Literature and a Meta-Regression', *Clinical Gastroenterology and Hepatology*, 5(1), pp. 103–110. doi: 10.1016/j.cgh.2006.09.033.

Umetani, N., Hiramatsu, S. and Hoon, D. S. B. (2006) 'Higher amount of free circulating DNA in serum than in plasma is not mainly caused by contaminated extraneous DNA during separation.', *Annals of the New York Academy of Sciences*, 1075, pp. 299–307. doi: 10.1196/annals.1368.040.

Urata, M. *et al.* (2008) 'Short Report Platelet contamination causes large variation as well as overestimation of mitochondrial DNA content of peripheral', 5, pp. 513–514.

Varhaug, K. N. *et al.* (2016) 'Increased levels of cell-free mitochondrial DNA in the cerebrospinal fluid of patients with multiple sclerosis', *Mitochondrion*, pp. 10–13. doi: 10.1016/j.mito.2016.12.003.

Vavricka, S. R. *et al.* (2015) 'Extraintestinal manifestations of inflammatory bowel disease', *Inflammatory Bowel Diseases*, pp. 1982–1992. doi: 10.1097/MIB.0000000000000392.

Ventham, N. T. *et al.* (2013) 'Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics.', *Gastroenterology*. Elsevier, Inc, 145(2), pp. 293–308. doi: 10.1053/j.gastro.2013.05.050.

Vermeire, S., Van Assche, G. and Rutgeerts, P. (2004) 'C-reactive protein as a marker for inflammatory bowel disease.', *Inflammatory bowel diseases*, 10(5), pp. 661–665. doi: 10.1097/00054725-200409000-00026.

Vermeire, S., Van Assche, G. and Rutgeerts, P. (2006) 'Laboratory markers in IBD: useful, magic, or unnecessary toys?', *Gut*, 55(3), pp. 426–31. doi: 10.1136/gut.2005.069476.

Verschoor, C. P. *et al.* (2015) 'Circulating TNF and mitochondrial DNA are major determinants of neutrophil phenotype in the advanced-age, frail elderly', *Molecular Immunology*, 65(1), p. 148–56. doi: 10.1016/j.molimm.2015.01.015. Epub 20. doi: 10.1016/j.molimm.2015.01.015.

Viglianti, G. a *et al.* (2003) 'Activation of autoreactive B cells by CpG dsDNA.', *Immunity*, 19(6), pp. 837–47. doi: 10.1016/s1074-7613(03)00323-6.

Vitali, R. *et al.* (2011) 'Fecal HMGB1 is a novel marker of intestinal mucosal inflammation in pediatric inflammatory bowel disease.', *The American journal of gastroenterology*, 106(11), pp. 2029–40. doi: 10.1038/ajg.2011.231.

Waldenström, A. *et al.* (2012) 'Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells', *PLoS ONE*, 7(4), p. e34653. doi: 10.1371/journal.pone.0034653.

Walko 3rd, T. D. *et al.* (2014) 'Cerebrospinal fluid mitochondrial DNA: a novel DAMP in pediatric traumatic brain injury', *Shock.* United States, 41(6), p. 499–503. doi: 10.1097/SHK.0000000000000160. doi: 10.1097/SHK.0000000000000160.

Walmsley, R. S. *et al.* (1998) 'A simple clinical colitis activity index', *Gut*, 43(1), pp. 29–32. doi: 10.1136/gut.43.1.29.

Wang, H. *et al.* (1999) 'HMG-1 as a late mediator of endotoxin lethality in mice', *Science*, 285(5425), pp. 248–251. doi: 10.1126/science.285.5425.248.

Wang, H. *et al.* (2015) 'Neutrophil Extracellular Trap Mitochondrial DNA and Its Autoantibody in Systemic Lupus Erythematosus and a Proof-of-Concept Trial of Metformin.', *Arthritis & rheumatology (Hoboken, N.J.)*. United States, 67(12), pp. 3190–3200. doi: 10.1002/art.39296.

Wang, H.-C. *et al.* (2012) 'The value of serial plasma nuclear and mitochondrial DNA levels in acute spontaneous intra-cerebral haemorrhage.', *European journal of neurology : the official journal of the European Federation of Neurological Societies*, 19(12), pp. 1532–8. doi: 10.1111/j.1468-1331.2012.03761.x.

Wang, H.-C. *et al.* (2013) 'The value of serial plasma and cerebrospinal fluid nuclear and mitochondrial deoxyribonucleic acid levels in aneurysmal subarachnoid hemorrhage.', *Journal of neurosurgery*, 118(1), pp. 13–9.

Wang, L. L. L. *et al.* (2015) 'Plasma nuclear and mitochondrial DNA levels in acute myocardial infarction patients', *Coron Artery Dis.*, 26(4), p. 286–8. doi: 10.1097/MCA.0000000000000244. doi: 10.1097/MCA.0000000000000231.

Wei, X. *et al.* (2015) 'Cationic nanocarriers induce cell necrosis through impairment of Na(+)/K(+)-ATPase and cause subsequent inflammatory response.', *Cell research*, 25(2), pp. 237–53. doi: 10.1038/cr.2015.9.

Welz, P.-S. *et al.* (2011) 'FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation.', *Nature*, 477(7364), pp. 330–4. doi: 10.1038/nature10273.

West, a P. *et al.* (2015) 'Mitochondrial DNA stress primes the antiviral innate immune response', *Nature*, 520(7548), pp. 553–557. doi: 10.1038/nature14156.

West, a P., Shadel, G. S. and Ghosh, S. (2011) 'Mitochondria in innate immune responses.', *Nature reviews. Immunology*. Nature Publishing Group, 11(6), pp. 389–402. doi: 10.1038/nri2975.

Whitaker, R. M. *et al.* (2015) 'Urinary mitochondrial DNA is a biomarker of mitochondrial disruption and renal dysfunction in acute kidney injury.', *Kidney international*. United States, 88(6), pp. 1336–1344. doi: 10.1038/ki.2015.240.

White, M. J. *et al.* (2014) 'Apoptotic Caspases Suppress mtDNA-Induced STING-Mediated Type I IFN Production', *Cell*, 159(7), pp. 1549–1562. doi: 10.1016/j.cell.2014.11.036.

Wiesner, R. J. *et al.* (1992) 'Counting target molecules by exponential polymerase chain reaction: copy number of mitochondrial DNA in rat tissues', *Biochemical and Biophysical Research Communications*, 46(5), pp. 553–559. doi: 10.1016/0006-291X(92)90517-O.

Williams, C. D. *et al.* (2011) 'Role of the Nalp3 inflammasome in acetaminophen-induced sterile inflammation and liver injury.', *Toxicology and applied pharmacology*. United States, 252(3), pp. 289–297. doi: 10.1016/j.taap.2011.03.001.

Williams, J. G. *et al.* (2016) 'Infliximab versus ciclosporin for steroid-resistant acute severe ulcerative colitis (CONSTRUCT): a mixed methods, open-label, pragmatic randomised trial', *The Lancet. Gastroenterology & Hepatology*. Elsevier B.V, 1(1), pp. 15–24. doi: 10.1016/S2468-1253(16)30003-6.

Won, J.-H. *et al.* (2015) 'Rotenone-induced Impairment of Mitochondrial Electron Transport Chain Confers a Selective Priming Signal for NLRP3 Inflammasome Activation', *Journal of Biological Chemistry*, 290(45), pp. 27425–27437.

Wright, E. K. *et al.* (2014) 'Fecal Biomarkers in the Diagnosis and Monitoring of Crohn's Disease', *Inflammatory Bowel Diseases*, 20(9), pp. 1668–1677. Available at: http://journals.lww.com/ibdjournal/Fulltext/2014/09000/Fecal_Biomarkers_in_the_Diagnosis_and_Monitoring.19.aspx.

Xia, P. *et al.* (2009) 'Simultaneous quantitative assessment of circulating cell-free mitochondrial and nuclear DNA by multiplex real-time PCR', 24, pp. 20–24.

Xia, C-Y. *et al*. (2017) 'Reference Intervals of Mitochondrial DNA Copy Number in Peripheral Blood for Chinese Minors and Adults', *Chinese Medical Journal*, pp. 2435-2440. doi: 10.4103/0366-6999.216395

Xie, J. *et al.* (2008) 'Structural basis for pattern recognition by the receptor for advanced glycation end products (RAGE)', *Journal of Biological Chemistry*, 283(40), pp. 27255–27269. doi: 10.1074/jbc.M801622200.

Xie, L. *et al.* (2017) 'Exogenous administration of mitochondrial DNA promotes ischemia reperfusion injury via TLR9-p38 MAPK pathway', *Regulatory Toxicology and Pharmacology*, 89, pp. 148–154. doi: https://doi.org/10.1016/j.yrtph.2017.07.028.

Xin, G. *et al.* (2016) 'Metformin Uniquely Prevents Thrombosis by Inhibiting Platelet Activation and mtDNA Release', *Scientific Reports*. The Author(s), 6, p. 36222. Available at: http://dx.doi.org/10.1038/srep36222.

Xue, X. *et al.* (2009) 'Optimizing the yield and utility of circulating cell-free DNA from plasma and serum.', *Clinica chimica acta; international journal of clinical chemistry*. Elsevier B.V., 404(2), pp. 100–4. doi: 10.1016/j.cca.2009.02.018.

Yasui, K. *et al.* (2016) 'Mitochondrial damage-associated molecular patterns as potential proinflammatory mediators in post–platelet transfusion adverse effects', *Transfusion*, 56(5), pp. 1201–1212. doi: 10.1111/trf.13535.

Ye, W. *et al.* (2017) 'Plasma-derived exosomes contribute to inflammation via the TLR9-NF-κB pathway in chronic heart failure patients', *Molecular Immunology*, 87, pp. 114–121. doi: 10.1016/j.molimm.2017.03.011.

Yu, M. *et al.* (2012) 'Cell-free Circulating Mitochondrial DNA in the Serum: A Potential Non-invasive Biomarker for Ewing's Sarcoma', *Archives of Medical Research*. Elsevier Inc, 43(5), pp. 389–394. doi: 10.1016/j.arcmed.2012.06.007.

Yuan, W. *et al.* (2014) 'An integrated epigenomic analysis for type 2 diabetes susceptibility loci in monozygotic twins.', *Nature communications*, 5, p. 5719. doi: 10.1038/ncomms6719.

Zachariah, R. R., Schmid, S. and Buerki, N. (2008) 'Levels of Circulating Cell-Free Nuclear and Mitochondrial DNA in Benign and Malignant Ovarian Tumors', 112(4), pp. 843–850.

Zanetti-Dallenbach, R. *et al.* (2008) 'Positive correlation of cell-free DNA in plasma/serum in patients with malignant and benign breast disease.', *Anticancer research*. Greece, 28(2A), pp. 921–925.

Zeevi, D. *et al.* (2015) 'Personalized Nutrition by Prediction of Glycemic Responses.', *Cell*. United States, 163(5), pp. 1079–1094. doi: 10.1016/j.cell.2015.11.001.

Zhang, J.-Z. *et al.* (2017) 'Plasma mitochondrial DNA levels were independently associated with lung injury in elderly hip fracture patients', *Injury*, 48(2), pp. 454–459. doi: 10.1016/j.injury.2017.01.009.

Zhang, Q. *et al.* (2010) 'Circulating mitochondrial DAMPs cause inflammatory responses to injury.', *Nature*. Nature Publishing Group, 464(7285), pp. 104–7. doi: 10.1038/nature08780.

Zhang, Q., Itagaki, K. and Hauser, C. J. (2010) 'Mitochondrial DNA is released by shock and activates neutrophils via p38 map kinase.', *Shock (Augusta, Ga.)*, 34(1), pp. 55–59. doi: 10.1097/SHK.0b013e3181cd8c08.

Zhang, Q., Lenardo, M. J. and Baltimore, D. (2017) '30 Years of NF-κB: A Blossoming of Relevance to Human Pathobiology', *Cell*, pp. 37–57. doi: 10.1016/j.cell.2016.12.012.

Zhang, Y. *et al.* (2016) 'Associations of mitochondrial haplogroups and mitochondrial DNA copy numbers with end-stage renal disease in a Han population.', *Mitochondrial DNA. Part A. DNA mapping, sequencing, and analysis*, pp. 1–7. doi: 10.1080/24701394.2016.1177038.

Zhang, Z. *et al.* (2013) 'Gene deletion of Gabarap enhances Nlrp3 inflammasome-dependent inflammatory responses', *J Immunol*, 190(7), pp. 3517–3524. doi: 10.4049/jimmunol.1202628.

Zheng, W. *et al.* (2006) 'Evaluation of AGR2 and AGR3 as candidate genes for inflammatory bowel disease', *Genes and Immunity*, 7(1), pp. 11–18. doi: 10.1038/sj.gene.6364263.

Zhong, X.-Y. *et al.* (2007) 'Increased concentrations of antibody-bound circulatory cell-free DNA in rheumatoid arthritis.', *Clinical chemistry*. United States, 53(9), pp. 1609–1614. doi: 10.1373/clinchem.2006.084509.

Zhou, R. *et al.* (2011) 'A role for mitochondria in NLRP3 inflammasome activation.', *Nature*, 469(7329), pp. 221–5. doi: 10.1038/nature09663.

Ziello, J. E., Huang, Y. and Jovin, I. S. (2010) 'Cellular Endocytosis and Gene Delivery', *Molecular Medicine*. ScholarOne, 16(5–6), pp. 222–229. doi: 10.2119/molmed.2009.00101.

Zitvogel, L., Kepp, O. and Kroemer, G. (2010) 'Decoding Cell Death Signals in Inflammation and Immunity', *Cell*. Elsevier, 140(6), pp. 798–804. doi: 10.1016/j.cell.2010.02.015.

APPENDICES

Appendix A – Clinical disease activity indices in IBD

Variable	Variable Description
General well-being	Score 0 = very well Score 1 = slightly below par Score 2 = poor Score 3 = very poor Score 4 = terrible
Abdominal pain	Score 0 = none Score 1 = mild Score 2 = moderate Score 3 = severe
Number of liquid stools / day	Score 1 point for each liquid stool
Abdominal mass	Score 0 = none Score 1 = dubious Score 2 = definite Score 3 = definite and tender
Complications	(Score 1 point each manifestation) Arthralgia Uveitis Erythema nodosum Aphthous ulcers Pyoderma gangrenosum Anal fissure New fistula abscess

Remission < 5; mild disease 5-7; moderate disease 8-16; severe disease > 16

Table A1: Harvey Bradshaw Index (HBI) for clinical assessment of disease activity in CD

Variable	Variable Description
Bowel frequency (day)	Score 0 = 0-3 Score 1 = 4-6 Score 2 = 7-9 Score 3 = >9
Bowel frequency (night)	Score 0 = 0 Score 1 = 1-3 Score 2 = 4-6
Urgency of defecation	Score 0 = None Score 1 = Hurry Score 2 = Immediately Score 3 = Incontinence
Blood in stool	Score 0 = None Score 1 = Trace Score 2 = Occasionally frank Score 3 = Usually frank
General well-being	Score 0 = Very well Score 1 = Slightly below par Score 2 = Poor Score 3 = Very poor Score 4 = Terrible
Extracolonic features	(Score 1 per manifestation) Arthritis Uveitis Erythema nodosum Pyoderma gangrenosum

Remission <=2, clinical activity > 2

Table A2: Simple Clinical Colitis Activity Index (SCCAI) for clinical assessment of disease activity in UC

Bloody diarrhoea	**≥ 6 bloody stools per day**
and at least one of:	
Temperature	> 37.8 degrees Celsius
Pulse rate	> 90 beats per minute
Haemoglobin	< 105 g/L
ESR	> 30 mm/h

Table A3: Modified Truelove & Witts' criteria for diagnosis of ASUC

Appendix B – Patient Questionnaire

Patient Questionnaire

To be completed by research clinician

Patient ID | **Date of completion**

Gender **M / F** **please circle**

Ethnic origin

1. Which of the following best describes your ethnic origin?

White European ☐

Jewish ☐ Ashkenazi ☐ Sephardic ☐

South Asian ☐ (Bangladesh/India/Pakistan)

South East Asian ☐

Hispanic ☐

Afro-Caribbean ☐

African American ☐

Pacific Islander ☐ Native Hawaiian ☐

Native American ☐ Native Alaskan ☐

Other ☐ Please specify

Mixed ☐ Please specify

Country of birth

2. Where were you born (town and country)?
   ……

3. Where did you spend the majority of your childhood (town and country)? ……

4. Was this location Urban / Rural ? please circle

5. Where were your biological parents born (town and country)?

Mother …….
Father…….

Social History and Environmental factors

Education

6. Number of years of education at recruitment (primary, secondary and further education).

Smoking

7. Do you currently smoke every day?

(If no skip to Q 10)

- ☐ Yes
- ☐ No

8. If yes, what do you smoke?

- ☐ Cigarettes
- ☐ Cigars
- ☐ Pipe
- ☐ Roll-ups

9. How many do you smoke per day?

10. If you only smoke socially, how many do you smoke per month?

(If no skip to Q 12)

11. When did you start smoking? (Year)

Have you ever smoked? ☐ Yes (If no skip to Q 17)

☐ No

12. If yes, what did you smoke? ☐ Cigarettes

☐ Cigars

☐ Pipe

☐ Roll-ups

13. When did you start smoking? (Year)

14. When did you stop smoking? (Year)

15. How many did you smoke per day?

16. Does your partner smoke?

☐ Yes
☐ No

17. Did anyone smoke in the house while you were growing up?

☐ Yes
☐ No

18. Smoking status at diagnosis of IBD

☐ Smoker
☐ Non-smoker
☐ Ex-smoker
☐ Unsure

Alcohol

19. Do you consume alcohol? ☐ Yes

☐ Never

If yes, how often? ☐ Most days ☐ Once or twice a week

☐ At weekends only ☐ Once or twice a month

☐ Once or twice a year

20. If yes, how many drinks per day?

☐ 0-1
☐ 2-3
☐ More than 3

21. If more than 3, how many usually? ……..

22. What do you usually drink? ☐ Beer ☐ Cider

☐ Wine ☐ Spirits

Childhood exposure

23. Did you have a pet in the house as a child?

☐ Yes ☐ No ……..…… ……
(type of pet)

24. Did you receive all your recommended vaccinations?

☐ Yes ☐ No ☐ Unsure

25. Did you require frequent antibiotics as a child?

☐ Yes ☐ No ☐ Unsure

26. If yes, what were these mainly required for?
……..

27. Were there any concerns over poor growth in childhood? ☐

Or delayed puberty? ☐

Please ask if you have any queries about any of the questions above.

The following sections should be completed with the aid of a doctor or nurse.

Family and Medical History

28. In total, how many biological brothers and sisters do you have? ☐

29. How many biological children do you have? ☐

30. Is there a family history of IBD?

☐ Yes ☐ No (skip to Q.32)

	Unknown	Crohn's Disease	Ulcerative Colitis	IBD (unclear type)	Age at diagnosis
Father	☐	☐	☐	☐	
Mother	☐	☐	☐	☐	
Siblings	☐	☐ number	☐ number	☐ number	
Children	☐	☐ number	☐ number	☐ number	

Are any other relations affected? – please specify below and also whether maternal or paternal.	Unknown	Crohn's disease	Ulcerative Colitis	IBD (unclear type)	Age at diagnosis
	☐	☐	☐	☐	
		☐	☐	☐	
		☐	☐	☐	

31. Have you or anyone in your family had any of the following conditions?

	Personal History	Family History (if yes, please specify relation)
Multiple Sclerosis	☐ No ☐ Yes	☐ No ☐ Yes Relation
Psoriasis	☐ No ☐ Yes	☐ No ☐ Yes Relation
Coeliac Disease	☐ No ☐ Yes	☐ No ☐ Yes Relation
Ankylosing Spondylitis	☐ No ☐ Yes	☐ No ☐ Yes Relation
Colorectal Cancer	☐ No ☐ Yes Age at diagnosis	☐ No ☐ Yes Relation Age at diagnosis
Any Cancer Please specify	☐ No ☐ Yes	☐ No ☐ Yes Relation
Other Immune Disorders Please specify		

32. Do you have any other medical problems? (Please list below)

Present Diagnosis

☐ Crohn's disease ☐ Ulcerative Colitis ☐ IBD unspecified ☐ Unknown

33. When did your symptoms start (month and year)

……

34. What symptoms did you have? (Tick all that apply)

☐ growth failure ☐ diarrhoea ☐ blood in stool ☐ abdominal pain
☐ fatigue/tiredness

☐ fistula/abscess ☐ nausea ☐ vomiting ☐ none ☐ other

If "none" please specify the clinical history that led to diagnosis of IBD.

……

If "other" please specify what other symptoms you had.

……

35. Have you lost any weight since the onset of your symptoms?

☐ Yes ☐ No

36. If yes, how much? …….....

37. When were you diagnosed (month and year)? ……

38. What was your weight at diagnosis? ……
and your height? ……...……

39. How many times have you been admitted to hospital for your IBD (other than for planned investigations)?

……

40. Have you had any operations for your IBD? ☐ No ☐ Yes (Please list below)

Operation	Year

41. Do you currently have an
☐ ileoanal pouch ☐ ileostomy ☐ colostomy ?

42. Have you had your tonsils removed?
If yes, what year? ……..

43. Have you had your appendix removed?
If yes, what year? ……

44. Have you had any abdominal surgery other than for IBD? Please specify below.

Medications

Medication	Treated with	Tolerated	On now
Oral 5-ASA	☐	☐	☐
Rectal 5-ASA	☐	☐	☐
Sulphasalazine	☐	☐	☐
Oral steroid	☐	☐	☐
Rectal steroid	☐	☐	☐
Azathioprine	☐	☐	☐
6-Mercaptopurine	☐	☐	☐
Methotrexate	☐	☐	☐
Ciclosporin	☐	☐	☐
Infliximab	☐	☐	☐
Adalimumab	☐	☐	☐
Certolizumab	☐	☐	☐
Natalizumab	☐	☐	☐
Antibiotics for IBD	☐	☐	☐
Elemental diet	☐	☐	☐

Other IBD therapies?.....

45. Oral contraceptive status ☐ Current ☐ Never ☐ Ex

46. Have you taken any NSAIDS (eg.ibuprofen, diclofenac, naproxen**)** in the last 6 months?

☐ Yes ☐ No

47. If yes, how often? ☐ Seldom ☐ Frequently

48. Have you taken any aspirin in the last 6 months? ☐ Yes ☐ No

49. If yes, how often? ☐ Seldom ☐Frequently

50. Have you taken any paracetamol in the last 6 months? ☐ Yes ☐ No

51. If yes, how often? ☐ Seldom ☐Frequently

52. What was your usual diet during the 6 months to a year prior to diagnosis?

☐ Mixed meat/fish/veg ☐ No red meat ☐ Vegetarian ☐ Vegan
☐ Pescetarian ☐ Gluten free ☐ Dairy free ☐ Lactose free

☐ Diabetic ☐ High fibre ☐ Low residue ☐ Low FODMAP

☐ Other, please specify

…….

Pregnancy

53. Have you been pregnant since you were diagnosed with IBD?

☐ Yes ☐ No

End of questionnaire

Appendix C – Clinical Activity Form

☐ Sample Collection ☐ Clinic Assessment (please tick)

To be completed by research clinician

Patient ID		Date of sample/clinic		Temp / Pulse	
Current weight (kg)		Usual weight (kg)		Unwanted weight loss(kg)	
Time to weight loss(months)		Height (metres)		BMI	
Fasting state	☐ fasted ☐ non-fasted		Type of bowel prep		

List all current meds including non-IBD drugs. Please note doses and duration of treatment especially steroids.	Any recently stopped?	When?

Have you had any courses of antibiotics in the last 6 months?(Please specify - what for, which antibiotic(s), when taken and how long for)

Smoking status ☐ never ☐ current, cigs = ☐ ex, stop date =

SCCAI / HBI

Please describe the following for the day and night prior to taking bowel prep (or previous day if none taken).

General well-being ☐ Very well ☐ Slightly below par ☐ Poor ☐ Very poor ☐ Terrible

Bowel frequency during day no of formed stools = no of liquid stools =

Bowel frequency during night no of formed stools = no of liquid stools =

Description of bowel movements

Urgency of defecation ☐ None ☐ Hurry ☐ Immediate ☐ Incontinence

Blood in stool ☐ None ☐ Trace ☐ Occasionally frank ☐ Usually frank

Abdominal pain ☐ None ☐ Mild ☐ Moderate ☐ Severe

Abdominal mass ☐ None ☐ Possible ☐ Definite ☐ Tender

EIMS ☐ Arthralgia ☐ Uveitis ☐ Erythema Nodosum ☐ Pyoderma Gangrenosum ☐ Anal fissure ☐ New fistula ☐ Abscess ☐ Aphthous ulcers

Current diet	☐ Mixed meat/fish/veg ☐ No red meat ☐ Vegetarian ☐ Vegan ☐ Pescetarian ☐ Gluten free ☐ Dairy free ☐ Lactose free ☐ Diabetic ☐ High fibre ☐ Low residue ☐ Low FODMAP ☐ Polymeric diet ☐ Elemental diet ☐ Clear fluid diet ☐ TPN ☐ IV fluids only ☐ Other –please specify below ……
Alcohol	Do you consume alcohol? ☐ Yes ☐ Never ☐ Most days ☐ At weekends only ☐ Once or twice a week ☐ Once or twice a month ☐ Once or twice a year How many drinks per day? ☐ 0-1 ☐ 2-3 ☐ More than 3 If more than 3, how many? …… What do you usually drink? ☐ Beer ☐ Cider ☐ Wine ☐ Spirits Notes ……

Clinician's assessment of patient's health status :
0 is worst, 10 is best (perfect health)

Please circle one number below

0 1 2 3 4 5 6 7 8 9 10

Each recruiting centre should specify the units used locally for each test below

Blood test	Date	Result	Stool test	Date	Result
Haemoglobin			Calprotectin		
WCC			Stool culture		
Neutrophils			Please specify pathogenic bacteria identified		
Lymphocytes					
Platelets					
ESR			C.diff toxin		
Albumin			CMV(specify test below)		
CRP					

Appendix D – Primer sequences used for quantification of mtDNA by qPCR

Name	Forward	Reverse	Ref
Cytochrome C oxidase subunit III (COXIII)	5'-ATGACCCACCAATCACATGC-3'	5'-ATCACATGGCTAGGCCGGAG-3'	(Zhang *et al.*, 2010)
NADH-dehydrogenase 2 (ND2)	5'- CACAGAAGCTGCCATCAAGTA-3'	5'- CCGGAGAGTATATTGTTGAAGAG-3'	(Lu *et al.*, 2010)

Table A4: Primer sequences used for quantification of mtDNA by qPCR

Appendix E – N-terminal hexapeptide sequences of mitochondrial formylated peptides*

Formylated peptide	N terminus	Uniprot number
NADH 1	Formyl-MPMANL	P03886
NADH 2	Formyl-MNPLAQ	P03891
NADH 3	Formyl-MNFALI	P03897
NADH 4L	Formyl-MPLIYM	P03901
NADH 4	Formyl-MLKLIV	P03905
NADH 5	Formyl-MTMHTT	P03915
NADH 6	Formyl-MMYALF	P03923
COX I	Formyl-MFADRW	P00395
COX II	Formyl-MAHAAQ	P00403
COX III	Formyl-MTHQSH	P00414
Cytochrome b	Formyl-MTPMRK	P00156
ATP synthase subunit 6	Formyl-MNENLF	P00846
ATP synthase subunit 8	Formyl-MPQLNT	P03928

**As described in (Rabiet, Huet and Boulay, 2005)*

Table A5: N-terminal hexapeptide sequences of mitochondrial formylated peptides.

Appendix F – Mayo endoscopic classification of UC severity

Mayo Score	Description	Detailed description
0	Normal	No friability or granularity Intact vascular pattern
1	Mildly active	Erythema Decreased vascular pattern Mild friability
2	Moderately active	Marked erythema Absent vascular pattern Friability Erosions
3	Severely active	Marked erythema Absent vascular markings Granularity Friability Spontaneous bleeding Ulcerations

Table A6: Mayo endoscopic classification of UC severity (Schroeder, Tremaine and Ilstrup, 1987)