Bacteria-Macrophage interactions in the Pathogenesis of Crohn's Disease

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

by Paul Kevin Flanagan

May 2017

Declaration

I declare this thesis to be comprised of my own work and that all experiments were performed by myself with the exceptions of: genomic analysis of patients' DNA, undertaken by the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh, UK; quantification of highly sensitive CRP and serum Vitamin D concentrations, undertaken by the Biochemistry Department, Royal Liverpool University Hospital, UK.

This thesis has not been submitted, either wholly or in part, in support of any other degree or qualification at this or any other institute of learning.

Acknowledgements

This work was initially supported by funding from the National Institute for Health Research (NIHR) Biomedical Research Centre, Liverpool and latterly by an NIHR Biomedical Research Fellowship and a Shire Innovation Fund for registrars. Sincere thanks are given for this support, without which the work would not have been possible.

I would like to thank Professor J.M. Rhodes who has acted not only as a supervisor on this project but was an inspirational mentor, both academically and clinically, before I entered the lab, a role he continues to fill and which has left an enduringly positive influence on my career. I would also like to thank Professor B.J. Campbell for the invaluable support and guidance he has provided throughout - I am indebted to him for his high calibre teaching and unremittingly rigorous pursuit of scientific method to the very highest of standards; without him I could not have completed this work.

I am grateful to Prof. S.W. Edwards and his group, in particular Dr. Helen Wright, who kindly allowed access to, and provided training for, flow cytometry experiments. Dr. Violaine Sée generously facilitated the use of the luminometer and a confocal microscope and Prof. Satsangi and Dr. Eileen Marks kindly ran genetic and serum assays in their labs for which I am enormously grateful. Dr. Sree Subramanian has been an invaluable friend and colleague, supporting me with the recruitment of patients. I am thankful to all my colleagues in the Gastroenterology department who gave a free listening ear and were generous with practical advice and moral support.

Finally, I would like to thank my family, and in particular my wife Jenni, for their forbearance, for their unquestioning support and for giving me the strength to complete this project.

Abstract: Bacteria-Macrophage interactions in the Pathogenesis of Crohn's Disease

Paul Kevin Flanagan

Crohn's disease (CD) is associated with defective innate immunity, including impaired neutrophil chemotaxis, and mucosal invasion by bacteria, particularly *E. coli* that replicate inside macrophage phagolysosomes.

In this thesis several hypotheses were tested:

(i) that CD macrophages might be defective at killing and or responding to Gram-negative bacteria, particularly *E. coli*;

(ii) that killing of phagocytosed bacteria within macrophages might be enhanced by drugs such as hydroxychloroquine (HCQ) (previously shown to raise intravacuolar pH), and vitamin D (previously shown to enhance macrophage function).

I assessed CD peripheral blood monocyte-derived macrophages (MDM) for their abilities to kill *E. coli*, with and without HCQ and vitamin D, and to generate neutrophil chemoattractants.

MDM from patients with CD were similar to those from healthy controls (HC) in allowing replication of phagocytosed CD-derived *E. coli*: HM605 [CD N=10, mean fold replication in 3h: 1.08, (95% confidence interval (CI) 0.39-1.78); HC N=9, 1.50, (95%CI 1.02-1.97); P=0.15] and also in generation of neutrophil chemoattractants in response to *E. coli* (mean fold chemotaxis relative to control: CD 2.55, 95%CI 2.31-2.80; HC 2.65, 95%CI 2.46-2.85, P=0.42). The only possible exception was reduced bacterial killing in macrophages from the relatively rare patients homozygous for *ATG16L1* polymorphisms, reported previously and confirmed in the single example in this series. HCQ and 1,25 OH2-vitamin D3 both caused dose-dependent inhibition of intra-macrophage *E .coli* replication 3h post-infection, HCQ: 73.9% inhibition (P<0.001) at 1µg/mL, accompanied by raised intra-phagosomal pH, and 1,25OH2-Vitamin D3: 80.7% inhibition (P<0.05) at 80nM. HCQ had synergistic effects with doxycycline and ciprofloxacin on killing of phagocytosed *E. coli.*

Thus: CD and HC macrophages generally perform similarly in allowing replication of phagocytosed *E. coli* and generating neutrophil chemoattractants. Replication of phagocytosed *E. coli* was substantially decreased by HCQ and vitamin D. These warrant further therapeutic trials in CD in combination with relevant antibiotics.

Contents

Preface

Chapters

List of Figures

List of Tables

List of abbreviations

5ASAs	5-aminosalicylates
6-TGN	6-thioguanine
ACCA	Antibodies to the microbial cell wall carbohydrates chitobioside
AIEC	Adherent and invasive E. coli
ALCA	Antibodies to the microbial cell wall carbohydrates laminaribioside
AMCA	Antibodies to the microbial cell wall carbohydrates mannobioside
ANOVA	One-way analysis of variance
APC	Antigen presenting cells
AS	Ankylosing spondylitis
ASCA	Anti-Saccharomyces cerevisiae antibody
ATG16L1	Autophagy-related 16-like 1
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CaCl2	Calcium chloride
CARD15	caspase recruitment domain-containing protein 15
CD	Crohn's disease
CDAI	Crohn's disease activity index
CFU	Colony forming units
CGD	Chronic granulomatous disease
CI	Confidence interval
Cipro	Ciprofloxacin
Cmax	Peak serum concentration achieved with standard oral dosing
CMV	Cytomegalovirus

CO_2	Carbon dioxide
CRP	C reactive protein
CT	Computerised tomography
DHR123	Dihydrorhodamine 123
DMSO	Dimethylsuphoxide
DNA	Deoxyribonucleic acid
Doxy	Doxycycline
DSS	Dextran sulphate sodium
E. coli	*Escherichia coli*
ECCO	European Crohn's and Colitis Organisation
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F. prau	*Faecalibacterium prauznitzii*
FAE	Follicle associated epithelium
FBS	Foetal bovine serum
FeNTA	Ferric nitrilotriacetic acid
FITC	Fluorescein isothiocyanate
g	Gram
g	gravity
GFP	Green fluorescent protein
GI	Gastrointestinal
GP2	Glycoprotein 2
GWAS	Genome wide association studies

HBI	Harvey Bradshaw index
HBSS	Hank's Balanced Salt Solution
HC	Healthy controls
HCl	Hydrochloric acid
HCQ	Hydroxychloroquine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMDMs	Human monocyte-derived macrophages
hsCRP	High sensitivity CRP
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IL-6	Interleukin 6
IL-8	Interleukin 8
IRGM	Immunity-related GTPase M
LB	Luria Bertani
lpfA	Long polar fimbriae
Lucigenin	N,N'-dimethyl-9,9'-biacridinium dinitrate
M cells	Microfold cells
MAP	*Mycobacterium avium* subsp paratuberculosis
MDM	Monocyte-derived macrophages
MDP	Muramyl dipeptide
$MgCl_2$	Magnesium chloride

MIC	Minimum inhibitory concentration
MMP12	Macrophage elastase
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
n/a	not applicable
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH_4Cl_2	Ammonium chloride
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NRES	National Research Ethics Service
NSAIDs	Non-steroidal anti-inflammatory drugs
OCP	Oral contraceptive pill
OD	Optical densitometry
OFG	Orofacial granulomatosis
OmpC	Outer membrane porin protein C
OR	Odds ratio
PAB	Pancreatic autoantibodies
pANCA	Peri-nuclear anti-neutrophil antibodies
PBS	Phosphate buffered saline
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PSC	Primary sclerosing cholangitis

RLUH	Royal Liverpool University Hospital
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RR	Relative risk
S. aureus	*Staphylococcus aureus*
SCAD	Segmental colitis associated with diverticular disease
SEM	Standard error of the mean
SES-CD	Simple Endoscopic Score for Crohn's Disease
SLAM	Signalling lymphocyte activation molecule
SNP	Single nucleotide polymorphisms
TNFα	Tumour necrosis factor alpha
UC	ulcerative colitis
UHQ	Ultra-high quality

1 Introduction

1.1 Inflammatory bowel disease

1.1.1 Definition

Inflammatory bowel disease (IBD) is a general term used to describe a chronic relapsing-remitting condition typified by inflammation of the gastrointestinal tract. It comprises two main clinical phenotypes, Crohn's disease and ulcerative colitis (UC). The two conditions share some common features but are commonly differentiated by disease location - Crohn's disease can affect any part of the gastrointestinal tract; by disease behaviour - Crohn's disease commonly causes stricturing and fistulation; and by histological features - Crohn's disease causes transmural inflammation and classically leads to granuloma formation (Abraham 2009). This thesis and the introduction herein is concerned primarily with Crohn's disease.

1.2 Crohn's disease

1.2.1 History of Crohn's disease

Historical descriptions of intestinal disorders can be found both in classical literature and again in the 19th century (Combe 1813), latterly reinterpreted as perhaps the first reports of inflammatory bowel disease, but it was not until the 20th century that ulcerative colitis and Crohn's disease were formally described. In his paper "Chronic Interstitial Enteritis" Dalziel detailed nine cases of chronic

colonic and/or small bowel inflammation, now taken as one of the earliest modern descriptions of Crohn's disease, bearing a striking similarity to the "pseudo-tuberculous" chronic enteritis affecting cattle, Johne's disease (Dalziel 1913). However it was not until the 1932 publication of a paper in *JAMA* that Crohn's disease acquired its eponymous name and the different disease phenotypes, still recognisable to a modern physician, were accurately reported (Crohn 1932), although Crohn's disease affecting only the colon was not widely accepted until 1960 (Lockhart-Mummery 1960).

1.2.2 Definition

Crohn's disease is a condition characterised by a range of common clinical, radiological, histological and endoscopic features as defined by Lennard-Jones (Lennard-Jones 1989), and affirmed by the European Crohn's and Colitis Organisation (ECCO) (Van Assche 2010).

1.2.3 Clinical features

1.2.3.1 Symptoms

Symptoms may vary dependent on disease location but commonly include diarrhoea (in around 90%), rectal bleeding (around 50%) (Lennard-Jones 1997), abdominal pain (70%) and weight loss (60%) (Stange 2006). Up to 20% of patients will develop a perianal fistula at some point in their disease course and the overall lifetime risk for some form of penetrating disease is 20-40% (Nordgren 1992; Schwartz 2002).

Extra-gastrointestinal problems occur more commonly than in ulcerative colitis and are seen in around one third; typical manifestations include oral aphthous ulcers, uveitis, erythema nodosum, pyoderma gangrenosum, psoriasis, peripheral arthropathy, axial arthropathy and primary sclerosing cholangitis (PSC), the latter almost exclusively in those with colonic disease. Extra-intestinal problems are multiple in 39% of affected patients and in 26% present before the diagnosis of IBD (Vavricka 2015).

1.2.3.2 **Histopathology**

Differentiation between Crohn's disease and ulcerative colitis can be challenging and there is no single defining pathological feature of Crohn's disease. Crohn's disease is strongly favoured by biopsies showing evidence of small bowel or patchy colonic disease, non-cryptolytic non-necrotic granulomas, segmental crypt distortion and an absence of mucin depletion (Feakins 2013). In surgical specimens transmural inflammation, transmural lymphoid hyperplasia and submucosal nerve fibre hyperplasia are considered typical, whilst granulomas, fibrotic submucosal thickening and fissures are strongly supportive (Van Assche 2010).

1.2.3.3 **Endoscopic features**

Colonoscopy remains one of the mainstays of investigation in Crohn's disease, both for diagnosis and disease assessment. Two main scoring systems are described for determining disease activity via colonoscopy, the Crohn's disease Endoscopic Index of Severity (Mary 1989) and the Simple Endoscopic Score for Crohn's Disease (SES-CD) (Daperno 2004). Both objectively quantify the severity of mucosal ulceration and any strictures present and both are prospectively validated but neither are widely used in routine clinical practice (Tontini 2014). For post-operative disease assessment the Rutgeerts' score, a simple measure of the number of ulcers and severity of inflammation at an ileo-colonic anastomosis (disease relapse is defined as a score of ≥2) is widely used and prospectively validated (Rutgeerts 1990).

Small bowel disease may also be assessed and quantified using capsule endoscopy (for patients with non-stricturing disease- the capsule can be retained in narrowed segments of bowel and may lead to obstruction), where the observed mucosal changes can be classified into normal, mild or moderate-severe disease using the Lewis score, now shown to correlate with the need for treatment and hospitalisation (Dias de Castro 2015).

1.2.3.4 **Radiological findings**

A wide range of radiological modalities are now available for imaging Crohn's disease including small bowel MRI, small bowel MR enteroclysis (MRI enhanced by the distension of the small bowel with a contrast medium instilled via a naso-gastric tube), CT scanning, barium studies (either a small bowel follow through or a small

bowel enteroclysis), white cell scanning and ultrasound. Choice of modality is still determined in large part due to local availability (Hafeez 2011) and the need to limit radiation burden which can be excessive in up to 10% of patients (Chatu 2012). For diagnosis MRI has a sensitivity and specificity of >90%, while CT has high specificity (>90%) but much lower sensitivity and contrast studies perform less well on both counts (Gatta 2012). When assessing for complications of Crohn's disease CT and MRI perform comparably but CT will often be used first line due to the speed and availability of scanning. Ultrasound is reported to have sensitivity and specificity of >90% but it is highly operator dependent and availability of intestinal ultrasound is patchy in the UK (Gatta 2012).

1.3 Classification

Crohn's disease is a heterogeneous condition in which there are groups of common phenotypes. The Montreal classification (detailed in Table 1), a modification of the earlier Rome and Vienna classifications, is the currently accepted system for describing this variation in disease location and behaviour according to the observed clinical characteristics (Silverberg 2005). Classification is not however a fixed entity and may evolve over time, most commonly in relation to disease behaviour (B) (changing in 45.9% of patients by 10years) and less frequently regarding location (L) (changing in 15.9% of patients by 10 years) (Louis 2001).

Age at Diagnosis (A)	
A1	≤16 years
A2	17-40 years
A3	>40 years
Location (L)	
L1	Terminal ileum
L2	Colon
L3	Ileocolon
L4	Upper GI (May also be added to L1-3 as a disease modifier)
Behaviour (B)	
B1	Non-stricturing, non-penetrating
B2	Stricturing
B3	Penetrating
p	Perianal disease modifier which may be added to B1-3

Table 1: Montreal classification of Crohn's disease. Adapted from (Silverberg 2005)

1.4 Disease markers

1.4.1 Serological markers

1.4.1.1 Circulating antibodies

Atypical peri-nuclear anti-neutrophil antibodies (pANCA) are more strongly associated with ulcerative colitis but around 20-30% of patients with Crohn's disease are pANCA positive although its presence is strongly associated with colonic disease, with one study demonstrating that 100% of patients testing positive for pANCA had colonic involvement (50% ileocolonic, 50% isolated colonic) (Vasiliauskas 1996). In UC pANCA positivity has been associated with resistant left sided colitis, early surgery and increased disease duration but no significant association with disease behaviour has been demonstrated in Crohn's disease (Elkadri 2013).

Anti-*Saccharomyces cerevisiae* antibody (ASCA) is detectable in up to 68% of patients with Crohn's disease (Reese 2006), is more strongly associated with small bowel disease (Odds ratio (OR) 2.2) and positive IgG serology is associated with stricturing/penetrating disease (OR 3.01) (Ryan 2013). ASCA antibodies are directed against microbial cell wall mannan, an epitope also expressed by *Candida albicans* and *Mycobacterium avium* subsp paratuberculosis (MAP) (Mpofu 2007). A similar mannan is also known to be the receptor for bacterial FimH on its receptor (Uroplakin) in bladder epithelial cells and it is conceivable that a similar structure may also act as receptor for FimH on glycoprotein 2 (GP2), its receptor on the luminal surface of microfold (M) cells overlying Peyer's patches (Hase 2009; Wellens 2008).

Antibodies to *Escherichia coli* outer membrane porin protein C (anti-OmpC) are found in 37-46% of Crohn's disease patients and are associated with fistulating disease, disease progression and need for surgery (Arnott 2004; Mow 2004).

Pancreatic autoantibodies (PAB), the autoantigen for which is the major zymogen granule membrane glycoprotein 2 (GP2) (Roggenbuck 2009), are found significantly more frequently in Crohn's disease patients (around $^{1}/_{3}^{rd}$) than in unaffected family members or patients with ulcerative colitis (both around 23%) (Joossens 2004). No consistent associations with disease phenotype or behaviour have been shown, with data conflicting (Desplat-Jego 2007; Lakatos 2009), but their presence overlaps with the presence of ASCA and has been linked to a possible microbial pathogenic mechanism (Lakatos 2009). It is not yet clear if PAB positivity represents a primary or secondary phenomenon but it may arise as a consequence of bacterial mediated small bowel inflammation; GP2 is found on the apical membrane surface of small intestinal Microfold (M) cells, binds to the FimH protein component of bacterial outer membrane type 1 pili, and mediates translocation of bacteria across M-calls (Hase 2009) – it follows, but remains unproven, that PAB may arise as a consequence of bacterial induced inflammation (Pavlidis 2012)

Antibodies to *Pseudomonas fluorescens* associated sequence I2 (anti-I2) are seen in 50-54% of patients (Landers 2002), are associated with stricturing disease, disease progression and the need for surgery (Prideaux 2012).

Antibodies to the microbial cell wall carbohydrates mannobioside (AMCA), laminaribioside (ALCA) and chitobioside (ACCA), collectively referred to as anti-glycan antibodies, are positive in around 40% of patients and high titres are

associated with a more aggressive disease course and an increased likelihood or having steroid dependent disease (Paul 2015). Similarly, antibodies to the Flagellin, CBir1, are detectable in 50-55% of patients and are associated with small bowel, stenosing and penetrating disease (Targan 2005).

Studies conducted on the stored sera of military personnel who later develop Crohn's disease have demonstrated the presence of these antibodies, including ASCA, anti-flagellin antibodies and anti-OmpC, in up to two thirds of patients with a median interval of antibody formation to disease development of between 3 and 6 years (Choung 2016; Israeli 2005). Furthermore, a panel of serological markers may also identify patients from a normal population at higher risk of developing IBD (van Schaik 2013).

Whilst no single serological marker has sufficient sensitivity or specificity to be used for diagnostic purposes (Prideaux 2012), combining assays can differentiate Crohn's disease and UC with positive predictive values of 92.5-96% (Quinton 1998). Despite this apparently impressive figure, their main current utility is as a diagnostic aid in difficult cases, but an emerging role in predicting disease behaviour (Ryan 2013), particularly when combined with genetic tests, hints at a more clinically useful future use (Lichtenstein 2011; Plevy 2013).

1.4.1.2 **Biological markers**

C reactive protein (CRP) is a non-specific marker of inflammation commonly used to help differentiate inflammatory and non-inflammatory causes of gastrointestinal symptoms, as well as to monitor disease activity in patients with established inflammatory bowel disease where an elevated level correlates with endoscopic evidence of inflammation (Mosli 2015). However it is not sensitive and normal levels are seen in 10-25% of patients with active disease, most commonly in patients with isolated small bowel disease (Florin 2006; Henriksen 2008; Karoui 2007). Further, adiposity may lead to a false positive result due the role of mesenteric fat in producing CRP (Peyrin-Biroulet 2012). Despite these limitations it remains a useful tool for disease assessment and may be used to predict treatment efficacy as low levels are associated with lower response rates to biologics (Jurgens 2011).

Calprotectin is a neutrophil protein released from inflamed tissue and detectable in the faeces of patients. It is supported by the National Institute for Health and Care Excellence (NICE 2013) to be used as a screening test to differentiate irritable bowel syndrome (IBS) from inflammatory bowel disease where it has a pooled sensitivity of 93% and specificity of 96% (van Rheenen 2010). It is a well established test for colonic and ileocolonic disease. Early studies raised questions about its reliability in isolated ileal disease (Sipponen 2008) but more recent studies have shown good correlation between calprotectin levels and small bowel inflammation as determined by MRI or surgical resection (Cerrillo 2015) although sensitivity is lower than in colonic disease (Stawczyk-Eder 2015). Calprotectin can also be used to predict the risk of relapse in patients with well controlled disease. In the STORI

trial, elevated calprotectin (>300µg/g) predicted relapse following withdrawal of infliximab, a finding confirmed in a recent meta-analysis (Louis 2012; Mao 2012) and, in a prospective trial, a level of <240µg/g in quiescent patients had a 96.8% negative predictive value for the risk of relapse (Naismith 2014). In post-surgical patients levels of <100µg/g have been shown to have a 91% negative predictive value for endoscopically detectable disease recurrence at 6 months (Wright 2015). Finally, much interest surrounds its use a as a surrogate for endoscopic assessment of mucosal healing, with one study showing a 94.1% sensitivity to do so with levels of <250µg/g (D'Haens 2012) and another reporting a 79% sensitivity with levels of <121µg/g (Guidi 2014) but a more recent study has reported that any level above 50µg/g is predictive of endoscopic disease activity (Falvey 2015). The use of calprotectin in this area requires further studies. The neutrophil protein lactoferrin has comparable diagnostic utility and performance but is less stable at room temperature and consequently its use has not translated into widespread clinical use (Mosli 2015).

1.4.1.3 **Serological overlap between colonic Crohn's disease and ulcerative colitis**

Colonic Crohn's disease (Montreal L2) is currently considered a separate entity to ulcerative colitis, but recent data suggests there is significant overlap, raising the possibility they may be spectrums of the same disease process. Both are more often negative for ASCA , commonly positive for pANCA and additionally both are negative for the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene association and strongly associated with the HLA DRB1*0103 haplotype (Hugot 2001; Reese 2006; Silverberg 2003). A recent very large international multicentre genotype-phenotype association study has shown that isolated colonic Crohn's disease is genetically separate from Crohn's disease with ileal involvement, lying approximately midway between the latter and ulcerative colitis (Cleynen 2016).

1.5 Epidemiology

1.5.1 Incidence and Prevalence

In the UK, the incidence of Crohn's disease has risen progressively over the last 75 years and varies between 7 and 9.56 per 100,000 population per year, with a prevalence of around 150 per 100,000 (Gunesh 2008; Rubin 2000; Steed 2010). The UK has some of the highest rates recorded and it should be noted that significant world wide variation in incidence and prevalence is seen (Burisch 2013). Within this variation two notable patterns have emerged; a north-south gradient, with the highest rates seen in northern latitudes, and an east-west European gradient, with populations of western European countries demonstrating a far higher disease burden than those in East despite similar population characteristics (Burisch 2014a; Shivananda 1996). It is not however, contrary to the historical view, a condition of Europe and North America, as there has been a clear emergence of the disease in Asia-Pacific where studies over the last two decades have demonstrated a rapidly rising incidence (Ouyang 2005; Yang 2008). This rise in Asia may in part be due to increasing westernisation, a view that is given further credence by the observation that patients who emigrate from low to high prevalence countries have an increased rate of IBD relative to controls (Ahuja 2010; Barreiro-de Acosta 2011).

1.5.2 Age

Crohn's disease has a bimodal pattern of incident age with the majority of patients presenting between the ages of 20 and 30years (with an average age of onset 5-10 years earlier than ulcerative colitis) but up to 15% occurring after 65 years (Ananthakrishnan 2009; Cosnes 2011; Duricova 2014). The latter tend more commonly to have colonic disease and it is possible that some of these represent cases of segmental colitis associated with diverticular disease (SCAD) that has a similar histology (Gisbert 2014). Some uncertainty exists over the proportion of patients presenting in childhood, largely due to variations in age cut offs for paediatric services, but it is thought between 7 and 25% present in childhood or adolescence (Cosnes 2011; Henderson 2012).

1.5.3 Sex

For adult Crohn's disease in North America and Northern and Western Europe there is a slight predominance in females, whereas the reverse holds true in Asia, and females more commonly develop extraintestinal manifestations of their disease (Molodecky 2012; Vavricka 2015). In the UK females are affected 1.3-1.6 more frequently than males (Gunesh 2008; Rubin 2000). In patients with a family history of Crohn's disease this female preponderance hold true (ratio 1.5:1) and additionally the index case is more commonly the mother, suggesting there may be sex-specific mechanisms affecting the risk of progeny developing Crohn's disease (Zelinkova 2012). The picture in paediatric disease is different however where a

clear male predominance is seen, a pattern which persists until after adolescence (Sagiv-Friedgut 2010).

1.5.4 Ethnicity

In addition to the world wide variations in incidence and prevalence, certain ethnic groups within national populations are at elevated risk. Jewish people have an increased risk of Crohn's disease relative to non-Jewish white people (relative risk 5.4-7.7) (Mayberry 1986), a risk that is higher in Ashkenazi Jews relative to Sephardic Jews (Roth 1989) and the life time risk of a first degree relative of a Jewish patient with Crohn's disease also developing the disease is 7.8%, compared to 5.2% in non-Jewish families (Yang 1993). The observed risk in Jewish patients, at least in part, has a genetic basis (Kenny 2012), but other migrant populations are also at increased risk relative to Caucasian populations, perhaps due to environmental factors - patients with Indian sub-continent ancestry living in the UK and the USA both have an elevated incidence of Crohn's disease (Bhopal 2014; Malhotra 2015). Conversely a nationwide study of disease prevalence in the USA has clearly shown Crohn's disease to be less common in non-white sub-populations (Nguyen 2014) and further, contrary to previous studies suggesting more severe disease in African-American patients, disease behaviour appears similar to white American patients (Mahid 2008).

1.5.5 Health/social class

Given the higher rates of disease in the western world, it might be expected that higher economic standing may also increase risk. In a retrospective study of paediatric Crohn's disease in Scotland an association was seen with higher social class (Armitage 2004)but in a prospective French study this was not shown to be the case, nor was deprivation associated with poorer disease outcomes, perhaps due to the high quality French healthcare system (Nahon 2009).

1.6 Disease risk factors

1.6.1 Genetics

Direct evidence for a genetic component to Crohn's disease pathogenesis came in the 1990s with the genome wide search of groups of disease affected families and subsequent identification of a disease susceptibility locus on chromosome 16 (Hugot 1996). In a seminal paper this was then shown to be due to two missense variants and a frameshift variant in the *NOD2*/*CARD15* (nucleotide- binding oligomerization domain-containing 2/ caspase recruitment domain-containing protein 15) gene (Hugot 2001) expressed in paneth cells, macrophages and epithelial cells and associated with ileal Crohn's disease (Ahmad 2002).

Genome wide association studies (GWAS) have now identified 206 distinct single nucleotide polymorphisms (SNP) associated with IBD disease susceptibility, mostly shared across diverse ethnic groups although with some geographical variation (Ellinghaus 2016; Liu 2015). Where present, genetic risk is shared with other immune mediated diseases including psoriasis, ankylosing spondylitis, type 1

diabetes mellitus and leprosy (Barrett 2008; Franke 2010; Jostins 2012; Zhang 2009), and recent publications have suggested the observed genetic overlap may in fact explain some of the variation in disease phenotype seen in IBD. Specifically it has been suggested that patients with primary sclerosing cholangitis (PSC) or extraintestinal manifestations of IBD may represent a sub-population genetically distinct from unaffected IBD patients and that these complications may arise as a consequence of polymorphisms shared with other auto-immune conditions (Ellinghaus 2016).

Arguably, the most significant impact of GWAS studies has been to implicate specific immune and cellular pathways leading to a greater understanding of underlying disease mechanisms. For Crohn's disease these can be grouped broadly into two key categories: innate immunity (autophagy, microbial recognition – *NOD2*, *ATG16L1*, *IRGM*) and acquired immune response regulation (lL23 signalling and Th17 cells – *IL23R*) (Jostins 2012). Significantly there is overlap with infectious diseases, specifically a shared risk with *Mycobacterial* disease (*NOD2*, *IL23R*, *TNTSF15*) (Jostins 2012; Zhang 2009). Mucosal barrier function, important in UC, does not appear so relevant for Crohn's disease (Lees 2011).

Individually the majority of identified risk loci have small odds ratios (Jostins 2012) but composite models demonstrate a cumulative effect with an increased genetic risk of disease for both affected patients and their first degree relatives in comparison to healthy controls (Kevans 2016). However there is marked overlap – many patients have a genetic risk comparable to the normal population and 14% of healthy controls without any family history of disease have a significant genetic risk

for Crohn's disease. These findings, comparable to an earlier study in which 70 of the then known 71 Crohn's disease specific risk alleles were typed, suggest that disease development is due to genetic risk in only a minority of patients (Hedin 2014).

Findings from family and twin studies also suggest that genetics only play a partial role in disease pathogenesis. Only 6-12% of Crohn's disease patients have a family history of the disease (Moller 2015), <1% of the offspring of affected patients develop IBD (Orholm 1999), and, in the latest analysis, the Swedish twin registry has shown concordance in monozygotic twins to be as low as 27% (Halfvarson 2011).

Indeed only 13.6% of disease variance is explained by the currently known disease associated polymorphisms and it is possible environmental factors alone might be sufficient to trigger disease, perhaps by inducing alterations in gene expression or innate immune function (Jostins 2012). Epigenetic factors, such as alterations in DNA methylation, histone proteins and chromatin, are one such possible mechanism through which environmental factors may exert functional changes which ultimately lead to disease development (Ventham 2013). Intriguingly DNA hyper- and hypo-methylation may be observed in both peripheral immune cells and intestinal mucosa, and this has been shown to alter mRNA production suggesting a functional role (McDermott 2016).

1.6.2 Environmental factors

1.6.2.1 **Diet**

Epidemiological and interventional studies as well as animal models and *in vitro* assays all provide evidence for a dietary influence in the development of Crohn's disease. These effects may begin at an early age. A systematic review of the impact of breastfeeding demonstrates a reduced risk of IBD seen in breast fed infants (OR 0.69) (Barclay 2009). Effects may also be seen later in life and it has been hypothesized that the observed northern and western gradients in IBD prevalence, and the emergence of IBD in traditionally low prevalent counties, relate in part to the adoption of a westernised diet. Epidemiological studies of migrant populations support this assertion, which is given further credence by the observed associated between disease incidence and components of a western diet, specifically emulsifiers and refined sugars (Lee 2015; Richman 2013; Roberts 2013). It therefore follows that dietary manipulation should influence disease severity. This hypothesis holds true both in animal models (where high fat and iron worsen disease but certain amino acids, vitamin D and several components of plant based feeds temper the inflammatory response) and in human trials where exclusive enteral nutrition induces remission in patients with active Crohn's disease (Lee 2015; Zachos 2007).

1.6.2.2 **Smoking**

Both current (OR 1.65-2.47) and former smokers (OR 1.33-2.51) have an elevated risk of Crohn's disease, the risk in the latter persisting for 5-10 years after smoking cessation (Calkins 1989; Tobin 1987). Early studies reported rates of smoking amongst patients with Crohn's disease to be as high as 50% but this is falling (perhaps due to the effects of taxation and public health policy) and more recent studies report rates of 19.2-33.2% (Lindberg 1988; Lunney 2015; Severs 2016). In addition to their elevated disease risk, smokers also run a more severe disease course with higher rates of relapse, a greater need for first time and re-do surgery, higher rates of penetrating disease and a greater prevalence of extra-intestinal disease manifestations (Severs 2016; To 2016). Light smokers have a risk equivalent to heavy smokers suggesting this is an all or nothing phenomenon, rather than a dose-dependent effect (Seksik 2009). However, smoking cessation reverses risk and patients who stop have disease flare rates comparable to non-smokers and reduced need for escalation of medical therapy (Cosnes 2001; Johnson 2005).

1.6.2.3 **Oral Contraception**

Following on from earlier smaller studies a large meta-analysis of 14 trials, containing in excess of 75,000 women, demonstrated a 1.46 pooled relative risk (95% confidence interval 1.26-1.70) for developing Crohn's disease in current users of the oral contraceptive pill (OCP). Risk increases with time exposed to the OCP (Cornish 2008) .

1.6.2.4 **Vitamin D**

Much recent interest has focussed on the role of vitamin D, in part due the high rates of disease in northern latitudes. Low vitamin D levels are common, are associated with active disease, an increased risk of surgery and hospitalisation, and low predicted levels are associated with an increased risk of developing disease (Ananthakrishnan 2013a; Ananthakrishnan 2012; Jorgensen 2013; Suibhne 2012). A therapeutic trial of vitamin D supplementation to prevent relapse showed a trend toward benefit but just failed to reach statistical significance (Jorgensen 2010).

1.6.2.5 **Westernisation and diet**

As discussed earlier, epidemiological studies inform us of a western predominance both in terms of Crohn's incidence and prevalence. The ECCO population-based inception cohort study, ECCO-EpiCom, report a two-fold increase in Crohn's disease incidence in western Europe relative to the east (Burisch 2014a). This difference could not be accounted for by differences in smoking, contraception use or family history and, paradoxically, other putative environmental risk factors appeared more prevalent in eastern Europe (high sugar intake, low fibre intake, low fruit and vegetable consumption, low rates of childhood infections) although the study was limited by problems with accurate dietary information (Burisch 2014b). Data from Asia-Pacific countries have more strongly suggested an effect from westernised lifestyles with a western diet before the age of 20 and a sedentary lifestyle both associated with an increased risk of Crohn's disease in Asian patients (Ng 2015). Supporting the Asia-Pacific findings are the results from the USA Nurses' Health

Study in which a dietary intake of fruit fibre in the highest quintile led to a 40% reduction in the risk of developing Crohn's disease (Ananthakrishnan 2013b).

1.6.2.6 **Hygiene**

Many studies have identified increased rates of immune mediated diseases in countries with high per-capita incomes, a phenomenon correlated with the falling incidence of infectious diseases seen in resource rich locations. This has led to the hygiene theory of disease pathogenesis in which it is proposed that naturally occurring exposure to microbes in early life leads to alterations in T helper cell activity, perhaps via changes in regulatory T cell function and circulating cytokines, and thereby protects against immune related disease (Bach 2002). Population based studies have given credence to this theory with a striking increase in the risk of Crohn's disease following childhood antibiotic use, a risk which increases with each subsequent course and is highest in the 3 months following treatment. Data however have been conflicting with some studies reporting a protective effect from infection, others failing to show a clear correlation between hygiene and the risk of Crohn's disease and studies from Asia and Italy failing to demonstrate any correlation with childhood antibiotic use and the risk of Crohn's disease (Castiglione 2012; Chu 2013; Ng 2015).

1.6.2.7 **Occupation**

In a retrospective study of the U.S. national occupational mortality database, patients with an established diagnosis of IBD were shown to have higher mortality rates in white collar industries relative to manual workers, when assessed using a proportional mortality ratio model (in which observed deaths are compared to expected standardised mortality rates). These findings have been consistent across three studies by the same authors covering a 22 year span, and have suggested a role for occupational environmental factors in disease associated mortality (Sonnenberg 2012). However the effect of occupation on the need for hospitalisation with Crohn's disease was less consistent and of a lower magnitude when studied using Swedish national databases, suggesting the effect of occupation is weak at most (Li 2009).

1.6.2.8 **Appendicectomy**

The apparent protective effect of appendicectomy in ulcerative colitis and some studies in Crohn's disease have raised the possibility that appendicectomy may be a risk factor for Crohn's disease. However a Scandinavian cohort study of more than 700,000 patients has failed to show a major effect, with only a modest increase in Crohn's disease seen post surgery, a finding most pronounced in the first six months post-appendicectomy. Crohn's disease is well recognised to mimic the signs and symptoms of appendicitis and the authors conclude that any association is probably an artefact brought about by diagnosis bias (Kaplan 2007).

1.6.2.9 **Psychological stress**

In a large meta-analysis looking at the effect of work-related stress on the risk of developing IBD, no association was seen for either UC or Crohn's disease (Heikkila 2014). However prospective studies have shown stress to be associated with the risk of Crohn's disease flares, probably via a number of different mechanisms including effects on the hypothalamus, gut microbiota and mast cells (Mardini 2004; Mawdsley 2005).

1.6.2.10 **Environmental factors precipitating disease flares**

GI infection may precipitate relapse in around 10% of patients with *Clostridium difficile* the cause in around half of these cases (Mylonaki 2004). Seropositivity for cytomegalovirus (CMV) occurs commonly in patients with Crohn's disease but its relevance and role in disease is much debated – a recent study of 249 patients did not find an association with disease objective markers of disease activity and concluded that CMV is likely to be a bystander (do Carmo 2014).

Use of non-steroidal anti-inflammatory drugs (NSAIDs) is commonly avoided due to a perceived risk of inducing disease flare although evidence for this is weak and a systematic review demonstrated only a small effect. The same study suggested paracetamol, commonly used as an alternative, may also carry some risk (Forrest 2004).

1.7 Disease associations

1.7.1 Orofacial granulomatosis

Orofacial granulomatosis (OFG) is an umbrella term to describe a chronic granulomatous condition affecting the oral cavity and/or the face and encompasses a range of conditions, namely; isolated oral Crohn's disease, oral sarcoidosis, cheilitis granulomatosa and Melkersson-Rosenthal syndrome (Challacombe 1997). It's presence in childhood has a stronger association with intestinal Crohn's disease (40% of patients) whereas in adult onset disease only a quarter of patients are similarly affected (Lazzerini 2014). In around half of patients a diagnosis of OFG pre-dates the later development of Crohn's disease (Campbell 2011; Gale 2016).

1.7.2 Rheumatological disease

Around one third of patients with Crohn's disease develop rheumatological symptoms falling into one of three main groups; type 1 peripheral arthropathy in which <5 joints are affected and which classically mirrors intestinal disease activity, type 2 peripheral arthropathy in which >5 joints are affected and which is generally independent of IBD activity, and axial disease which may manifest as inflammatory back pain, sacroiliitis or ankylosing spondylitis (AS) (Ott 2013).

HLA-B27 seropositivity in Crohn's disease is slightly more common than the general population and is strongly associated with the development of sacroiliitis although interestingly appears to be less important in disease associated ankylosing spondylitis than in idiopathic AS (Orchard 2009). Of patients with idiopathic AS less

than 10% will develop Crohn's disease although nearly three quarters have subclinical intestinal inflammation (Reveille 2012).

There may be shared pathological mechanisms and indeed Crohn's disease shares genetic factors with psoriatic arthropathy and ankylosing spondylitis, including polymorphisms in IL-12, IL-23R and the NFκB pathway (Reveille 2012). Moreover similar patterns of T cell upregulation and cytokine production may be observed in Crohn's disease and the spondyloarthropathies leading to the hypothesis that the rheumatological disease arises as a consequence of primed immune cells circulating to the joint space from the gut (Fantini 2009).

1.7.3 Chronic granulomatous disease and other rare single gene disorders

Chronic granulomatous disease is a rare disorder arising from genetic mutations in the NADPH oxidase complex and is x-linked in 65% of cases. This leads to an immunodeficiency state as a consequence of impaired neutrophil function which then results in recurrent infection. Many will develop GI symptoms and nearly half develop a Crohn's disease like illness (Marks 2009).

Very early onset IBD, infantile IBD and neonatal IBD (i.e. those variants of IBD presenting under the age of 6) are commonly monogenic and frequently due to defects in immune function including impaired phagocyte function, T-cell defects, B-cell defects and failures of immune regulation. These IBD variants are typified by a predominance of colonic disease, higher rates of complex perianal disease and relative resistance to conventional treatments (Uhlig 2014).

1.8 Natural history

1.8.1 Effect of age on disease progression

The age of presentation is an important factor in disease behaviour. Paediatric Crohn's disease more commonly affects the colon and the proximal GI tract (perhaps a consequence of higher pick up rates due to routine use of upper GI endoscopy in these patients) and tends to run a more aggressive course (Duricova 2014). In contrast, elderly patients (over 65 years) more commonly have isolated colonic disease, are less likely to extend their disease to other locations after diagnosis, less frequently suffer extraintestinal manifestations of their disease and are less likely to have complicated disease (Charpentier 2014). Despite this it should be noted that elderly patients requiring inpatient care for their disease have higher admission-related adjusted mortality rates despite lower rates of surgery, fistulating disease, perianal disease and obstruction (Ananthakrishnan 2009).

1.8.2 The role of genetics in predicting disease behaviour and treatment response

NOD2 variants have long been associated with a greater risk of complicated disease and more recent models now suggest risk may be cumulative with additional gene variants conferring a progressively higher risk of complications (Gerich 2014). More complex models using a combination of genetics, immune cell gene expression and circulating serological markers show some early promise but have yet to be prospectively validated in a real world population.

In an emerging field it seems likely genetic factors will have an increasing role in predicting the risk of adverse reactions to treatments for IBD. Patients with the HLA-DQA1*02:01–HLA-DRB1*07:01 haplotype have a significantly elevated risk of thiopurine induced pancreatitis (Heap 2014) and HLA-DRB1*03:01 is associated with the risk of 5-aminosalicylate induced nephropathy (Heap 2016).

Predicting treatment response is less certain but inconsistent data suggests a weaker response to tumour necrosis factor α (TNFα) antagonists in patients with a *NOD2* variant and an enhanced response in patients with variants of the Fas ligand (Vermeire 2010b). Glucocorticoid resistance is associated with genetic variation in steroid receptors (NR3C1 gene), altered intracellular drug concentration (MDR1 gene) and possibly genetically programmed over-production of pro-inflammatory cytokines (Gabryel 2016).

1.8.3 Natural history of disease phenotypes

Disease behaviour changes over time in the majority of patients although disease location generally remains fixed (Cleynen 2016; Cosnes 2002; Louis 2001). Overall penetrating complications are commoner than stricturing, particularly in younger patients and those with perianal disease, with the latter most common in patients with small bowel disease and an absence of perianal involvement (Cosnes 2002). At diagnosis 70-80% of patients have non-stricturing non-penetrating disease, with a 22% risk of progression to either stricturing or penetrating disease by 1 year, 33-43% by 5 years and 50% by 20 years (Cleynen 2016; Thia 2010).

1.8.4 Effect of treatment on disease progression

Longitudinal studies, as might be expected, have shown increasing use of immunosuppressants and biological drugs over time, and also show an overall reduction in the need for surgery at 1 year, 5 years and 9 years after diagnosis, with a meta-analysis showing thiopurines to be associated with a lower risk of surgery, although this apparent treatment effect has not been consistent across all studies (Chatu 2014; Ramadas 2010; Rungoe 2014).

It is conceivable this finding arises as a consequence of poorly defined treatment endpoints, and separate data shows patients who achieve mucosal healing (achievable with immunosuppressants and biologics) have reduced need for surgery, hospitalisation and steroids (Neurath 2012). This, and lessons learnt from rheumatoid arthritis, has led to the concept of "treat to target" in which medical therapy is optimising to achieve hard end points (mucosal healing, normalisation of biochemical tests) with the aim of controlling active disease and preventing long term complications (Bouguen 2015). Whilst logical this approach lacks evidence from prospective trials and efficacy remains unproven.

1.8.5 Prognostic factors

Factors associated with an increased risk of long term disease complications are: peri-anal disease, steroid use at diagnosis, age under 40 at diagnosis, upper GI involvement, smoking, extensive small bowel disease, ileal involvement and stricturing or penetrating disease (Thia 2010).

1.9 Treatment

1.9.1 5-aminosalicylates

5-aminosalicylates (5ASAs) target the epithelial cell and are efficacious in UC but Cochrane reviews showed no benefit for the induction or maintenance of remission in Crohn's disease (Akobeng 2005; Lim 2010) with a subsequent meta-analysis finding similar results (Ford 2011b) although there may be some benefit in preventing post-operative relapse (Ford 2011c).

1.9.2 Steroids

Well established in treating active Crohn's disease, corticosteroids inhibit T-cell activation and cytokine production, are highly efficacious, induce remission in 60% and are recommended in many international guidelines (Summers 1979). Budesonide is effective in inducing remission in active Crohn's disease and although less efficacious than prednisolone or hydrocortisone does have a more favourable side effect profile (Rezaie 2015). Despite their efficacy steroids have short term effects only, relapse is common on stopping treatment and they are ineffective for maintenance of remission (Ford 2011a).

1.9.3 Immunosuppressants

Thiopurines block Rac1 activation which leads to apoptosis of T-cells and thereby imparts their well documented immunosuppressive effects (Tiede 2003). Efficacy in maintaining remission of Crohn's disease was confirmed in a Cochrane review

(Prefontaine 2009), however in a further systematic review were not shown to be superior to placebo in inducing remission (Chande 2013). In their 2002 paper Dubinsky *et al* investigated why nearly half of patients with active disease and one third of patients with quiescent disease fail to induce/maintain remission with thiopurines. They found response to be associated with achieving a therapeutic 6-thioguanine (6-TGN) concentration (>235pmol/8x10^8 red cells), that only 28% of non-responders gain benefit from dose escalation, and in the remainder that dose increase does not raise 6-TGN levels (Dubinsky 2002). Co-prescription of allopurinol with thiopurines shifts drug metabolism, has been shown to increase 6-TGN levels, and 66% of patients with previous thiopurine failure respond to treatment (Smith 2012). Routine early use of azathioprine does not lead to better outcomes than conventional use in terms of: time spent in remission, need for biologic treatment or the need for surgery (Cosnes 2013).

1.9.4 Anti TNFs

TNFα antagonists bind both soluble and transmembrane bound TNF and leading to 2 main modes of action; i) induction of M2 wound healing macrophages and ii) induction of T-cell apoptosis and downregulation of CD40 dependent immune response (Danese 2006; Levin 2016). T-cell apoptosis is dependent on the concurrent presence of CD14+ macrophages expressing membrane bound TNF and only occurs with infliximab, adalimumab and certolizumab (drugs with proven clinical efficacy) but not with etanercept (previously shown only to be equivalent to placebo in treating active Crohn's disease) (Atreya 2011).

Infliximab and adalimumab remain the main two anti-TNFα drugs in clinical use in the UK, have proven efficacy in inducing and maintaining remission, can lead to mucosal healing and reduce complications of Crohn's disease including hospitalisation and surgery (Terdiman 2013). Combination with thiopurines is superior to monotherapy in achieving clinical response, steroid free remission and mucosal healing (Colombel 2010). Standard dosing is weight based and much interest surrounds the use of trough drug levels to guide treatment, but clinical utility remains uncertain with a recent prospective trial of dose adjustment based on trough levels failing to show any superiority in achieving disease remission (Vande Casteele 2015).

1.9.5 Newer biologic drugs

Vedolizumab, a monoclonal antibody which blocks alpha4beta7 interaction with MAdCAM-1 on endothelial cells and thereby prevents leucocyte trafficking to the gut, is superior to placebo in inducing clinical remission (defined by Crohn's disease activity index (CDAI) ≤150) in a mixed population of patients with active Crohn's disease (the Gemini II study, which included a proportion of anti-TNF refractory patients) at 6 weeks (14.5% vs 6.8%), maintained through to week 52 in 36-39% (Sandborn 2013). In a group of patients all of whom were refractory to anti-TNF treatment (Gemini III) efficacy was again seen, although the onset of action was slower with superiority over placebo in achieving clinical remission seen only at 10 weeks (but not at 6 weeks) (Sands 2014).

Ustekinumab, a monoclonal antibody directed against the common p40 subunit of IL12 and Il23, achieved clinical response in patients with anti-TNF treatment failure in a phase 2b trial and a recent follow on phase 3 trial has demonstrated efficacy relative to placebo with response rates of around 33% and remission in 15-20% (Sandborn 2016; Sandborn 2012).

1.9.6 Diet and nutritional therapies

Exclusive enteral nutrition is effective in inducing remission in Crohn's disease and on a per-protocol basis achieves response rates equivalent to steroids (Zachos 2007). Tolerance remains a significant issue but even when only used to provide 50% of calorie intake it is effective in maintaining remission (Akobeng 2007).

Manipulation of a normal diet including exclusion diets (milk, dairy products, wheat, foods high in insoluble fibre), diets low in sugar and diets low in fats have not consistently been shown to confer clinical benefit (Richman 2013). In complex Crohn's disease exclusive total parenteral nutrition may achieve disease control (Ostro 1985), possibly analogous to the benefit seen in distal disease following defunctioning surgery (Burman 1971).

1.9.7 Antibiotics

Antibiotics are quite commonly used to treat Crohn's disease, and in light of the observed changes in gut microbiota might be expected to have therapeutic benefit. Multiple clinical trials have assessed this issue (summarised in Table 2-5) but overall there is little evidence to support their use (Khan 2011) except for the following; nitroimidazoles are effective at preventing post-operative disease recurrence (Rutgeerts 1995), ciprofloxacin and metronidazole benefit perianal disease (Khan 2011) and may benefit colonic but not ileal disease (Perencevich 2006). The choice of antibiotic used in clinical trials has historically been largely empiric but *in vitro* studies show efficiency of targeted therapy against intra-macrophage bacteria and further trials are now ongoing (Subramanian 2008).

Treatment	Control	Treatment duration	Blinding	N=	Endpoint	Treatment response	Control response	Significance	Reference
Metronidazole	Placebo	2 months	Double	22	Point score	Equal to placebo	Equal to treatment	NS	(Blichfeldt 1978)
Metronidazole	Placebo	16 weeks	Double	105	CDAI <150	36%	25%	NS	(Sutherland 1991)
Ciprofloxacin	Pentasa	6 weeks	Nil	40	CDAI <150	56%	55%	NS	(Colombel 1999)
Ciprofloxacin	Placebo	6 months	Nil	47	Mean CDAI	Mean CDAI 111	Mean CDAI 205	P<0.001	(Arnold 2002)
Ciprofloxacin + Metronidazole	Nil	10 weeks	Nil	72	HBI≤3	68%	n/a	n/a	(Greenbloom 1998)
Metronidazole + Ciprofloxacin + Budesonide	Budesonide	8 weeks	Double	134	CDAI <150	33%	38%	NS	(Steinhart 2002)
Metronidazole + Ciprofloxacin	Nil	4 weeks	Nil	7	Improvement	n/a	n/a	n/a	(Ishikawa 2003)
Metronidazole OR Cotrimoxazole OR Metronidazole + Cotrimox	Placebo	2 weeks	Nil	72	Clinical Improvement	67% 17% 71%	35%	NS	(Ambrose 1985)
Clarithromycin	Nil	4-12 weeks	Nil	25	HBI≤3	4 week 48% 12 week 44%	n/a	n/a	(Leiper 2000)
Clarithromycin	Nil	4-24 weeks	Nil	14	CDAI <150	4 weeks 36% 24 week 29%	n/a	n/a	(Inoue 2007)
Clarithromycin	Placebo	3months	Nil	41	CDAI <150	26%	27%	NS	(Leiper 2008)
Rifaxamin bd OR Rifaxamin od	Placebo	12 weeks	Double	83	CDAI <150	52% 32%	33%	NS	(Prantera 2006)
Rifaximin 400mg bd OR Rifaximin 800mg bd OR Rifaximin 1200mg bd	Placebo	12 weeks	Double	402	CDAI <150	54% 62% 47%	43%	800mg dose only, p<0.005	(Prantera 2012)

Table 2: Trials of antibiotics in active Crohn's disease

Treatment	Control	Treatment duration	Blinding	N=	Endpoint	Treatment response	Control response	Significance	Reference
Clarithromycin + Rifabutin + Clofazimine	Placebo	2 years or until relapse	Double	213	Relapse	12 months 39% 24 months 26% 36 months 59%	52% 43% 50%	NS NS NS	(Selby 2007)
Rifabutin + Clarithromycin +/- Ciprofloxacin, Ofloxacin or Clofazimine	Nil	Mean of 18 months	Nil	46	HBI <5	93.5%	n/a	n/a	(Gui 1997)
Rifampicin + Ethambutol + Dapsone + Clofazimine	Placebo	9 months	Double	40	Treatment failure	13% relapse	61% relapse	P<0.05	(Prantera 1994)
Ethambutol + Clarithromycin	Placebo	3 months	Double	31	Persistent active disease	33% active	44% active	NS	(Goodgame 2001)
Rifampicin + Isoniazid + Ethambutol	Placebo	2 years	Double	130	Composite effects	No difference	No difference	NS	(Swift 1994)
Rifampicin + Isoniazid + Ethambutol	Placebo	5 years follow up	Double	130	Relapse	39%	43%	NS	(Thomas 1998)
Ethambutol + Rifampicin	Placebo	1 year	Double	27	Mean CDAI	183	156	NS	(Shaffer 1984)
Rifabutin + Clarithromycin	Nil	4-17 months	Nil	36	Fall in CDAI	58.3%	n/a	n/a	(Shafran 2002)
Clarithromycin	Nil	3 months	Nil	15	Remission	71%	n/a	n/a	(Graham 1995)
Clofazimine + Corticosteroids	Placebo	12 months	Nil	49	Remission	64%	50%	NS	(Afdhal 1991)

Table 3: Trials of antibiotics targeted against MAP in Crohn's disease

Treatment	Control	Treatment duration	Blinding	N=	Endpoint	Treatment response	Control response	Significance	Reference
Ciprofloxacin + Metronidazole + Azathioprine	Nil	8 weeks	Nil	52	Response	35%	n/a	n/a	(Dejaco 2003)
Ciprofloxacin + Infliximab	Placebo + Infliximab	12 weeks	Double	24	50% reduced fistulae	73%	39%	NS	(West 2004)
Ciprofloxacin OR Metronidazole	Placebo	10 weeks	Nil	25	Remission	30% 0%	12.5%	NS	(Thia 2009)
Metronidazole ointment	Placebo	4 weeks	Double	74	Fall in PCDAI	Equal	Equal	NS	(Maeda 2010)

Table 4: Trials of antibiotics in perianal Crohn's disease

Treatment	Control	Treatment duration	Blinding	N=	Endpoint	Treatment response	Control response	Significance	Reference
Metronidazole	Placebo	3 months	Double	60	Recurrence	52%	75%	NS	(Rutgeerts 1995)
Ornidazole	Placebo	1 year	Double	81	Recurrence	7.9%	37.5%	P<0.005	(Rutgeerts 2005)
Metronidazole + Azathioprine	Metronidazole	3 months	Nil	81	Recurrence	55%	78%	P<0.05	(D'Haens 2008)

Table 5: Trials of antibiotics as post-surgery prophylaxis

1.9.8 Surgery and the risk of disease recurrence

Generally indicated for stricturing or penetrating disease unresponsive to medical therapy, surgery is commonly required but is not curative and endoscopic recurrence, quantifiable using the Rutgeerts' score, occurs in 85% by 3 years (Rutgeerts 1990). Smoking, penetrating disease behaviour, ileocolonic disease location, short interval between diagnosis and first surgery and young age are all associated with an increased risk of recurrence after surgery (Lautenbach 1998). The risk of recurrent disease is reduced by the use of immunomodulators in high risk patients and by performing routine colonoscopy at 6 months followed by treatment escalation in patients with early disease recurrence (De Cruz 2015).

2 Innate Immune system in Crohn's disease and its interaction with the microbiome

2.1 Implications of gene association studies for innate immune function

Arguably, the biggest contribution of GWAS studies to the understanding of Crohn's disease pathogenesis has not been to address issues of hereditability but to identify previously unknown or little considered biological pathways with relevance for disease pathogenesis. Of particular interest has been the light shone on the innate immune system including autophagy, the adaptive immune system and barrier function.

2.1.1 Autophagy and macrophage function

Autophagy, the process whereby a double membraned autophagosome is formed in the cytoplasm and subsequently fuses with a lysosome to degrade cytosolic components, is a key mechanism in cellular homeostasis both in health and disease and represents an important pathway for clearing intracellular bacteria. Variants in autophagy-related 16-like 1 (*ATG16L1*) and immunity-related GTPase M (*IRGM*) are associated with increased risk for Crohn's disease.

The strongest genetic association with Crohn's disease is with polymorphisms and deletions in *NOD2*/*CARD15*, conferring a relative risk for developing Crohn's disease of 2.13-3.03 (Liu 2015). The NOD2 protein acts as an intracellular receptor for muramyl dipeptide (MDP), a component of Gram-negative and Gram-positive

bacterial cell wall peptidoglycan, leading to production of Paneth cell defensins, which have a potent antimicrobial effect (Gaya 2006; Wehkamp 2005). MDP-engaged NOD2 also plays an important role in autophagosome generation, recruiting ATG16L1 to the cell-surface membrane where bacterial engulfment is occurs. Crohn's related mutations in *NOD2* have complex effects including impaired autophagy (via a failure to recruit ATG16L1) and bacterial killing by macrophages, reduced mononuclear cell IL-8 response to bacteria and reduced defensin production by Paneth cells (Wehkamp 2005). Loss of *ATG16L1* and *IGRM* function has also been shown to alter Paneth cell granule morphology (Cadwell 2008), to increase interleukin 1β production and allow enhanced intracellular bacterial survival (Lees 2009).

Patients who are homozygous for *NOD2* polymorphisms and patients who are homozygous for *ATG16L1* polymorphisms have macrophages that are deficient at killing phagocytosed bacteria and also have increased levels of circulating bacterial DNA, implying increased bacterial translocation (Gutierrez 2014).

2.1.2 Evidence for failure of adequate bacterial clearance in Crohn's disease

It is worth noting that as many as one third of patients with Crohn's disease in remission, most of whom have no *NOD2* or *ATG16L1* abnormality, have evidence of circulating bacterial DNA, particularly Gram-negative organisms, and that this correlates with substantially increased risk for subsequent relapse (Gutierrez 2014). This is in keeping with fact that macrophages are at best rather ineffective at killing bacteria in comparison with neutrophils (Hoidal 1981).

2.1.3 Intra-macrophage bacterial replication and the development of disease

There is growing evidence for intra-vesicular replication of bacteria such as *E. coli* within Crohn's tissue macrophages. Studies by Ryan et al , for example, used laser capture on Crohn's tissue sections to show that the majority of Crohn's disease granulomata contained *E. coli* DNA, whereas control granulomata did not (Ryan 2004). This reflects similarities with conditions such as Q fever and Whipple's disease, where intramacrophage replication of bacteria inside macrophage phagosomes - *Coxiella burnetii* and *Tropheryma whipplei* respectively - is central to pathogenesis. In these conditions the organisms exhibit different ways in which they circumvent killing by the macrophages. *Tropheryma whipplei* resides within a phagosome prevented from maturation by blocking the switch from Rab5 to Rab7 so that the phagosomes are unable to fuse with lysosomes (Mottola 2014). *C burnetii* however is able to replicate within phagolysosome-like vacuoles (Winchell 2014).

2.1.4 Macrophage function and neutrophil chemotaxis

It is important to note though that even healthy macrophages are very poor at killing some phagocytosed bacteria, including *E. coli* and *Mycobacterium spp*., and bacterial killing is performed much more efficiently by neutrophils. Segal and colleagues have suggested that much of the problem in Crohn's disease arises as a result of defective neutrophil chemotaxis towards sites of inflammation. They have shown very marked reduction in neutrophil chemotaxis into patient's serum within Perspex windows placed over abraded skin and have also shown slow neutrophil

recruitment into colonoscopic biopsy lesions as well as delayed recruitment of radiolabelled neutrophils to intradermally injected dead *E. coli*. In separate experiments monocyte-derived macrophages demonstrated attenuated cytokine production in response to *E. coli* infection and toll-like receptor stimulation suggesting that the delayed neutrophil chemotaxis may arise as the result of defective macrophage cytokine responses (Marks 2006; Sewell 2012; Smith 2009).

2.2 The role of the microbiome

There is general consensus that the gut microbiota are involved in some way in Crohn's disease pathogenesis even though there is no strong evidence for any specific pathogen. This is supported by the fact that a wide range of animal models of inflammatory bowel disease, many based on knockouts of specific immune-related genes, all require the presence of normal commensal bacteria to develop inflammation.

The total human gut microbiome consists of around 1,150 bacterial species, the majority of which cannot be cultured and have been identified using molecular techniques. Each individual typically hosts around 160 species (Qin 2010). Gut colonisation is established within the first two weeks of life with further changes on weaning and then usually remains remarkably stable over time (Hoogkamp-Korstanje 1979). It is important to note that the mucosal and faecal microbiomes consist of distinct bacterial populations and mucosa-associated bacteria may be more important in IBD (Eckburg 2005).

2.2.1 Faecal microbiota

Many independent groups have now consistently found differences in the faecal microbiome in patients with IBD compared to healthy controls. Firstly there is a significantly reduced biodiversity (reduced by about 25%) in Crohn's disease with changes in the dominant organisms. Secondly, whilst the microbiota in health remains stable over time, significant temporal variations are seen in IBD. Furthermore, significant inter-individual differences are seen in IBD more than in health (Joossens 2011; Manichanh 2006; Scanlan 2006; Seksik 2003).

In health the *Firmicutes* and *Bacteroidetes* phyla predominate and contribute to the production of epithelial metabolic substrates including butyrate (Backhed 2005). By contrast in Crohn's disease changes are observed in the microbiota characterised by a relative lack of *Firmicutes* (specifically a reduction in *Clostridium leptum*) and Bacteroidetes with an over-representation of Enterobacteria (including *Escherichia coli*), which may conceivably alter epithelial homeostasis. Relapses in Crohn's disease have been commonly reported in association with pathogenic infections such as *Campylobacter* and *Salmonella* gastroenteritis (Irving 2008).

It is conceivable that some of these observed variations arise as a result of environmental and dietary influences but family and twin studies suggest they are disease specific. In families with at least 3 members affected by Crohn's disease there were still significant differences in the faecal microbiota of patients compared to unaffected family members (Joossens 2011). These findings are supported by a study in which monozygotic twins discordant for Crohn's were shown to have significantly different faecal microbiota with a reduction in biodiversity seen in

Crohn's disease – a change most marked in patients with ileal disease (Dicksved 2008).

It is however notable that changes in faecal microbiota are also seen in infectious colitis and in models of inflammation such as dextran sulphate sodium (DSS) induced colitis (Nanda Kumar 2008) so the faecal dysbiosis may arise at least in part as a consequence of inflammation rather than as a primary event (Sokol 2006).

2.2.2 Mucosa-Associated Bacteria

More specific changes have been found in the mucosa-associated microbiota in Crohn's disease. In the healthy colon there is a continuous mucus coating consisting of two layers, the outer of which is loosely adherent and has a favourable environment for bacterial growth but the inner tightly adherent layer is normally sterile. In the small intestine the adherent layer is much thinner and probably discontinuous. In IBD, particularly Crohn's disease, there is a marked increase in bacteria associated with the colonic adherent mucus layer (Johansson 2008; Ott 2004; Swidsinski 2002).

In Crohn's disease consistent increases in mucosa-associated *Proteobacteria* (including *E. coli*) and reductions in *Firmicutes* are reported but results for *Bacteroidetes* vary between studies (Frank 2007; Martinez-Medina 2006; Ott 2004). There is strong evidence for an increase in mucosal associated *E. coli* in both the ileum and colon and their presence within Crohn's granulomas, as previously mentioned, argues for a primary pathogenic role (Ryan 2004). Crohn's disease *E.*

coli isolates tend to have an adherent and invasive (AIEC) phenotype typified by invasion of epithelial cells and replication within macrophages (Glasser 2001). However the AIEC phenotype lacks an associated genotype and is defined using invasion into an epithelial cell line, I407, which is now known to be a transformed cervical cancer cell line of uncertain relevance. Moreover replication within macrophages is seen with the majority of human intestinal *E. coli* isolates, not just those with the ability to invade I407 (O'Brien 2016). Mucosal *E. coli* have been shown to induce formation of multi-nucleated giant cells with granulomatous morphology *in vitro* and *E. coli* with a similar phenotype induce granulomatous colitis in Boxer dogs (Meconi 2007; Simpson 2006).

2.2.3 Faecalibacterium prauznitzii

In ileal Crohn's disease both mucosal and faecal bacterial studies have demonstrated reduced levels of the Firmicute, *Faecalibacterium prauznitzii* (*F. prau*), a member of the Clostridium leptum group. In patients with ileal Crohn's disease a strong association is seen with depleted *F. prau* in biopsies taken from the colon and ileum and this is not the case for colonic disease. This reduction in *F. prau* appears to be associated with a concomitant increase in ileal and colonic *E. coli* (Willing 2009). This may be clinically relevant as low levels of mucosa-associated *F. prau* are associated with a much greater risk of recurrent Crohn's disease following surgery and the bacteria and its supernatant are able to reduce severity of chemical (TNBS)- induced colitis in murine models (Sokol 2008).

2.2.4 Specific factors affecting the microbiome

Smokers have demonstrable differences in their faecal microbiome relative to non-smokers and healthy controls, perhaps of relevance when considering the increased risk of disease in current and previous smokers (Benjamin 2012).

Unaffected siblings of patients with Crohn's disease have been shown to have both a mucosa-associated microbiome and faecal microbiome significantly different to both affected relatives and healthy controls. This variation is typified by a reduction in diversity and fewer *Faecalibacterium prausnitzii* (*F. prau*) although it is notable that intestinal permeability was normal in healthy siblings (Hedin 2016; Hedin 2014).

2.3 M cells as a portal of entry

M (microfold) cells are specialised epithelial cells which account for about 5-10% of the dome epithelium overlying Peyer's patches in the distal ileum and lymphoid follicles in the colon. They represent the major portal of entry for invasive gut pathogens and it is likely that they are also the initial portal of entry by which *E. coli* are able to invade (Sansonetti 1999). In support of this, the aphthoid ulcers that are the earliest lesions of Crohn's disease are commonly seen to overly Peyer's patches and lymphoid follicles (Fujimura 1996). Moreover there is a striking correlation between the age-related incidence of Crohn's disease and the number of Peyer's patches in the small bowel, the latter peaking in late adolescence and then falling away (Van Kruiningen 1997). Crohn's disease mucosal *E. coli* isolates

more frequently express long polar fimbriae (lpfA) that are essential for translocation across M cells (Prorok-Hamon 2014).

2.4 Summary of possible interaction between immune system and infection in Crohn's disease

In conclusion, although convincing evidence for a specific pathogen is lacking, there is growing evidence to suggest that a combination of mucosa-associated *E. coli,* well adapted for replication within macrophages, and a lack of probiotic *Faecalibacterium prausnitzii*, combine with inborn or acquired defects in immune or barrier function to lead to the development of Crohn's disease.

3 Hypothesis and Aims

3.1 Hypothesis

Mucosal *E. coli* isolates, which have ability to survive and replicate within intracellular vesicles, are able to persist within macrophages and this may represent a primary pathogenic event in Crohn's disease. Gene associations with Crohn's disease also imply defective innate immunity including macrophage function. Hydroxychloroquine is known to enhance killing of other intracellular organisms and may similarly facilitate macrophage killing of *E. coli.* Vitamin D enhances immune cell function and may also improve macrophage killing of phagocytosed *E. coli.*

The hypotheses explored in this thesis are that:

1. Defective macrophage killing of bacteria, particularly *E. coli*, may be an underlying pathogenic mechanism in Crohn's disease.
2. Hydroxychloroquine treatment may enhance the ability of murine and human macrophages to kill intracellular *E. coli* and this effect may be synergistic with antibiotics.
3. Hydroxychloroquine may lead to enhanced killing of intracellular bacteria by favourably altering the macrophage intracellular environment.
4. Vitamin D supplementation may enhance the ability of macrophages to kill intracellular bacteria.

3.2 Aims

1. To determine the ability for *E. coli* to survive within murine and human (including Crohn's disease and control) macrophages and to assess their cytokine and chemokine responsiveness.
2. To determine the effect of hydroxychloroquine treatment on the survival of *E. coli* within murine macrophages
3. To determine the mechanisms by which hydroxychloroquine acts on intracellular organisms
4. To determine the effect of hydroxychloroquine on bacterial survival in human monocyte derive macrophages
5. To determine the effects of Vitamin D supplementation on intramacrophage bacterial survival

4 Materials and Methods

4.1 Materials

Unless otherwise stated all materials were obtained from Sigma-Aldrich (Poole, UK).

4.2 Ethical approval

Ethical approval for this study was obtained from the National Research Ethics Service (NRES) Committee North West (Study number 09/H1010/64).

4.3 Patient recruitment

Patients were recruited from the Royal Liverpool University Hospital (RLUH) and healthy controls recruited from both RLUH and the University of Liverpool. A summary of inclusion/exclusion criteria and patient characteristics is included supplementary appendix 1 (Table 8-10). Baseline characteristics varied only in the level of high sensitivity CRP (hsCRP) (higher in CD group) but were otherwise equivalent. Five of the CD patients had active disease as defined by Harvey Bradshaw index (HBI) >4 (Vermeire 2010a). None of the CD patients were receiving immunosuppressants, corticosteroids or anti-TNFα therapy and none had features suggestive of sepsis (median hsCRP 5.9mg/L, range 1.04-18.3).

4.4 Bacterial strains and culture

4.4.1 Bacterial strains

Two representative CD mucosa-associated *Escherichia coli* isolates were studied; both conform to the original AIEC phenotype: HM605, an isolate from the colonic mucosa of a patient with CD (Martin 2004), and LF82, isolated from an ileal lesion of a French patient with CD (Boudeau 1999) (kindly gifted by the late Prof. A. Darfeuille-Michaud, Pathogénie Bactérienne Intestinale, Clermont-Ferrand, France). Both have been shown to survive and replicate within the phagolysomes of murine and peripheral blood monocyte-derived macrophages (Bringer 2006; Mpofu 2007; Subramanian 2008). In addition, two laboratory strains of *E.coli*; K-12 (ATCC 29425, Manassas, VA, USA) and K-12 derivative EPI300™-T1R (Epicentre BioTechnologies, Madison, WI, USA), and *Staphylococcus aureus* (*S. aureus*) Oxford strain (NCTC 6571, Public Health England, Porton Down, UK), acted as controls.

4.4.2 Bacterial culture

Isolates were cultured overnight on Luria Bertani (LB) agar (Table 6) at 37^{o}C in air. After overnight culture, isolates were washed three times, resuspended in sterile phosphate buffered saline (PBS) pH 7.3, and adjusted by optical densitometry (OD_{550nm}) to a desired final concentration (for macrophage killing assays; 1x10^{9}/mL, for flow cytometry; 1x10^{10}/mL).

Bacteria were freshly cultured and placed in suspension for each experiment. On each occasion the concentration of bacterial in suspension was confirmed by plating in triplicate onto LB agar, incubating overnight at 37^{o}C in air, and finally enumerating colony forming units (CFU).

Constituent (made up to 1000mls with deionised water)	**Manufacturer**
10 g Bacto™ Tryptone	Becton Dickinson (New Jersey, USA)
5 g Bacto™ Yeast	Becton Dickinson (New Jersey, USA)
5 g NaCl	Sigma-Aldrich
15g Agar	Sigma-Aldrich

Table 6: Constituents of LB agar

4.4.3 Standard curves of bacterial concentration in solution

In order to reliably produce accurate concentrations of bacteria in suspension using optical density (OD), standard curves of bacterial concentration against OD_{550nm} were generated for each strain used (Figure 4-1-4-3).

Following overnight culture, washing and suspension in PBS, serial dilutions of concentrated bacterial suspensions were performed. For each dilution the OD_{550nm} was measured before plating in triplicate onto LB agar and then enumerating colony forming units (CFUs) after overnight culture. Each experiment was performed in triplicate and average values used to generate the curves.

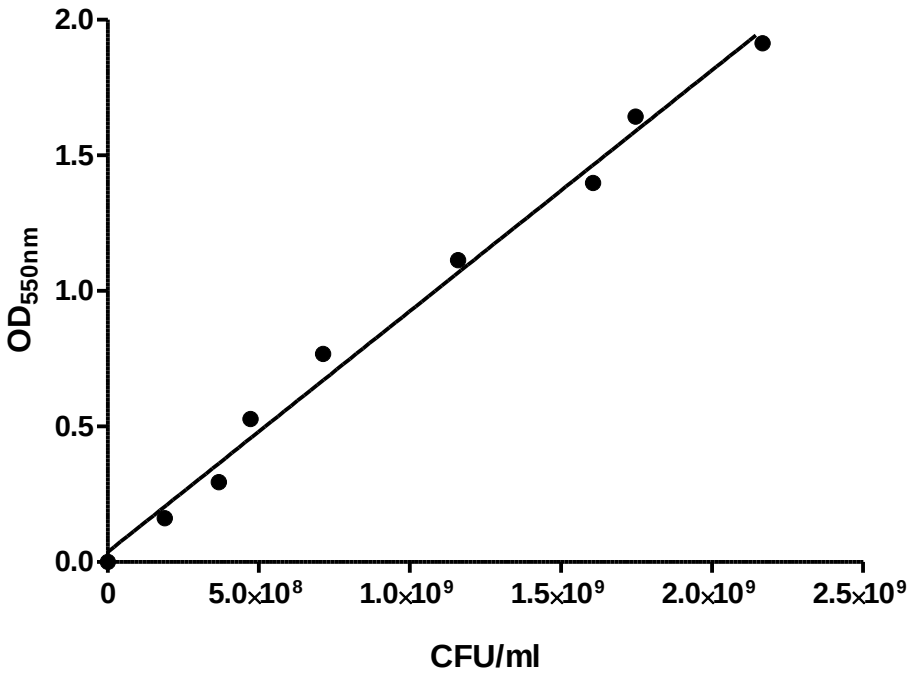

Figure 4-1: Standard curve for Escherichia coli HM605 against OD_{550nm}

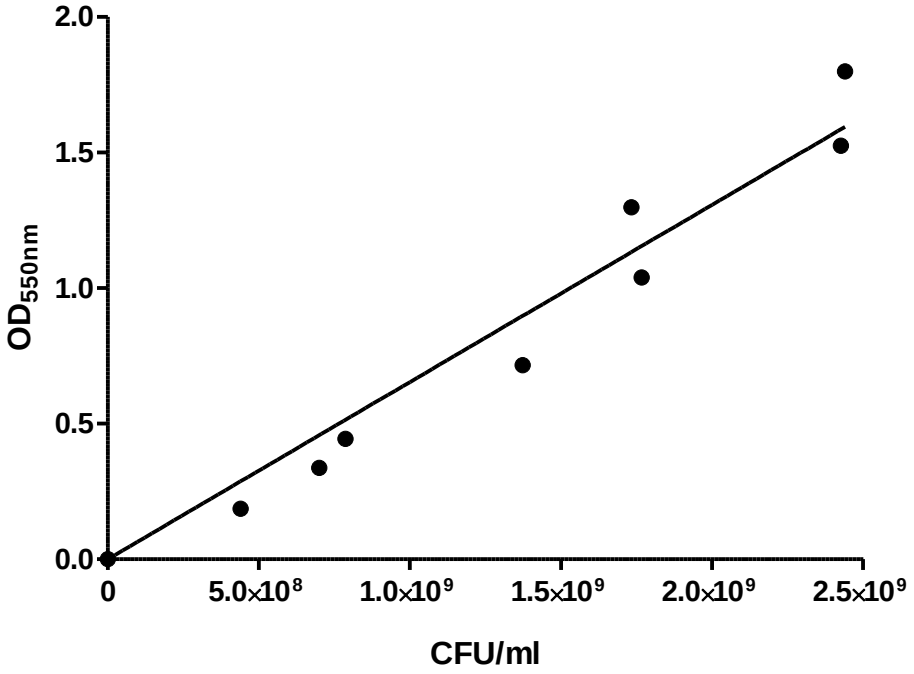

Figure 4-2: Standard curve for Escherichia coli K12 against OD_{550nm}

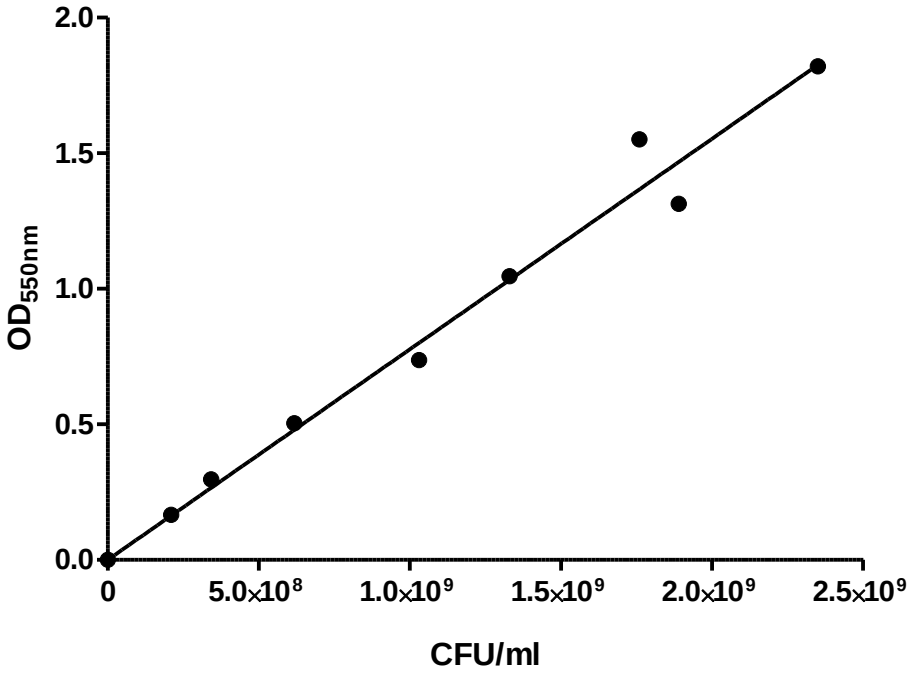

Figure 4-3: Standard curve for Staphylococcus aureus Oxford strain against OD_{550nm}

4.4.4 Bacterial growth in broth

Bacterial growth dynamics were determined by incubation of bacteria in broth. $1x10^7$ bacteria were added to RPMI-1640 supplemented with 10% vol/vol FBS and 2mM Glutamine plus hydroxychloroquine (0-10µg/mL) or vehicle control, and maintained at 37^{o}C on an orbital incubator at 150rpm in air. Optical density (OD_{600nm}) was determined each hour. In separate experiments samples were obtained every two hours and plated onto LB agar plates to confirm bacterial viability.

4.4.5 Minimum inhibitory concentration testing

For each *E. coli* strain the minimum inhibitory concentrations (MIC) for ciprofloxacin and doxycycline were determined using Etest® stable gradient antibiotic strips (Biomerieux, Basingstoke, UK). Following overnight culture on LB agar at 37^{o}C in air, *E. coli* isolates were washed three times, resuspended in sterile PBS, adjusted to MacFarlane standard 0.5 and streaked onto LB agar plates. Etest® strips were applied, plates incubated overnight and results read the following day according to the manufacturer's instructions (Figure 4-4 and 4-5).

Figure 4-4: Representative image of minimum inhibitory concentration testing for ciprofloxacin against E. coli strains using Etest® strips

Figure 4-5: Representative image of minimum inhibitory concentration testing for doxycycline against E. coli strains using Etest® strips

4.5 Mammalian Cell culture

Unless otherwise stated Corning Costar tissue culture (Tewksbury, MA, USA) plates were used, obtained from Fisher Scientific (Loughborough, UK).

4.5.1 J774A.1 murine macrophages

J774A.1 murine macrophages, (# 91051511) were obtained from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK). Cells were cultured in 75cm^2 flasks and maintained at 37^{o}C in Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% vol/vol foetal bovine serum (FBS), 2mM glutamine, 50U/mL Penicillin and 50µg/mL Streptomycin (complete RPMI), in a humidified atmosphere of 5% CO_2/95% air. Macrophages were passaged by scraping twice weekly, up to passage 19.

4.5.2 Human monocyte-derived macrophages

50mls of peripheral blood was obtained from consenting volunteers and mixed immediately with unfractionated heparin sodium (Wockhardt UK Ltd, Wrexham, Wales) to a final concentration of 5units/ml. Heparinised blood was mixed with an equal volume of sterile PBS (Life Technologies, Paisley, UK), layered over Lymphoprep™ density gradient medium (Alere, Stockport, UK) in 50ml polypropylene tubes (Greiner Bio-One, Stonehouse, UK) at a ratio of 2 parts blood/PBS to 1 part Lymphoprep. A mononuclear cell layer was then generated by centrifugation at 800 x *g* for 20minutes at room temperature with brakes minimised (Figure 4-6). The resulting mononuclear cell layer was carefully aspirated, transferred to a clean 50ml tube and twice washed in sterile PBS by centrifugation at 400 x *g* for 10mins at room temperature. Cells were resuspended in RPMI-1640 medium supplemented with 20mM Hepes, 100U/mL Penicillin and 100µg/mL Streptomycin, adjusted to $5x10^6$/ml, seeded into 100mm Nunc™ cell culture dishes (VWR International, Lutterworth, UK) and cultured at 37^oC in a humidified atmosphere of 5% CO_2, 95% air for 2hours. Non-adherent cells were removed by washing twice with sterile PBS and adherent monocytes then differentiated into macrophages by 5 day culture at 37^oC in RPMI-1640 medium supplemented with 10% vol/vol FBS, 20mM Hepes, 100U/mL Penicillin and 100µg/mL Streptomycin, in a humidified atmosphere of 5% CO_2, 95% air as per (Smith 2009).

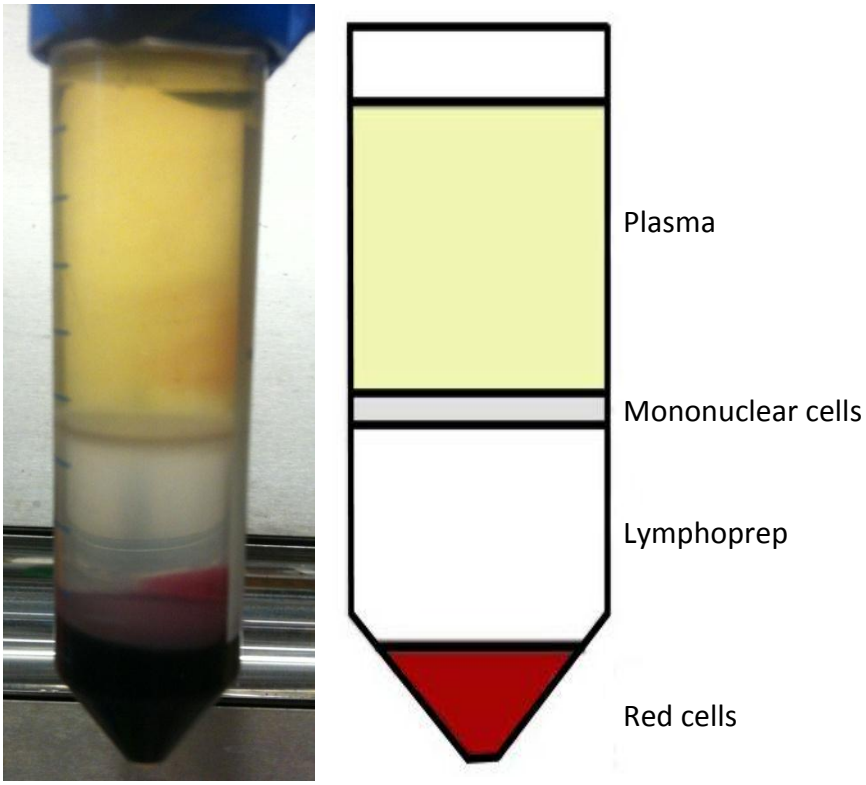

Figure 4-6: Photograph (left) and diagram (right) showing mononuclear cell isolation after centrifugation over Lymphoprep™ density gradient medium

4.5.3 Genomic analysis for CD-associated polymorphisms in *NOD2*, *ATG16L1* and *IRGM*

EDTA peripheral venous blood samples were taken from the CD patients and healthy controls in whom macrophage function studies were performed, genomic DNA extracted using a Nucleon® BACC3 kit (Gen-Probe, Livingston, UK), yield determined using PicoGreen® (Life Technologies) and samples normalised to final DNA concentration of 10ng/µL. DNA was analyzed by TaqMan® system (Applied Biosystems, Foster City, CA, USA) in the Wellcome Trust Clinical Research Facility Edinburgh, (Western General Hospital, Edinburgh, UK) for *NOD2* (rs2066844, rs2066845 and rs2066847) (Barrett 2008; WTCCC 2007), *ATG16L1* (rs2241880) (Cooney 2010; Rioux 2007) and *IRGM* (rs13361189) (Parkes 2007) as previously described (Van Limbergen 2008).

4.5.4 Human neutrophils

50mls of peripheral blood was obtained from healthy consenting donors and heparinised to a final concentration of 5units/ml. Heparinised blood was layered over Polymorphprep™ (Alere, Stockport, UK) in a ratio of 1 part blood to 1 part Polymorphprep in 50ml polypropylene tubes then centrifuged at 500 x *g* for 35minutes at room temperature with brakes minimised (Figure 4-7). After careful removal of the mononuclear cell layer neutrophils were aspirated, transferred to a clean 50ml tube, resuspended in RPMI supplemented with 20mM Hepes, 0.5% vol/vol bovine serum albumin (BSA), 2mM $CaCl_2$ and 2mM $MgCl_2$ and centrifuged at 500g for 5 min at room temperature. After careful removal of the supernatant,

cells were resuspended in 3mls of supplemented RPMI and 27mls of sterile red cell lysis buffer (13.4mM potassium bicarbonate, 155mM ammonium chloride, 96.7µM EDTA in deionised water), allowed to stand for 3minutes to lyse red cells and then centrifuged at 400 x *g* for 3minutes. Finally the supernatant was discarded, the isolated neutrophils were resuspended in supplemented RPMI and then used immediately.

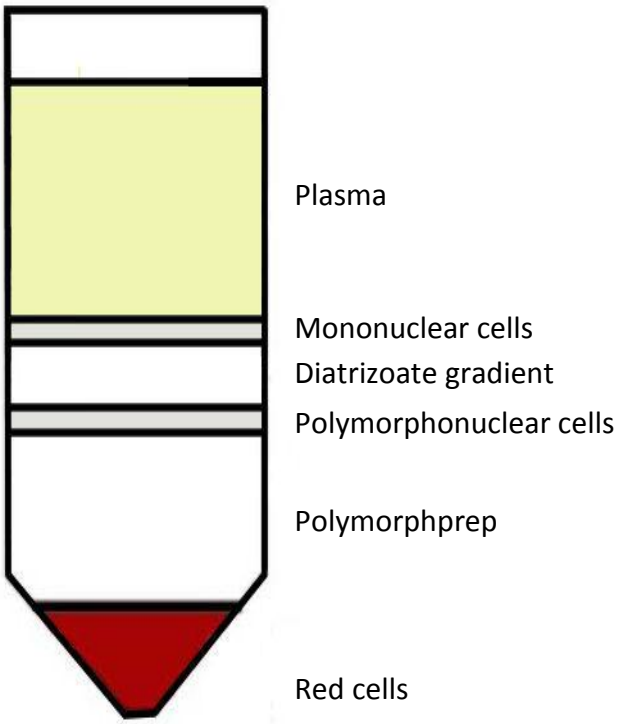

Figure 4-7: Diagram demonstrating resultant cellular layers allowing isolation of neutrophils after centrifugation of heparinised blood over Polymorphoprep™ density gradient medium

4.5.5 Assessment of cellular cytotoxicity

Release of intracellular adenylate kinase, as a marker of cell damage, was quantified over 6h following cellular treatment and/or bacterial infection using the Toxilight™ assay kit (Lonza, Slough, UK). Assays were performed according to the manufacturer's instructions in white walled, clear bottom, 96 well plates. Luminescence was measured on a Tecan F200 microplate reader (Tecan, Männedorf, CH). Total cellular levels of adenylate kinsae were established using a Total Lysis kit (Lonza). Release of adenylate kinase into the culture media was expressed as a proportion of total adenylate kinase release and was compared to untreated, vehicle-treated or uninfected controls.

4.6 Intramacrophage bacterial replication

4.6.1 J774A.1 murine macrophages

Replication of Crohn's associated *E. coli* within J774A.1 murine macrophages was assessed by culture and lysis after gentamicin killing of non-internalised bacteria. Cells were seeded in triplicate into 24 well plates at 10^5 cells per well in fully supplemented RPMI-1640 medium and cultured for 24h at 37^0C, 5% CO_2, 95% air. Prior to infection, monolayers were washed 3 times with sterile PBS, 800µL antibiotic-free RPMI added to each well and macrophages infected at a multiplicity of infection (MOI) of 10. Following a 2h infection, macrophages were washed three times with sterile PBS and then treated for 1h in RPMI containing 20µg/mL gentamicin to kill non-internalised bacteria. After 1h incubation macrophages were washed three times with sterile PBS then lysed with 1% vol/vol Triton X-100 in

deionised water for 5 min to release internalised bacteria. Lysates were plated to LB agar and colony forming units (CFU) enumerated after overnight culture at 37^{o}C in air.

In order to assess relative killing (or replication) of phagocytosed bacteria, parallel plates were performed where macrophages were cultured for a further 3h in fresh medium following the 1h gentamicin treatment step (6h total), again followed by washing, lysis, overnight culture and colony counting.

To calculate fold replication of *E. coli* within macrophages, the viable counts at the end of the final 3h incubation period (total 6h) were compared to viable CFU obtained from parallel plates lysed immediately after 1h gentamicin treatment (3h).

Data were expressed as fold change of recovered intramacrophage bacteria at 6h relative to intracellular numbers at 3h.

4.6.2 Human monocyte-derived macrophages

Macrophages were generated from adherent monocytes as described above. Monolayers were washed twice with sterile PBS, cells detached by scraping and resuspended in antibiotic free X-Vivo 15 medium (Lonza, Slough, UK) then seeded in triplicate into 24 well plates at 10^5 cells per well before overnight culture. Intra-macrophage replication was determined used the technique described above with the modification of using a MOI of 25.

4.6.3 Effect of hydroxychloroquine on bacterial growth in broth

Bacterial growth dynamics were determined by incubation of bacteria in broth. $1x10^7$ bacteria were added to RPMI supplemented with 10% FBS and 2mM glutamine and maintained at 37^oC on an orbital incubator at 150rpm in air. Optical density was determined each hour. In separate experiments samples were obtained every two hours and plated onto LB agar plates to confirm bacterial viability.

4.6.4 Effects of hydroxychloroquine, with and without antibiotics, and vitamin D on intra-macrophage survival of internalised Crohn's-associated *E. coli*

The effects of hydroxychloroquine, antibiotics and vitamin D on intra-macrophage bacterial survival were determined using the intra-macrophage bacterial replication assay described above.

Hydroxychloroquine undergoes steady-state pharmacokinetics and doses were selected based on published steady state concentrations (Carmichael 2003; Munster 2002).

Doxycycline and ciprofloxacin were tested both at Cmax (the peak serum concentration achieved with a standard oral dosing regimen) and 10% Cmax based on published data (Agwuh 2006; Gonzalez 1984; Saravolatz 2005) (Figure 6-4).

$1,25OH_2$Vitamin-D_3 concentrations were those defined as deficient in human serum (<20nM) (Hewison 2012; Rosen 2011) and that required for optimal immune cell function (>80nM) (Fabri 2011; Hewison 2012).

4.6.5 Influence of intracellular iron availability on the action of hydroxychloroquine

To determine whether the antibacterial actions of hydroxychloroquine (HCQ) were mediated through pH-mediated restriction of intramacrophage iron, macrophages were pretreated with either 10µM ferric citrate, which exhibits pH-dependent solubility and release of free ferric iron, or 10µM ferric nitrilotriacetic acid (FeNTA), which releases free ferric iron irrespective of pH, as per (Byrd 1991; Trinder 1998).

Ferric citrate was dissolved in sterile ultra-high quality (UHQ) water (18.2 MΩ-cm (0.05 µS) at 10 mg/mL, aliquoted and stored at -20^{o}C until needed. FeNTA was formed by combining ferric chloride (100 mg/mL in 1N HCl) and nitrilotriacetic acid disodium salt (100 mg/mL in UHQ water) in equimolar quantities, diluting to 1mM with sterile UHQ water and adjusting to pH 7.4 with 1N NaOH, yielding 85% of monomeric FeNTA as per (Bates 1971). FeNTA was fresh on the day of each experiment to avoid any instability observed during storage.

4.7 Phagolysosomal pH

4.7.1 Fluorescent microplate reader

To assess for intraphagolysosomal pH changes effected by hydroxychloroquine, macrophages were monitored using two pH-sensitive fluorophores; pHrodo™ conjugated to *E. coli* bioparticles, fluorescent at acidic pH (Deriy 2009) and fluorescein isothiocyanate (FITC)-dextran, fluorescent at alkaline pH (Ohkuma 1978). J774A.1 macrophages seeded into white walled, clear bottomed 96-well plates at $5x10^5$/mL, were maintained in supplemented RPMI-1640 media and cultured for 24h. They were then washed three times with sterile PBS, followed by addition of 100µL of pHrodo™ *E. coli* bioparticles (0.1 mg/mL in phenol red-free Hank's Balanced Salt Solution (HBSS) containing 20mM HEPES, pH 7.4), and infected for 2h with *E. coli* K-12 strain EPI300™-T1R at an MOI of 10. Macrophages were then washed twice with sterile PBS and maintained in HBSS containing hydroxychloroquine (0-20 µg/mL) for a further 3h. For FITC-dextran, this was added to macrophages wells prior to overnight culture to a final concentration of 1 mg/mL. Fluorescence was quantified using a Tecan F200 microplate reader using excitation/emission wavelengths of; 535nm excitation, 590nm emission for pHrodo™ and 485nm excitation, 535nm emission for FITC-dextran.

For both fluorophores, a pH calibration curve was generated following incubation of fluorophore loaded macrophages in pH-calibrated phosphate-citrate buffers (range pH 4-8) containing 20µM nigericin, an ionophore which equilibrates intracellular pH with extracellular pH (Reijngoud 1975) (Figure 4-8 and 4-9). Immediately after addition of calibration buffers, fluorescence emissions were measured as previously

detailed. Readings were taken every 5 min for 1h. Bafilomycin A1, a vacuolar H+ ATPase inhibitor (Bowman 1988) was used as a positive control for macrophage phagolysosome alkalinisation.

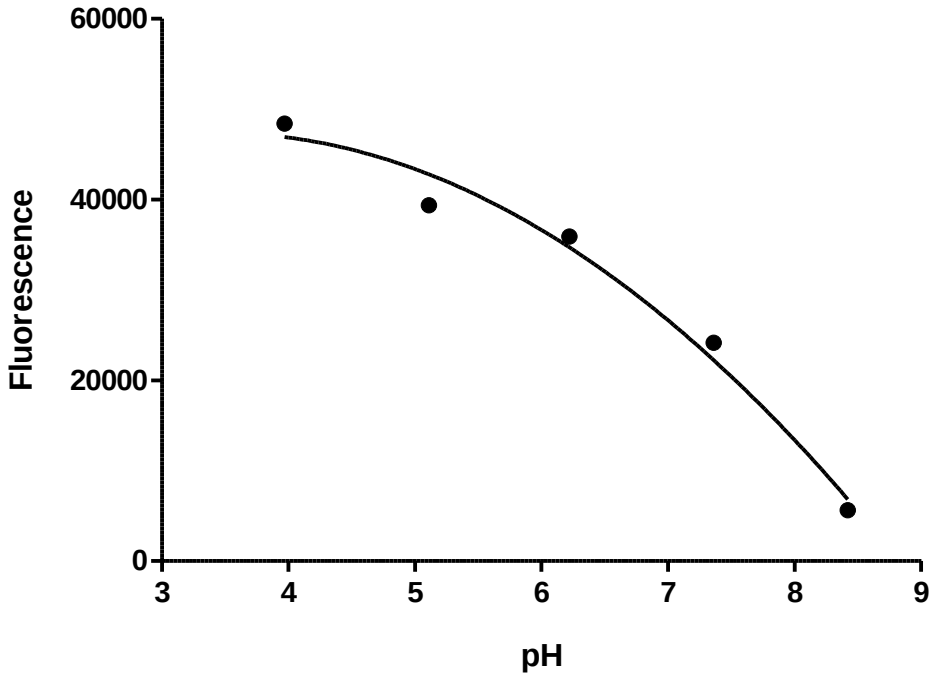

Figure 4-8: Standard pH calibration curve measuring fluorescence of intramacrophage pHrodo™ labelled E. coli bioparticles against solutions of known pH

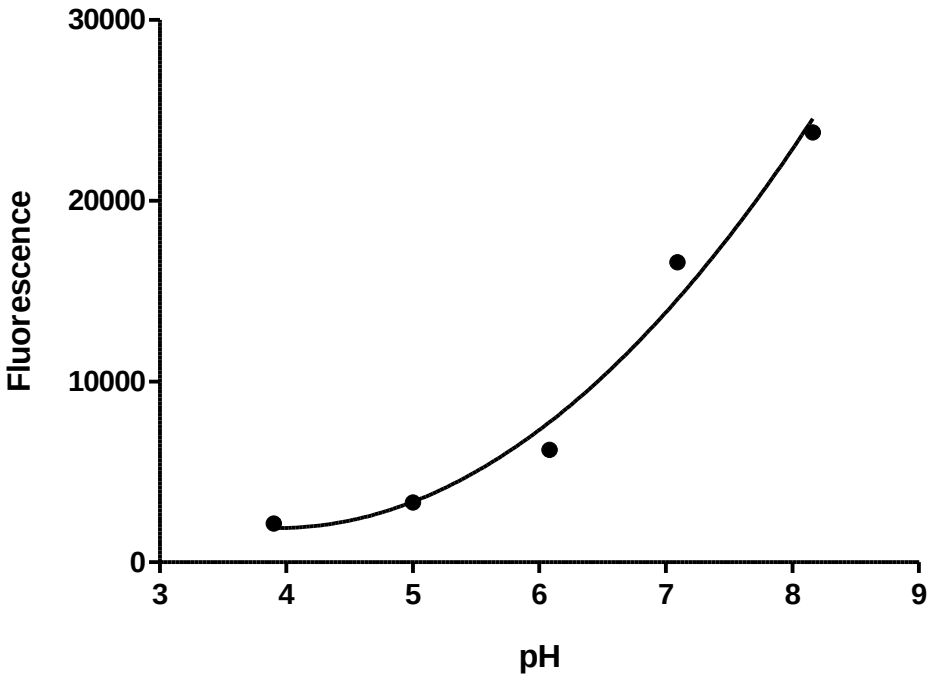

Figure 4-9: Standard pH calibration curve measuring fluorescence of intramacrophage FITC against solutions of known pH

4.7.2 Fluorescent microscopy

Phagolyosomal pH was further assessed by fluorescent microscopy using dual labelled *E. coli*. *E. coli* K12 (ATCC® 29425™, Manassas, VA, USA) grown overnight on LB agar were harvested, washed 3 times in sterile PBS, resuspended in 100mM Trehalose before freezing at -80^{o}C and subsequent overnight lyophilisation. Viability of organisms recovered after lyophilisation was confirmed by re-suspension and overnight culture. Lyophilized *E. coli* K12 were then dual-labelled with Alexa Fluor® 350 (a pH-insensitive flurophore) (Life Technologies, Paisley, UK) and pHrodo™ red, succinimidyl ester (pH-sensitive, increasing fluorescence at acidic pH) (Life Technologies, Paisley, UK). pHrodo™ in 100% anhydrous dimethylsuphoxide (DMSO) was added to *E. coli* re-suspended in 100mM sodium

bicarbonate (pH 8.5) to achieve a final concentration of 0.5mM and incubated on a rocking platform at room temperature for 45 min. Subsequently, *E. coli* were washed twice with sterile HBSS, resuspended in sterile PBS (pH 9) and Alexa Fluor® 350 in 100% anhydrous DMSO added to a final concentration of 0.5mM before incubation for 60 min. Labelled bacteria were then washed with HBSS and resuspended in sterile PBS (pH 9) and either stored at 4^{o}C and used with 3d, or frozen at -20^{o}C for later use.

Microscopy was performed on macrophages seeded onto 19mm coverslips in 12 well tissue culture plates at $5x10^{5}$ per mL, maintained in complete RPMI-1640 medium and cultured for 24h. Monolayers were washed three times with sterile PBS, treated with 1ml of antibiotic-free RPMI 1640 medium and infected with dual labelled *E. coli* K12 (MOI of 50). Following 3h infection, cells were washed twice with sterile PBS, fixed with 2% vol/vol paraformaldehyde in PBS (Taab, Berks, UK), mounted in Vectashield mounting media (Vector Labs, Peterborough, UK) and imaged using an Olympus BX51 fluorescence microscope using excitation/emission wavelength of; 560 excitation, 630 emission for pHrodo™ and 360 excitation, 460 emission for Alexa Fluor® 350. Image processing was performing using AQM 6 software (Kinetic imaging, Nottingham, UK).

4.7.3 Confocal microscopy

Confocal microscopy was used to measure intra-organelle pH following hydroxychloroquine treatment of macrophages. All confocal microscopy was performed using a Zeiss LSM 510 meta laser confocal microscope with an atmosphere- and temperature-controlled stage. Images were processed using LSM 5 software (Carl Zeiss AG, Oberkochen, Germany) and fluorescent intensity analysed with AQM 6 (Kinetic Imaging, Nottingham, UK).

$2x10^5$ J774A.1 murine macrophages were seeded into 35mm CELLview™ tissue culture dishes (Greiner Bio-One, Stonehouse, UK) in 2mls RMPI supplemented with 10% vol/vol FBS, 2mM Glutamine, 50U/mL Penicillin and 50µg/mL Streptomycin and cultured overnight. Monolayers were washed three times with sterile PBS before adding 1.9mls phenol red-free, antibiotic-free RPMI (Life Technologies, Paisely, UK) containing 10µg/mL pHrodo™ *E. coli* bioparticles (suspended in phenol red-free Hanks Balanced Salt solution (HBSS) supplemented with 20mM HEPES, pH 7.4) and incubated for 30mins at 37^oC (Figure 4-10 to 4-12). Monolayers were washed three times with sterile PBS then incubated in phenol red-free RPMI containing hydroxychloroquine (0-10µg/ml) for 3h. NH_4Cl(2mM) was used as an intra-phagolysosome alkalinisation control. Finally, 10 high powered fields of view were imaged for each plate using a 63x oil immersion objective with optimisation of the focal plane to maximise fluorescent intensity on each occasion.

To generate pH values from fluorescent intensity, standard curves were obtained. After overnight culture and 30min loading with pHrodo™ particles, monolayers were incubated for 30mins with calibration buffers containing 5mM Hepes, 120mM

KCl, 20mM NaCl, 1mM CaCl2, 1mM MgCl2, Nigericin 10µM, Vanilomycin 10µM, Bafilomycin 0.1µM adjusted to pH 4-7. Monolayers were imaged as detailed above (Figure 4-13).

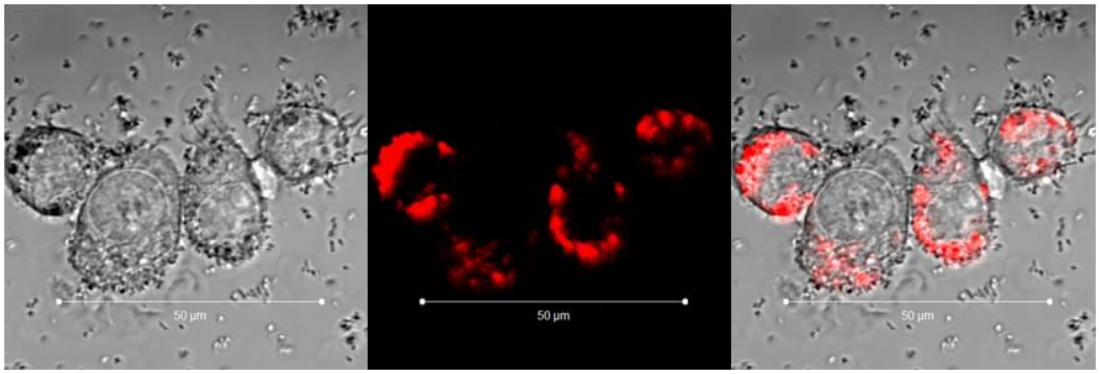

Figure 4-10: Representative images demonstrating uptake of 100µg/ml pHrodo™ E. coli bioparticles by J774A.1 macrophages at 30minutes. Panels show; brightfield image (left), fluorescent image (centre) and merged (right). Scale bar = 50µm

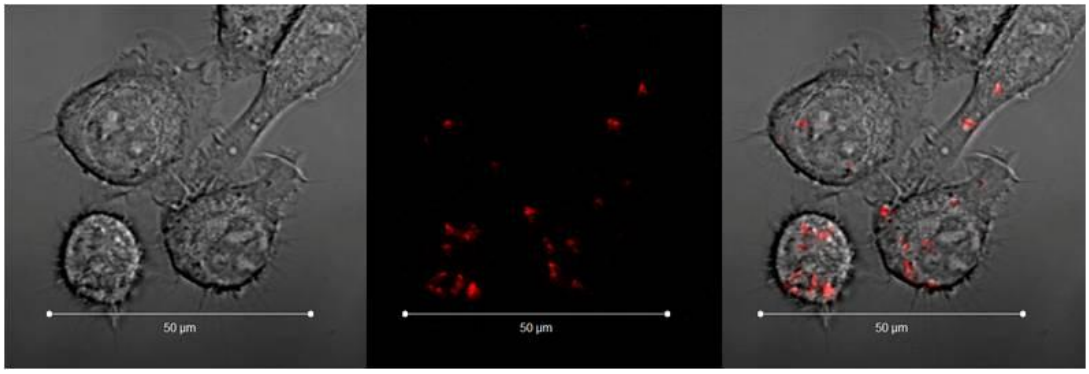

Figure 4-11: Representative images demonstrating uptake of 10µg/ml pHrodo™ E. coli bioparticles by J774A.1 macrophages at 30minutes. Panels show; brightfield image (left), fluorescent image (centre) and merged (right). Scale bar = 50µm

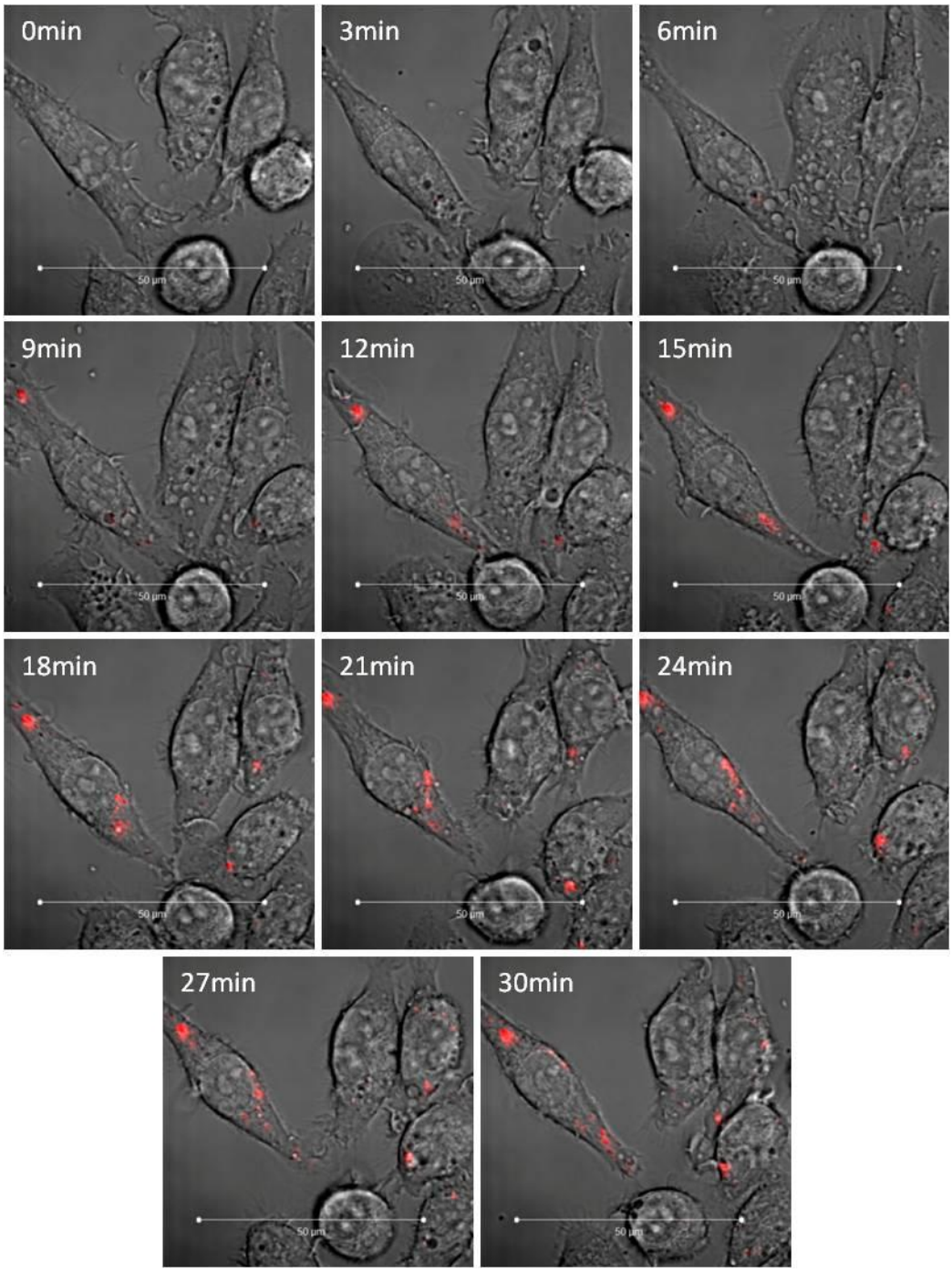

Figure 4-12: Time course of uptake of 10µg/ml pHrodo™ E. coli bioparticles by J774A.1 macrophages (merged images). Scale bar = 50µm

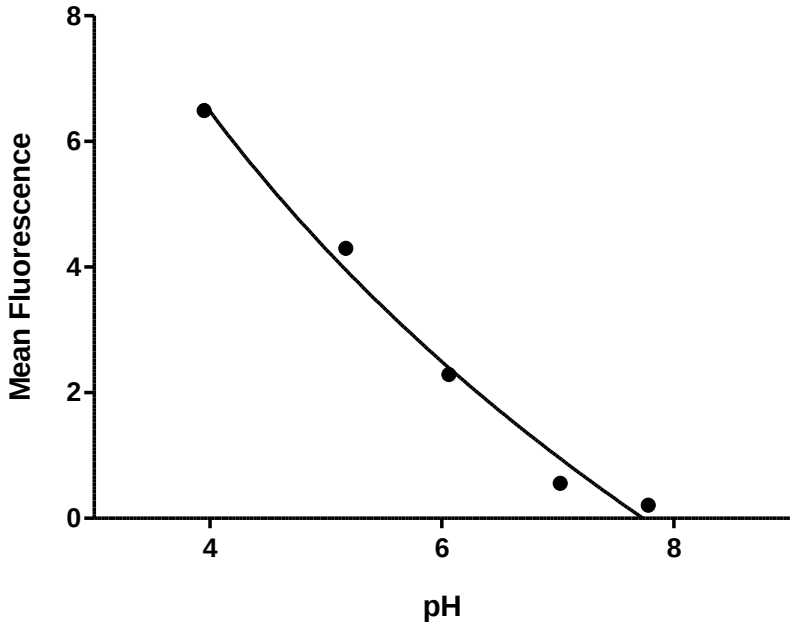

Figure 4-13: Standard pH calibration curve generated by confocal microscopy measuring fluorescence of intramacrophage pHrodo™ labelled E. coli bioparticles against solutions of known pH

4.7.4 Final method for determination of phagolysosomal pH

Confocal microscopy was preferred as the definitive technique for determination of phagolysosomal pH. This technique allowed high quality imaging, showed clear cellular uptake of the fluorophone therefore allowing definitive confirmation of intracellular fluorescence and produced little inter-assay variation.

Images from standard fluorescent microscopy were of insufficient quality to generate meaningful results. The fluorescent microplate reader technique exhibited large inter-assay variation, failed to demonstrate a result from the positive control and it could not reliably determine whether fluorescence was intracellular thereby leading to uncertainty over whether the data represented phagosomal pH.

4.8 Protease activity

4.8.1 Colorimetric measurement of enzymatic action

Protease activity was determined via enzyme-specific substrate degradation in a colorimetric assay according to published methodology (Liu 2012; Rivera-Marrero 2004; Standish 2009).

J774A.1 macrophages were seeded in triplicate into 24 well plates at $2.5x10^5$/well and cultured overnight. Monolayers were washed three times with sterile PBS, 800µL antibiotic-free RPMI added and cells infected at a MOI of 10. After 2h infection, cells were washed three times, treated with RPMI containing 20µg/ml gentamicin for 1h to remove extracellular bacteria, then incubated for a further 3h with media containing hydroxychloroquine (0-10µg/ml) or dimethylsulphoxide (DMSO) vehicle control. At the end of 6h cells were washed three times with sterile PBS and lysed with 50mM Hepes, 50mM NaCl, 0.1% Triton X in sterile water for 30minutes on ice. 100µL aliquots of lysate were transferred to white walled, clear bottom 96 well plates (Corning, Tewksbury, MA, USA) and specific substrates for each enzyme added: N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (in DMSO, final concentration 1mM) for Cathepsin G and N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (in DMSO, final concentration 0.85mM) for Macrophage elastase (MMP12). Plates were incubated for 24h in the dark and then OD_{410nm} read with a Sunrise™ microplate reader (Tecan).

4.8.2 Calculation of enzymatic (protease) activity

Enzyme activity was calculated from colourimetric enzyme specific substrate degradation using the Beer-Lambert equation (Wilson 2010).

4.8.2.1 **The Beer Lambert equation**

This states:

$$A = \varepsilon \times c \times d$$

A = Absorbance per time (change in optical density)

ε = Molar extinction coefficient

c = Concentration per time

d= Path length

Thus;

$$c = \frac{A}{(\varepsilon \times d)}$$

Values used:

A; change in optical density from baseline (per minute)

ε; Cathepsin G (8800), MMP12 (132800) (Sigma-Aldrich and (Palmier 2007))

d; path length was calculated as detailed below

4.8.2.2 **Path length calculation**

The path length in a 96 well plate can be calculated by rearranging the formula for the volume of a cylinder $h = \frac{V}{\pi r^2}$

h = Path length

V = filling volume

r = diameter

Path length was therefore calculated using the well bottom surface area ($0.32cm^2$) provided by the manufacturer (Corning) (from which r can be calculated as 3.19mm using area = πr^2), and the filling volume (100µl).

$$h = \frac{100\mu l}{\pi(3.19mm)^2}$$

Therefore;

$$h = \frac{100mm^3}{\pi(3.19mm)^2}$$

So;

$$h = \frac{100mm}{31.97} = 3.13mm = 0.313cm$$

4.8.2.3 **Calculation of enzymatic activity**

Enzymatic concentration per time was therefore calculated thus:

$$\text{concentration per time} = \frac{\text{change in OD from baseline per minute}}{\varepsilon \times 0.313}$$

Finally, enzymatic units were calculated from concentration per time on the following basis:

1 unit of active Cathepsin G as defined as c= 1nM/sec (Sigma)

1 unit of MMP12 defined as c=100pM/min (Sigma)

4.8.2.4 **Confirmation of linear relationship between OD and enzyme activity**

This method requires a linear relationship between enzyme activity and optical density. This was confirmed by co-incubating serial dilutions of purified Cathepsin G (0.5-20nM (Sigma-Aldrich, Dorset, UK)) and macrophage elastase (MMP12) (0.5-2.5nM (Enzo Life sciences, Exeter, UK)) with their relevent substrates for 24h in the dark then optical density determined as previously detailed (Figure 4-14 and 4-15).

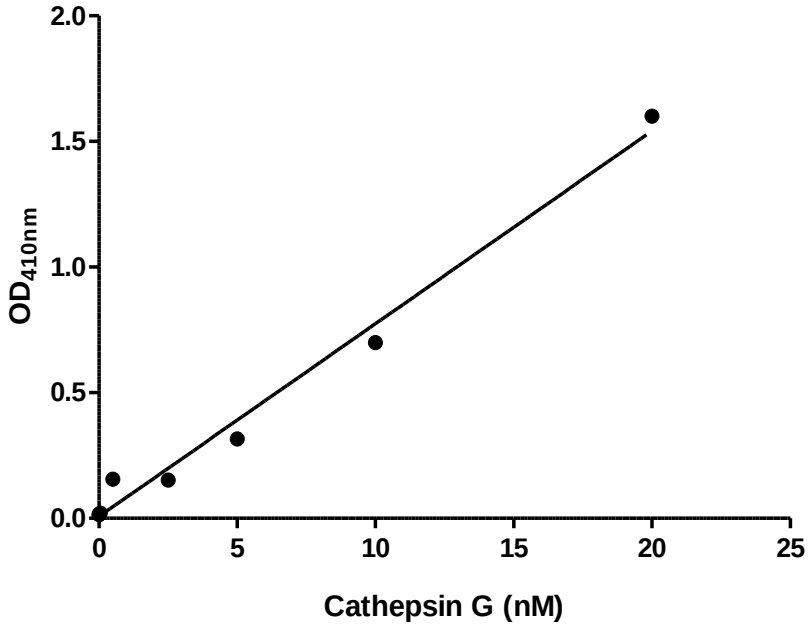

Figure 4-14: Confirmation of linear relationship between Cathepsin G enzymatic degradation of N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide and optical density

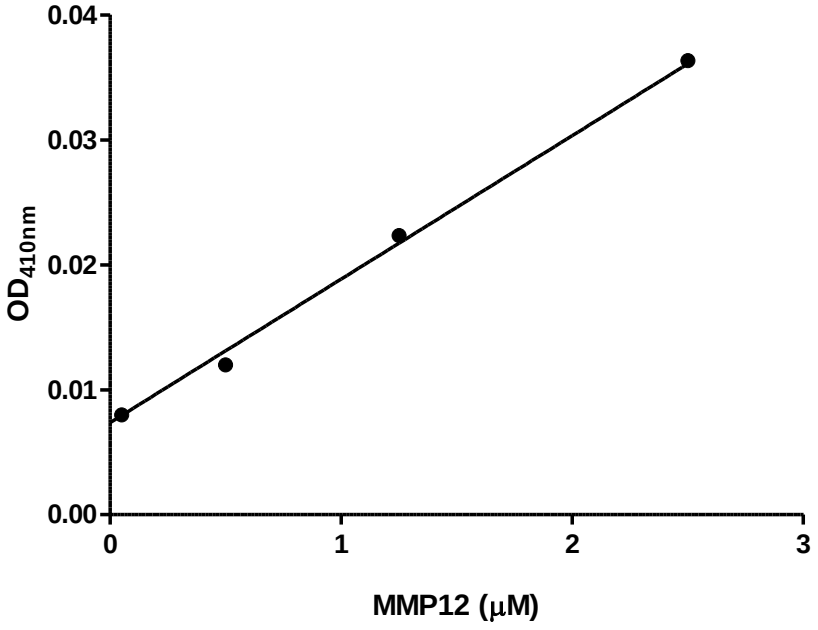

Figure 4-15: Confirmation of linear relationship between macrophage elastase (MMP12) enzymatic degradation of N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide and optical density

4.9 Respiratory burst

4.9.1 Flow cytometry

A Millipore Guava® EasyCyte plus flow cytometry system (Millipore; Billerica, MA, USA) was used for all experiments.

4.9.1.1 Measurement of intracellular respiratory burst

Following optimisation (as described below), intracellular respiratory burst was measured by flow cytometry using the fluorophore Dihydrorhodamine 123 (DHR123), fluorescent in the presence of reactive oxygen species (Emmendorffer 1990) (Figure 4-16). Macrophages were seeded into 24 well plates at $5x10^5$/well, primed with interferon gamma (IFNγ) and cultured overnight. Monolayers were washed 3 times with sterile PBS, and incubated for 3h in antibiotic-free RPMI-1640 medium containing 20mM HEPES, and hydroxychloroquine or DMSO vehicle control. Respiratory burst was initiated with either *E. coli*, *Staph. aureus* (at a multiplicity of infection of 100) or 1μM phorbol 12-myristate 13-acetate (PMA). After 15 min, 50nM DHR123 was added followed by a further 15 min incubation. Macrophages were then washed 3 times with sterile PBS and cells detached using 10mM EDTA in PBS.

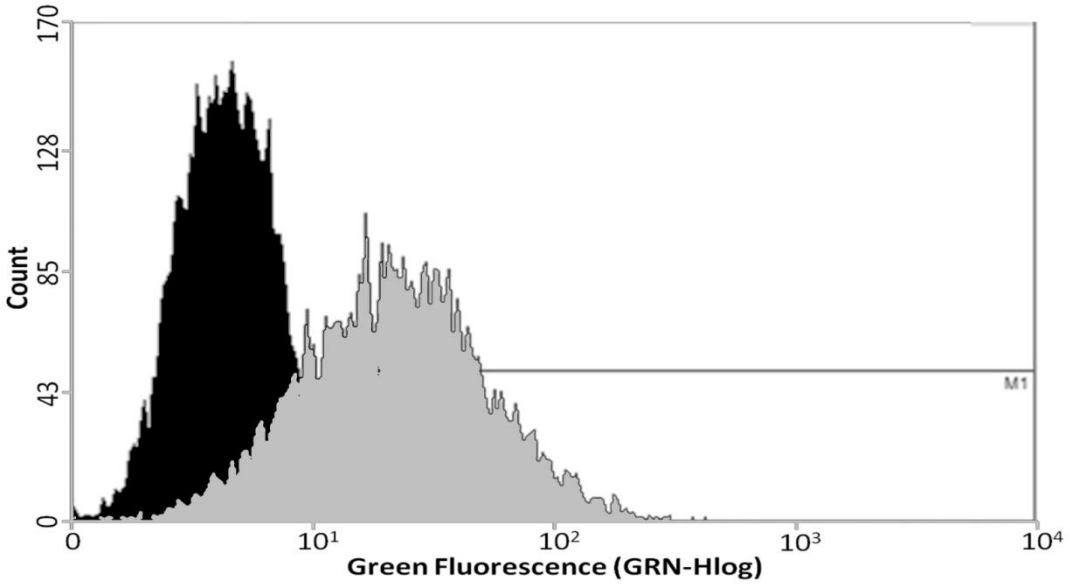

Figure 4-16: Representative flow cytometry histogram demonstrating respiratory burst following infection of J774A.1 macrophages with bacteria as demonstrated by an increase in green fluorescence (light grey) relative to uninfected controls (black).

4.9.1.2 **Flow cytometry data acquisition**

For each assay, gating of macrophages to exclude cellular debris was performed based on their scatter characteristics (Figure 4-17) and fluorescent thresholds adjusted as appropriate to control for background fluorescence. A minimum of 5000 gated events were acquired and analysed for each sample using a Guava® EasyCyte plus flow cytometer.

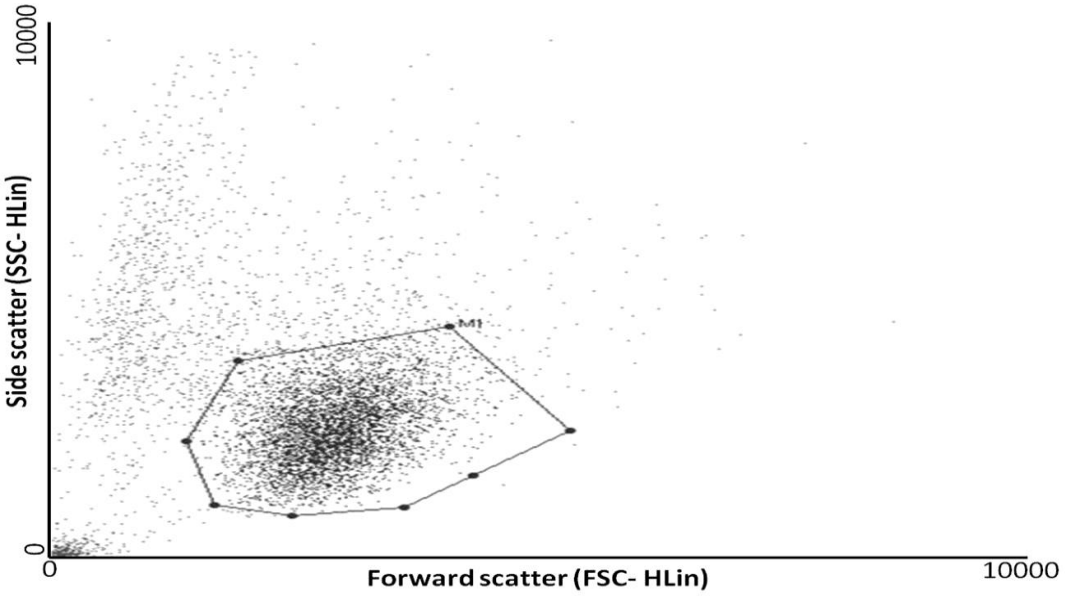

Figure 4-17: Representative flow cytometry scatter plot demonstrating gating of macrophages

4.9.1.3 **Optimisation of Flow cytometry**

4.9.1.3.1 Dissociation of monolayers from tissue culture plates

In order to perform flow cytometry it was necessary to rapidly generate a single cell suspension of viable cells from adherent monolayers. The EasyCyte system does not utilise sheath fluid, relying on capillary action to produce cell flow, meaning cell clumping must be avoided to prevent blockages of the flow chamber.

To achieve this several solutions were tested to ensure maximum possible cellular yield;

- Cell dissociation solution (Sigma)
- Citric saline solution (15mM Sodium citrate dihydrate, 135mM Potassium chloride in deionised water)
- 2mM EDTA in PBS
- 5mM EDTA + 5mg/ml lignocaine in PBS
- 10mM EDTA in PBS

Trypsin was avoided due to the potential for damage to the cell surface and consequent effect on respiratory burst (Bryant 1986).

Optimal dissociation of macrophages from tissue culture plates was achieved by co-incubating monolayers with 10mM EDTA in PBS at 37^{o}C, 95% air, 5%CO_2 for 10mins followed by gentle pipette mixing. Light microscopy on each occasion confirmed near complete dissociation of monolayers.

4.9.1.3.2 Confirmation and optimisation of bacterial phagocytosis

E. coli K12 was cultured overnight on LB agar at 37^{o}C in air, washed three times in PBS, adjusted to 1x10^{9}/ml in PBS, then heat killed by incubation at 60^{o}C for 30mins in a water bath. Bacteria were then labelled by first permeabilising with 1mM Ethyleneglycoltetraacetic acid (EGTA) pH7.4 followed by staining with the fluorophore Propidium iodide (PI) (120µM final concentration) for 2h at 4^{o}C on a

rocking platform in the dark (Lopez-Amoros 1995). Finally, bacteria were washed three times with 0.1% gelatin in HBSS, opsonised with 30% mouse serum at 37^{o}C in an orbital incubator (150rpm) for 1h, washed three times with PBS and stored in the dark at 4^{o}C until needed.

Staph. aureus Oxford was stained in an identical manner with the exception of omitting the EGTA permeablisation step.

E. coli XL1-blue expressing green fluorescent protein (GFP) (kindly donated by Prof. B. Campbell) was cultured, heat killed and opsonised as detailed above without the need for staining.

Flow cytometry was undertaken as previously described and confirmed a shift in fluorescence consistent with phagocytosis of bacteria by macrophages (Figure 4-18). Assays with variable MOI demonstrated an optimal phagocytosis signal with an MOI of 100 (Figure 4-19).

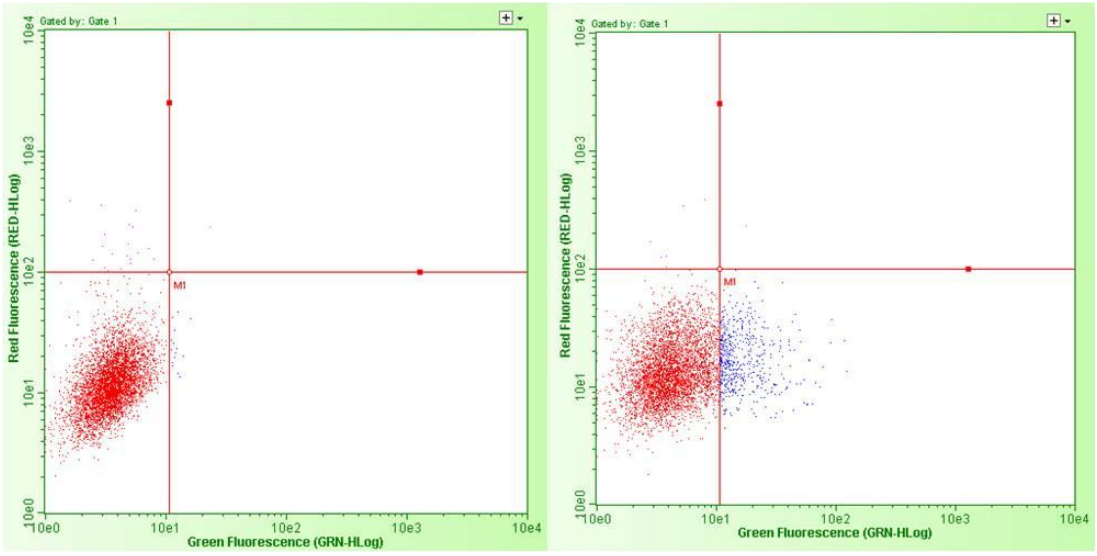

Figure 4-18: Phagocytosis of green fluorescent E. coli by macrophages as demonstrated by flow cytometry. Left panel shows uninfected macrophages. Right panel shows green shift of macrophages infected with GFP E. coli. Similar results obtained for PI labelled E. coli and S. aureus.

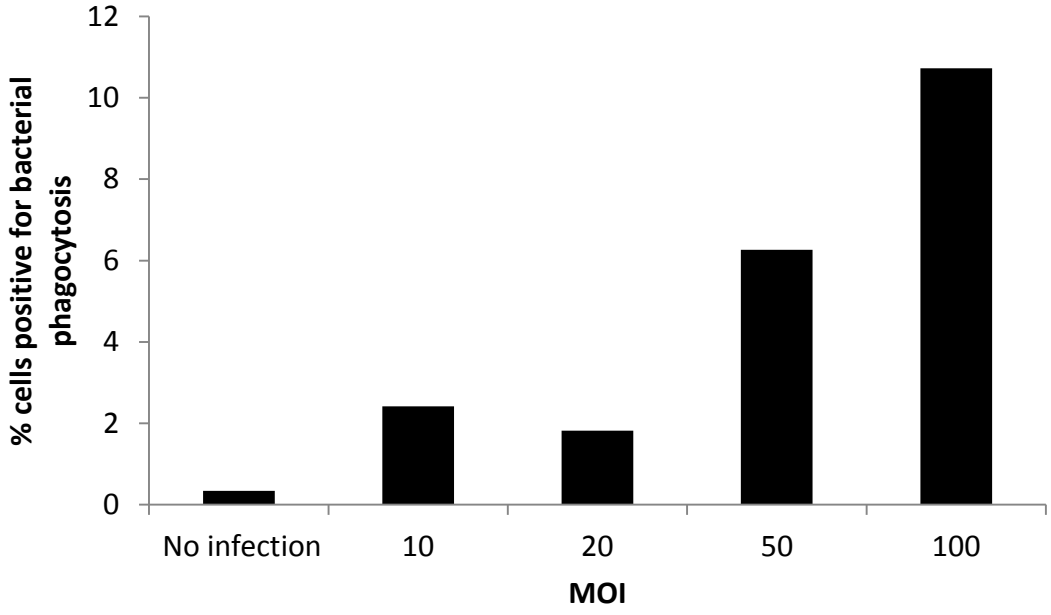

Figure 4-19: The number of macrophages returning a positive signal for phagocytosis as demonstrated by FACS increases with MOI. Data shown for GFP labelled E. coli. Similar data obtained with PI labelled E. coli and S. aureus.

4.9.1.3.3 Determination of optimal DHR concentration

Flow cytometry was performed as previously described with variable concentrations of DHR123. An optimal concentration should show low levels of background fluorescence with a measurable increase after induction of respiratory burst, which in this case was determined to be 50nM of DHR 123 (Figure 4-20 and 4-21).

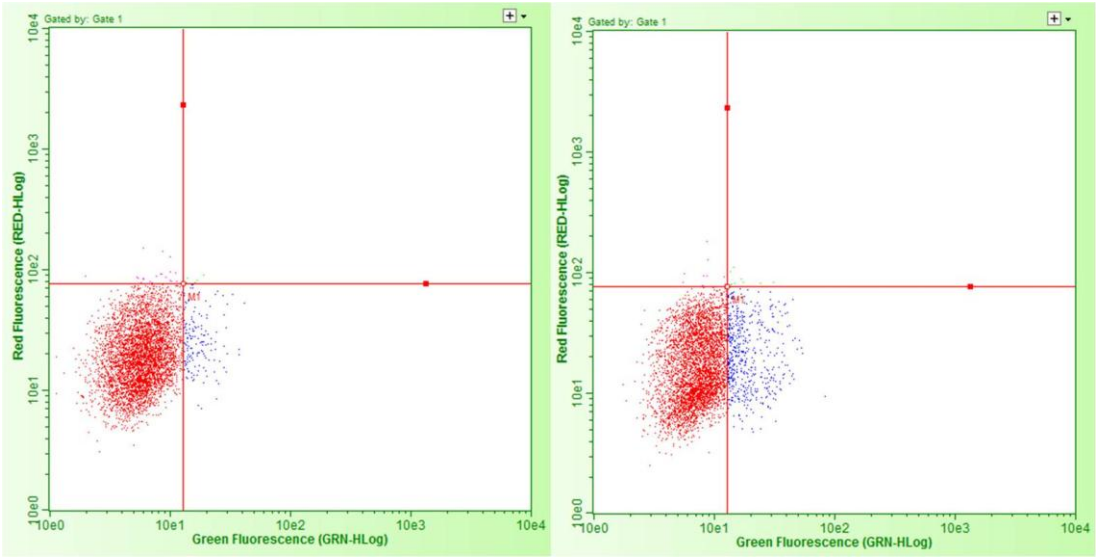

Figure 4-20: Optimisation of DHR123 concentration for flow cytometry. Uninfected macrophages showing minimal fluorescence (left panel) and infected macrophages showing a shift to the right (right panel, shift marked in blue) demonstrating intracellular respiratory burst. In this image 50nM DHR123 was used.

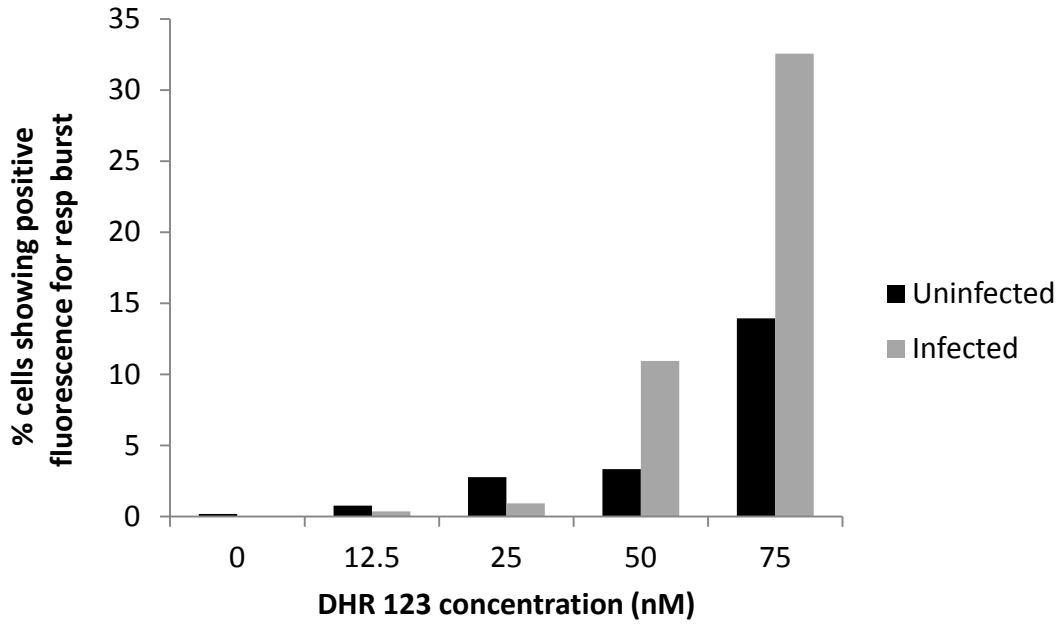

Figure 4-21: Percentage of cells returning a positive signal for respiratory burst at variable concentrations of DHR 123. Uninfected cells are shown in black and infected cells in grey. 50nM was optimal due to low levels of background fluorescence and a measurable increase after stimulation.

4.9.2 Chemiluminescence

Total respiratory burst in macrophages (pre-treated with or without hydroxychloroquine) was measured by a microplate luminescence assay using lucigenin (N,N'-dimethyl-9,9'-biacridinium dinitrate). Cells were seeded into white-walled, clear bottom 96 well plates at $5x10^5$/mL, primed with 100 U/ml interferon gamma (IFNγ) and cultured overnight. Following three sterile PBS washes, cells treated with 1-10μg/ml HCQ, 20-80nM Vitamin D or vehicle control for 3h in phenol red-free, antibiotic-free RPMI-1640 media supplemented with 10% vol/vol FBS and 2mM glutamine. Lucigenin was then added to final concentration of 5μM, (a level at which lucigenin does not undergo redox cycling (Li, 1998)), and respiratory burst initiated with 1μM phorbol-12-myristate-13-acetate (PMA). Luminescence was read immediately using a BMG Labtech Fluostar Omega microplate reader (BMG Labtech; Offenburg, Germany), and subsequently every 5 min for 1h.

4.10 Neutrophil migration assay

Macrophage-mediated human neutrophil chemotaxis was quantified using a CytoSelect™ 96-Well Cell Migration Assay, 3 μm pore diameter (Cambridge Bioscience, Cambridge, UK) according to the manufacturer's instructions. Chemotactant in the lower chamber was X-Vivo 15 medium from 10^5 MDMs either uninfected or infected for 6h with *E.coli* HM605 or K12 (MOI 25). CyQuant® GR fluorescence was measured using an F200 microplate reader (Tecan) using excitation at 485nm, emission detection at 535nm (Figure 4-22).

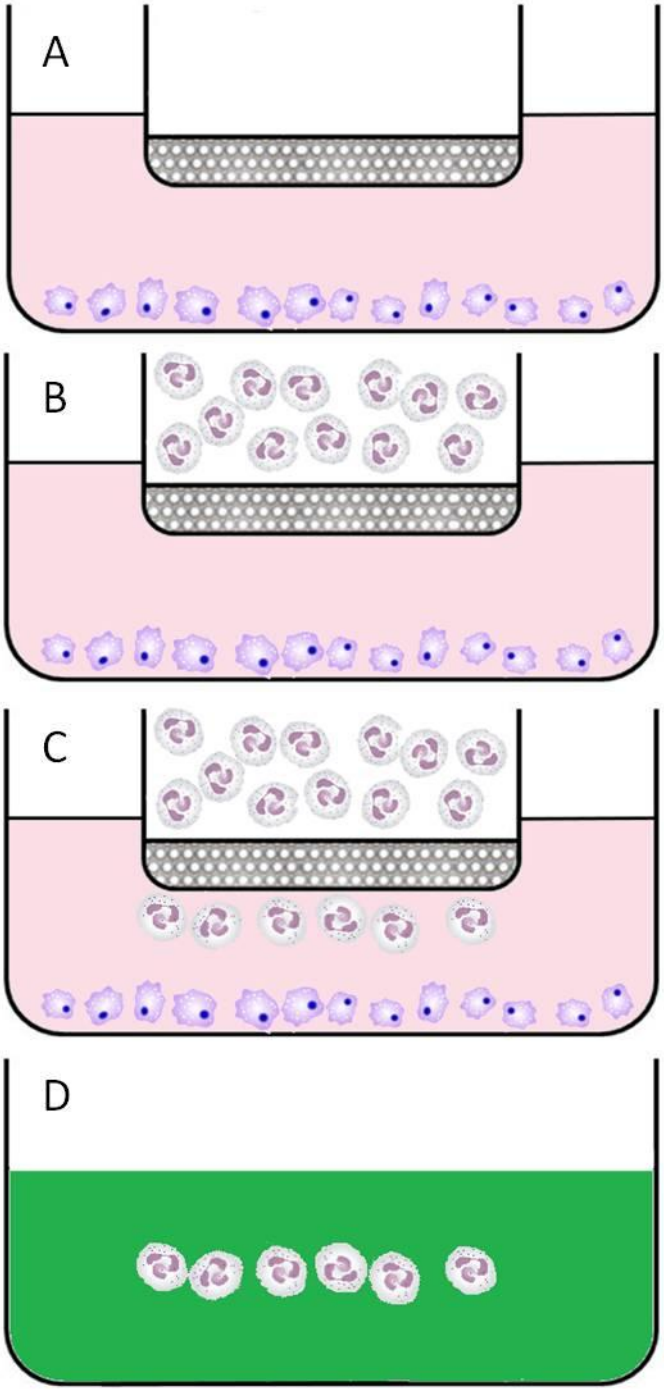

Figure 4-22: Schematic representation of human neutrophil migration assay. (A) Medium from human macrophages either infected or uninfected with E. coli is placed in the bottom chamber. (B) 2.5x10^5 human nuetrophils were added to the top chamber above a 0.3µm pore (C) Plates were incubated for 2h to allow migration of neutrophils through to the lower chamber with non-migratory cells retained in the upper chamber. (D) Migratory neutrophils were detached, lysed and CyQuant® GR dye added for detection

4.11 Cytokine release by macrophages

Secretion of human Interleukin 6 (IL-6), Interleukin 8 (IL-8) and tissue necrosis factor alpha (TNFα) from MDMs in response to bacterial infection was quantified by sandwich ELISA as per the manufacturer's instructions (R&D systems, Abingdon, UK (product codes: IL-6, D6050; IL-8, D800C; TNFα, DTA00C)) according to the manufacturer's instructions. Release of murine IL-6 and TNFα in response to HCQ-treated J774a.1 macrophages, with or without HM605 infection, was similarly determined by sandwich ELISA (R&D systems - product codes: IL-6, M6000B; TNFα, SMTA00B). OD_{450nm} was measured using a Sunrise™ microplate reader (Tecan). In each assay standard curves were obtained to allow quantification of cytokine release (Figure 4-23-4-27).

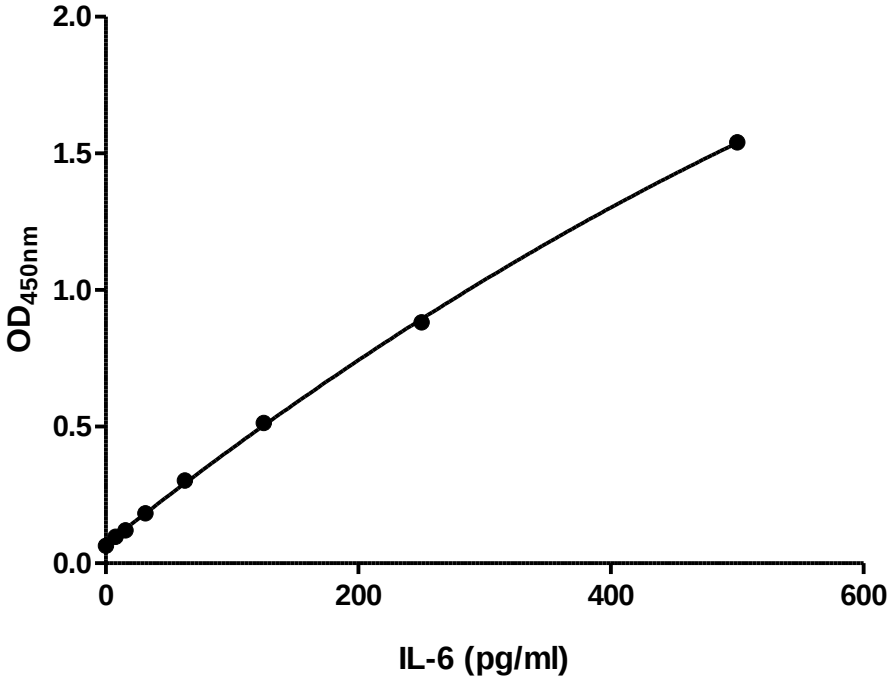

Figure 4-23: Standard curve for murine IL-6 concentration against OD_{450nm}

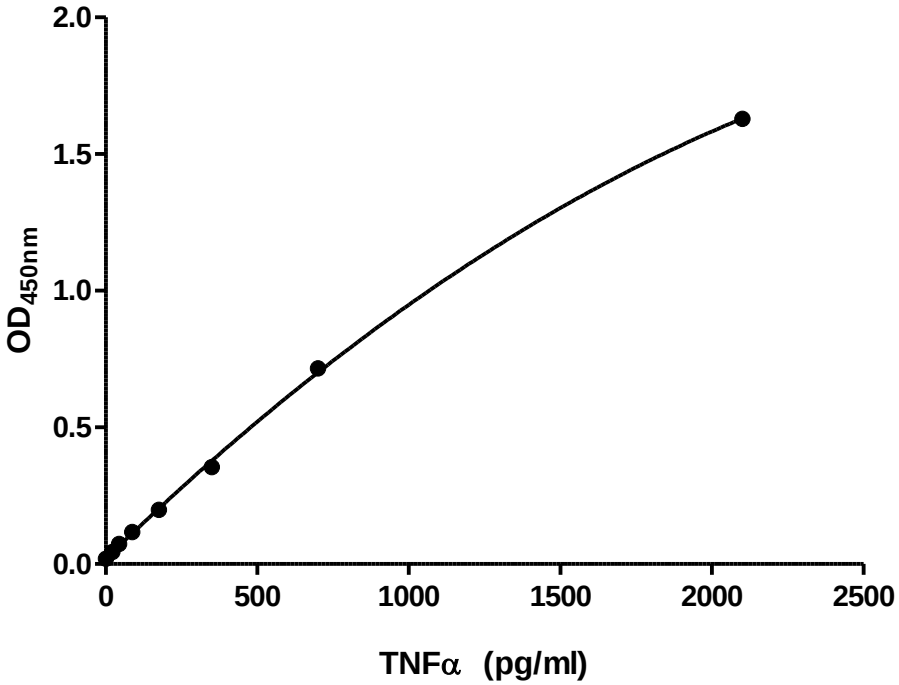

Figure 4-24: Standard curve for murine TNFα concentration against OD_{450nm}

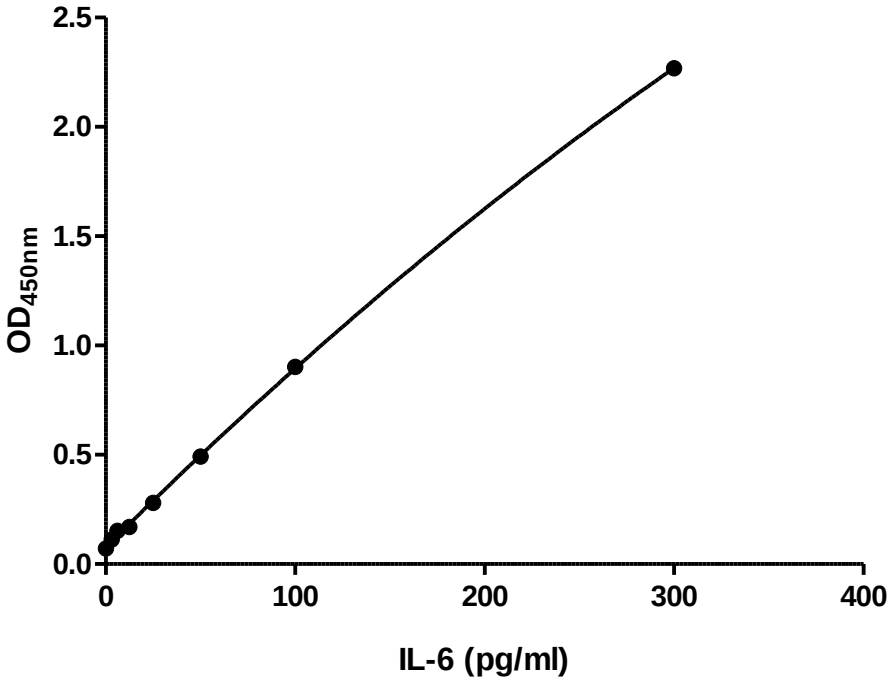

Figure 4-25: Standard curve for human IL-6 concentration against OD_{450nm}

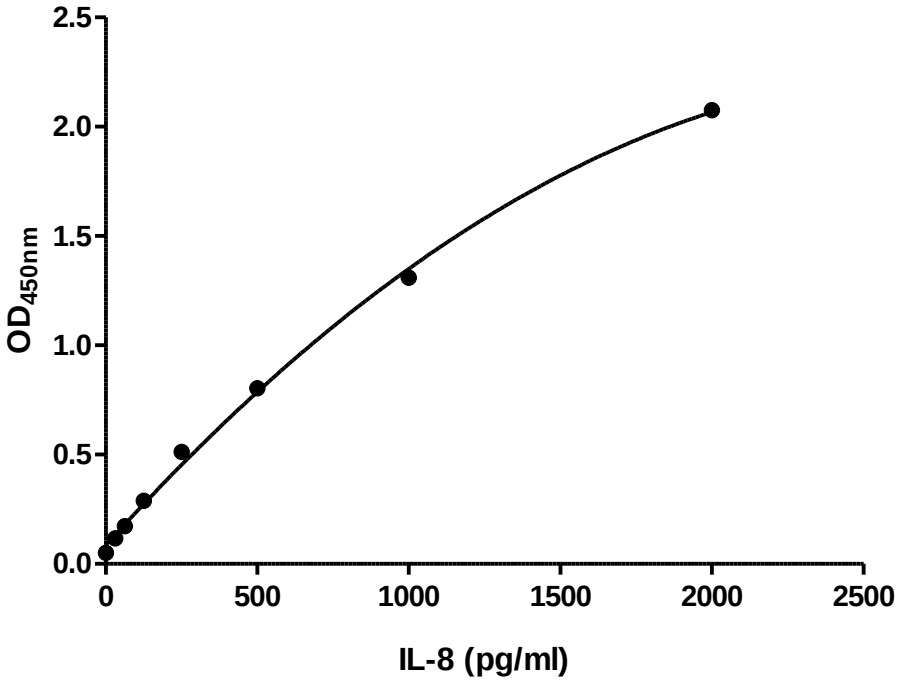

Figure 4-26: Standard curve for human IL-8 concentration against OD_{450nm}

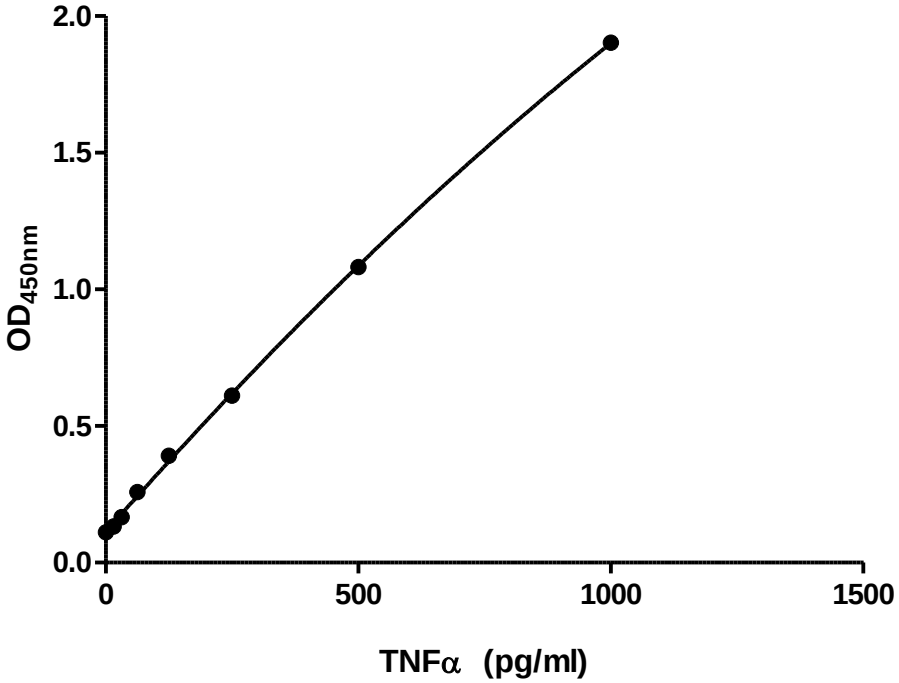

Figure 4-27: Standard curve for human TNFα concentration against OD_{450nm}

4.12 Statistics

N numbers indicate the total number of independent experiments performed with cells seeded in triplicate for each experiment unless otherwise indicated. Independent sample groups were assessed for normality and equality of variances. As appropriate, comparisons for data sets between CD and HC were analysed by unpaired t-test or Mann-Whitney U. Dose responses were analysed using Cuzick's test for trend, followed if significant by Dunnett's test. Multiple treatment groups were first analysed using either one-way analysis of variance (ANOVA) or a non-parametric Kruskal-Wallis test and subsequently followed by pair-wise comparisons of treatment means (StatsDirect v2.6.8; Sale, UK). Differences were considered significant when two-tailed $P<.05$.

4.13 Human Macrophage Project Sample size calculation

Sample size calculations were performed to take account of the potential for both type I and type II errors. Type I errors occur when the null hypothesis is rejected when it's true, the risk of which is determined by the critical p value (typically $p<0.05$) and is referred to as the significance level α. Type II errors occur with rejection of the null hypothesis when it's false, the probability of which is termed β. Statistical power is an expression of the chance of a type II error occurring and is defined as $1-\beta$. Sample size calculations were performed as per (Campbell 1995) and confirmed as per (Armitage 2005; Fleiss 2003) to give a 80% power at 5% significance.

The number of samples in each group is given by the formula:

$$n = \frac{((Z_{1-\alpha/2} + Z_{1-\beta})^2)*((Pa(1-Pa)+Pb(1-Pb))}{\delta^2}$$

Pa = Expected proportion in group A

Pb = Expected proportion in group B

δ = Difference between group A and group B (i.e. Pa – Pb)

$Z_{1-\alpha/2} = 1.96$ (from (Campbell 1995))

The anticipated difference between the proportion of Crohn's disease patients and controls in achieving complete killing of intramacrophage bacterial is 62.5% (Elliott 2011). Using the same data the mean change in intramacrophage *E. coli* counts between 1h and 4h was +394% and -65.13% for Crohn's disease patients and healthy controls respectively. However the standard deviation was not available

meaning it was not possible to perform sample size calculations using this data. I therefore estimated that a significant difference would be found if we recruited 5 patients and 5 controls to the study, although ultimately 10 patients and 10 controls were recruited to account for the possibility that the effect size may be smaller than previously reported (Table 7).

Power (β)	**Significance (α)**	**Difference (δ)**	**Sample Size needed (each group)**
80%	5%	90%	1
80%	5%	80%	2
80%	5%	70%	4
80%	5%	60%	5
80%	5%	50%	6
80%	5%	40%	8
80%	5%	30%	12

Table 7: a priori power calculation for human macrophage project.Based on previous studies a sample size of 5 was calculated to give 80% power at 5% significance.

5 Investigation into the ability of murine and human macrophages to kill Crohn's disease mucosa-associated *E. coli*

5.1 Introduction

Genome wide association studies implicate the innate immune system in Crohn's disease as a possible underlying pathogenic process, including pathways relevant to handling of intracellular infection. Indeed an increase in mucosa-associated *E. coli* has been repeatedly reported in patients with Crohn's disease, and these bacteria survive and replicate within macrophages leading to granuloma formation. Further, functional studies demonstrate altered macrophage cytokine release and impaired neutrophil chemotaxis in response to both mucosal injury and subcutaneous *E. coli* injection in patients with Crohn's disease, suggesting a failure of macrophage function and subsequent bacterial persistence may represent a common pathogenic process.

Early studies (Mee 1980) have shown monocytes from patients with Crohn's disease have a normal ability to kill Gram-positive organisms, but it is now clear that macrophages have specific pathways for dealing with Gram-negative organisms, specifically the SLAM (Signalling lymphocyte activation molecule) system (Berger 2010), and it is therefore still possible that patients with Crohn's disease may have a specific defect in the handling of Gram-negative infection. To date no study has adequately addressed this and the innate ability of macrophages from patients with Crohn's disease to handle Gram-negative infection remains unclear.

5.2 Hypothesis

Defective macrophage killing of bacteria, particularly *E. coli*, may be an underlying pathogenic mechanism in Crohn's disease.

5.3 Aims

1. To determine the ability for *E. coli* to survive within murine and human (including Crohn's disease) macrophages

2. To investigate patient factors which may affect the ability of human macrophages to kill intracellular bacteria

5.4 Materials and methods

Unless otherwise stated the material and methods are as described in chapter 4. Thus patient recruitment is as detailed in section 4.3, and mammalian cell culture technique as in section 4.5.

Comparison of baseline characteristics for healthy controls and Crohn's disease patients are detailed in Figure 5-1. Apart from a higher hsCRP level in patients with Crohn's disease (mean 7.8mg /L CD, 2.2mg/L HC, P=0.03) no significant differences were demonstrated between the two groups, with comparable age (mean 44.8y CD, 51.7y HC, P=0.297) and smoking prevalence (30% CD, 10% HC, P=0.29), equivalent serum Vitamin D levels (mean 26nmol/L CD, 30.8nmol/L HC, P=0.43) and equal prevalence of the Crohn's disease associated SNPs tested for in this study. No patients or controls were homozygote for *NOD2* or *IRGM* variants, with one Crohn's disease patient homozygote for *ATG16L1* variant.

Individual patient characteristics are summarised in Figure 5-2. Further details are available in Table 10 (Appendix 1) including individual results for intramacrophage replication assays.

	Healthy controls (N=10)	Crohn's disease (N=10)	*P* value
Male (%)	50%	20%	0.177
Age (y)	51.7 ± 3.8	44.8 ± 5.2	0.297
Smoker (%)	10%	30%	0.290
Harvey Bradshaw Index	n/a	5.0 ± 1.7	n/a
Serum Vit D3 (nmol/L)	30.8 ± 4.7	26.0 ± 3.7	0.433
hsCRP (mg/L)	2.2 ± 0.6	7.8 ± 2.0	0.03
***NOD2* homozygote w/t**	90%	80%	0.556
***NOD2* heterozygote**	10%	20%	0.556
***ATG16L1* homozygote w/t**	40%	40%	0.999
***ATG16L1* heterozygote**	60%	50%	0.677
***ATG16L1* homozygote variant**	0%	10%	0.343
***IRGM* homozygote w/t**	80%	70%	0.628
***IRGM* heterozygote**	20%	30%	0.628

Figure 5-1: Baseline characteristics of Crohn's disease patients and healthy controls from whom monocyte derived macrophages were generated. Data presented as % population affected or mean ± SEM as relevant. Specific SNPs analyzed were; NOD2/CARD15 (rs2066844, rs2066845 and rs2066847), ATG16L1 (rs2241880) and IRGM (rs13361189). Abbreviations used; hsCRP (high-sensitivity C-reactive protein), n/a (not applicable), wt (wild-type). P values as shown obtained by Mann-Whitney U.

	Sex	Age	Current smoker	Montreal	Disease duration (years)	Harvey Bradshaw Index	Vitamin D3 (nM)	hsCRP (mg/L)	No. of altered alleles NOD2 (rs2066844)	No. of altered alleles NOD2 (rs2066845)	No. of altered alleles NOD2 (rs2066847)	No. of altered alleles ATG16L (rs2241880)	No. of altered alleles IRGM (rs13361189)
HC 1	M	36	No	n/a	n/a	n/a	45	0.4	0	0	0	1	0
HC 2	F	59	Yes	n/a	n/a	n/a	20	3.07	0	0	0	1	0
HC 3	F	56	No	n/a	n/a	n/a	42	5.66	0	0	0	0	1
HC 4	M	51	No	n/a	n/a	n/a	46	0.57	0	0	0	0	0
HC 5	M	74	No	n/a	n/a	n/a	22	2.65	0	0	0	0	0
HC 6	F	34	No	n/a	n/a	n/a	10	0.35	0	0	0	1	0
HC 7	M	57	No	n/a	n/a	n/a	41	4.62	0	0	0	1	0
HC 8	F	43	No	n/a	n/a	n/a	29	3.37	0	0	0	1	0
HC 9	M	59	No	n/a	n/a	n/a	8	1.12	0	0	0	0	0
HC 10	F	48	No	n/a	n/a	n/a	45	0.63	1	0	0	1	1
CD 1	F	38	No	A2, L1, B3p	1-5	0	31	2.75	1	0	0	0	0
CD 2	F	64	Yes	A3, L1, B2	5-10	5	12	14.15	0	0	1	1	0
CD 3	F	45	No	A2, L1, B2	>10	0	23	1.46	0	0	0	1	1
CD 4	F	21	No	A1, L3, B1	>10	2	13	1.04	0	0	0	0	1
CD 5	F	48	No	A2, L1, B2	5-10	7	43	18.3	0	0	0	2	0
CD 6	M	39	No	A2, L1, B2	>10	10	16	10.14	0	0	0	0	0
CD 7	F	73	No	A3, L2, B1	>10	9	38	14.91	0	0	0	0	0
CD 8	M	24	No	A1, L1, B3p	>10	1	36	3.95	0	0	0	1	1
CD 9	F	40	Yes	A2, L1, B2	>10	16	33	4.84	0	0	0	1	0
CD10	F	56	Yes	A2, L3, B2p	>10	0	15	6.95	0	0	0	1	0

Figure 5-2: Individual characteristics of patients and healthy controls. rs numbers represent specific SNPs analysed. Abbreviations used; hsCRP (high-sensitivity C-reactive protein), n/a (not applicable)

5.5 Results

5.5.1 Crohn's disease associated *E. coli* replicate within murine macrophages

Initial assays confirmed the previously described ability for Crohn's disease associated *E. coli* to survive and replicate within both murine and human macrophages. *E. coli* K12 survived within J774a.1 murine macrophages (n=3, 1.43 ± 0.34 fold change in intracellular bacteria over 3h (mean ± SEM)) whereas Crohn's disease associated *E. coli* HM605 and LF82 both demonstrated net replication over the same period (HM605; n=3, 7.00 ± 1.14, P<0.001. LF82; 3.84 ± 0.42, n=3, P<0.001) (significance reported relative to K12, ANOVA) (Figure 5-3).

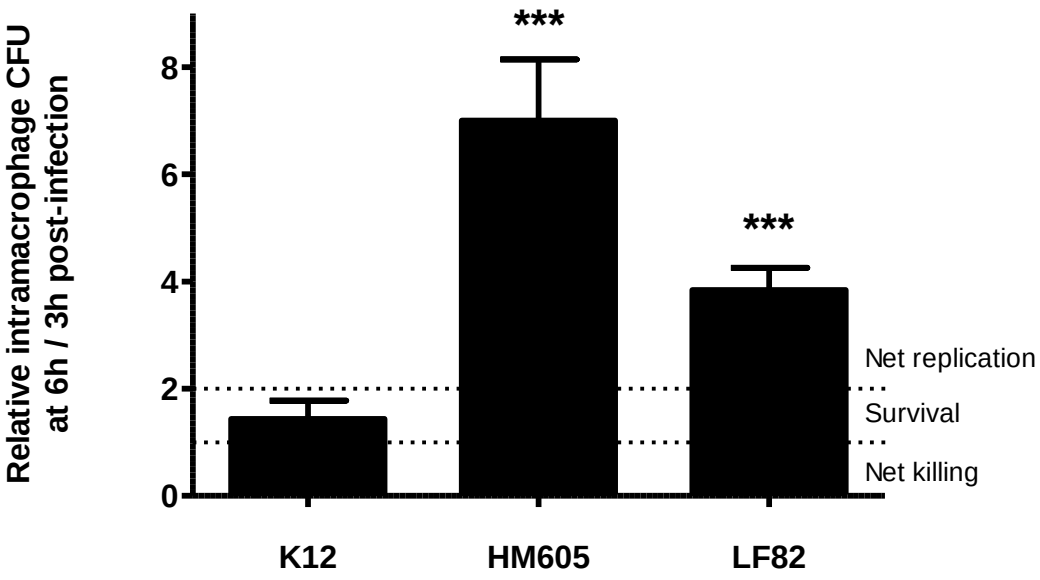

*Figure 5-3: J774a.1 murine macrophages are ineffective at killing internalised E. coli. Lab reference strain K12 demonstrated intra-macrophage survival whilst Crohn's disease associated E. coli (HM605 and LF82) demonstrated net replication (n=3, ***P<0.001, ANOVA).*

5.5.2 Replication of intracellular bacteria within human monocyte-derived macrophages

Neither Crohn's disease nor healthy control peripheral blood MDM were very effective at killing *E. coli*. Crohn's disease MDM allowed net replication of phagocytosed *E. coli* HM605 (n= 10 patients, 1.08 mean fold replication over 3 hours, 95% CI 0.39–1.78) as did healthy controls (n = 9, 1.50 mean fold replication, 95% CI 1.02–1.97, P = 0.15, Mann–Whitney U test). Similar results were obtained for replication of phagocytosed *E. coli* K12 (CD n = 10, 0.54 mean fold replication, 95% CI 0.24–0.84; HC, n = 10, 0.86 mean fold replication, 95% CI 0.47–1.26; P = 0.14) and also for phagocytosed *S. aureus* (CD n = 10, 0.37 mean fold replication, 95% CI 0.18–0.55; HC, n = 10, 0.48 mean fold replication, 95% CI 0.39–0.57; P = 0.09) (Figure 5-4).

In light of the lack of significant differences between the two study groups pooled results from both healthy controls and Crohn's disease patients were analysed and demonstrated a small net killing effect of *E. coli* K12 (n=20, 0.70 fold change in intracellular bacteria over 3h, 95% confidence interval (CI) 0.46-0.94) and net replication of *E. coli* HM605 (n=19, 1.28 fold change, 95% CI 0.85-1.71, $p<0.05$ relative to K12, 2-tailed T-test) in keeping with the phenotype of these bacteria observed seen within murine macrophages.

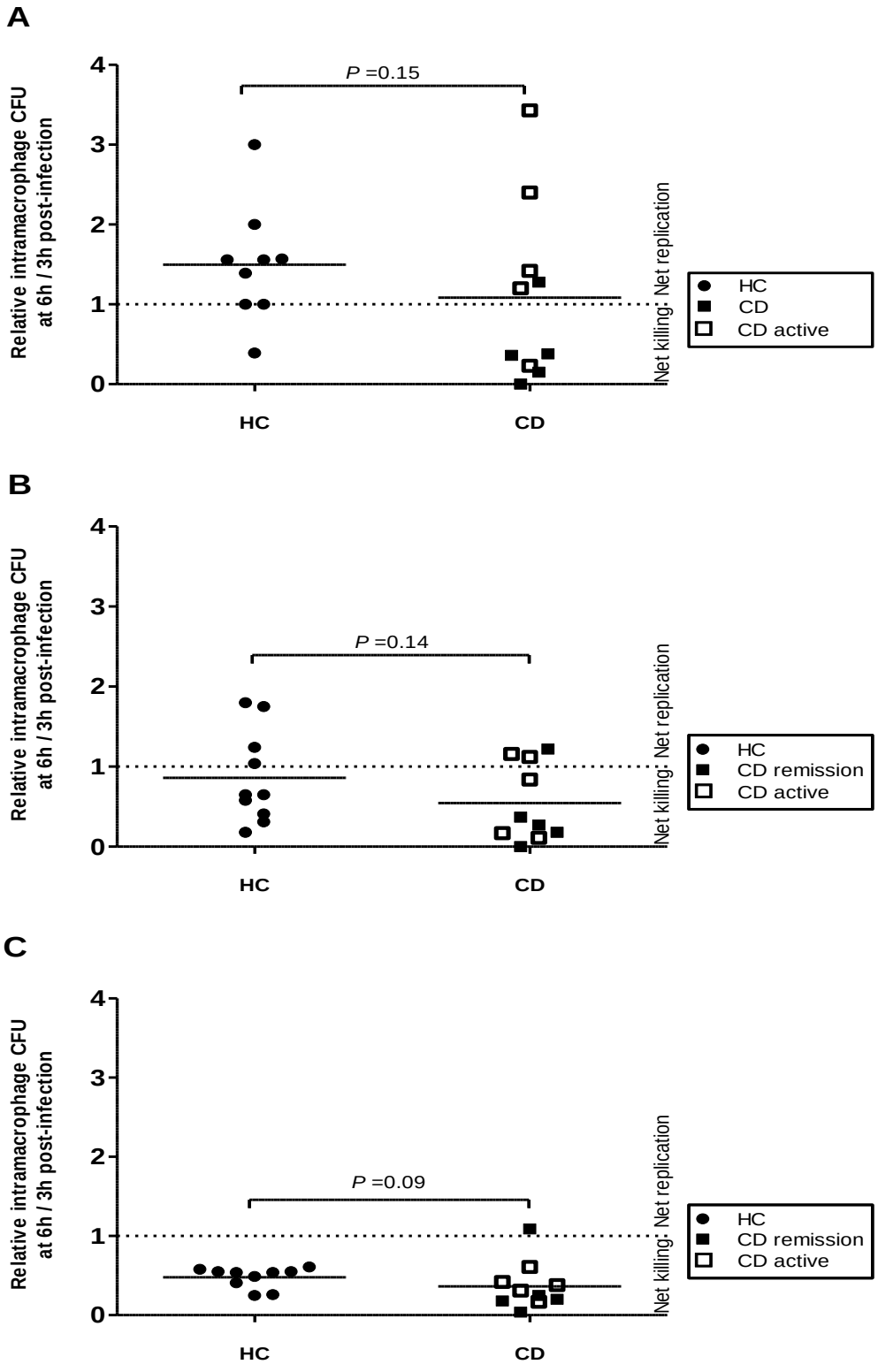

Figure 5-4: MDM isolated from patients with Crohn's disease (CD) and healthy controls (HC) possess similar ability to kill phagocytosed bacteria. No significant differences were seen between CD and HC in the killing of E. coli HM605 (A), E. coli K12 (B) or Staph. aureus Oxford strain (C). Each point represents individual patient mean of triplicate samples, horizontal bar indicates overall mean. P values obtained using Mann-Whitney U test.

5.5.3 Effect of possession of Crohn's disease associated *NOD2* and *ATG16L1* single nucleotide polymorphisms on killing of intramacrophage *E. coli* HM605

No correlation was seen between the total number of affected *NOD2*/*CARD15* and *ATG16L1* alleles and ability to kill intramacrophage HM605, but none of the patients with Crohn's disease studied were homozygous for affected *NOD2*/*CARD15* or *IRGM* alleles, and only 1 was homozygous for affected *ATG16L1* (Figure 5-5). It is however worthy of note that the single patient homozygous for variant *ATG16L1* had active disease and demonstrated the highest intramacrophage replication of HM605 (3.43 fold increase over 3h, upper empty square, right hand column, Figure 5-5).

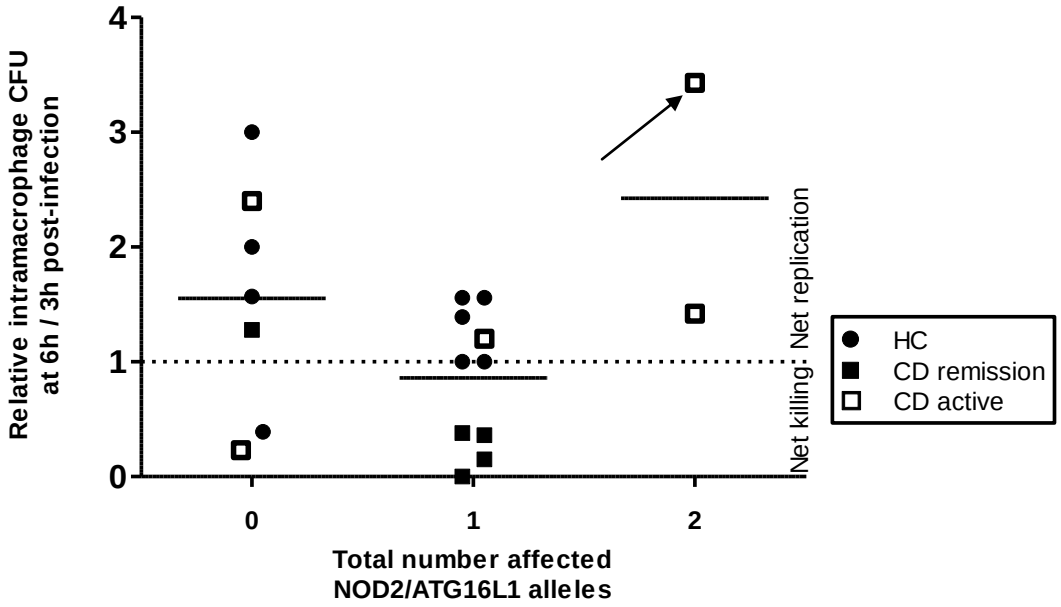

Figure 5-5: Presence of NOD2/CARD15 and ATG16L1 variants were not associated with altered ability to kill intra-macrophage Crohn's disease E. coli HM605. Patient NOD2/CARD15 and ATG16L1 status reported as number of disease associated alleles. Arrow indicates the single patient homozygous for variant ATG16L1

5.5.4 Effect of Crohn's disease activity on killing of intramacrophage *E. coli* HM605

There was a non-significant trend toward increased intramacrophage replication of HM605 among patients with Crohn's disease with active disease (mean fold replication = 1.74 [95% CI, 0.67–2.81]) compared with those with inactive disease (mean = 0.43 [95% CI, 0.0–0.87], P = 0.10), but replication of HM605 in patients with active disease was similar to that seen in healthy controls (Figure 5-6).

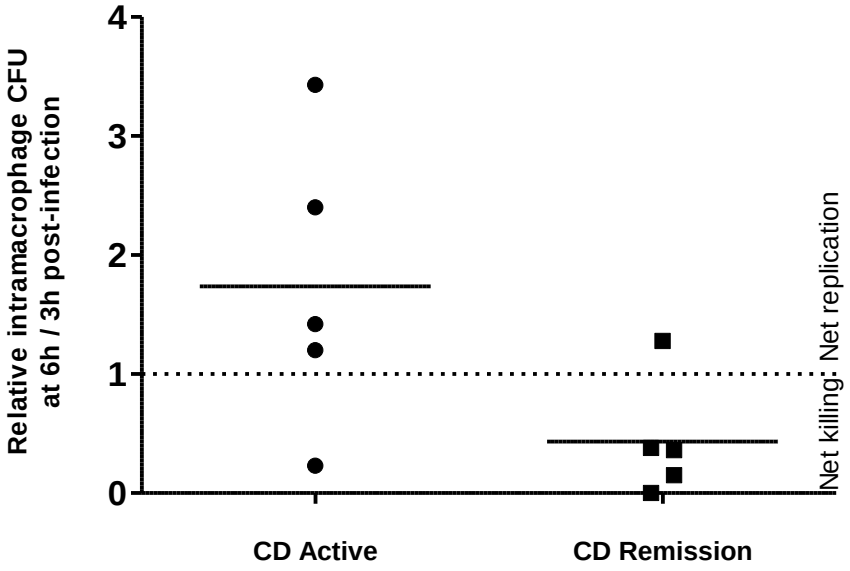

Figure 5-6: Crohn's disease activity defined by Harvey Bradshaw index did not significantly affect the ability of monocyte derived macrophages to kill intracellular Crohn's disease associated E. coli HM605. Each point represents individual patient mean of triplicate samples, horizontal bar indicates overall mean. (P=0.10, Mann-Whitney U).

5.5.5 Correlation between serum highly sensitive CRP levels and killing of intramacrophage bacteria

No correlation was seen between systemic inflammation, as measured by high sensitivity CRP (hsCRP), and the ability of monocyte derived macrophages to kill intracellular bacteria. Spearman's rank showed no correlation between hsCRP and killing of *E. coli* HM605 (rank -0.08, P=0.75, 2 tailed T-test), K12 (rank -0.35, P=0.13) nor *Staph aureus* Oxford strain (rank -0.31, P=0.19) (Figure 5-7)

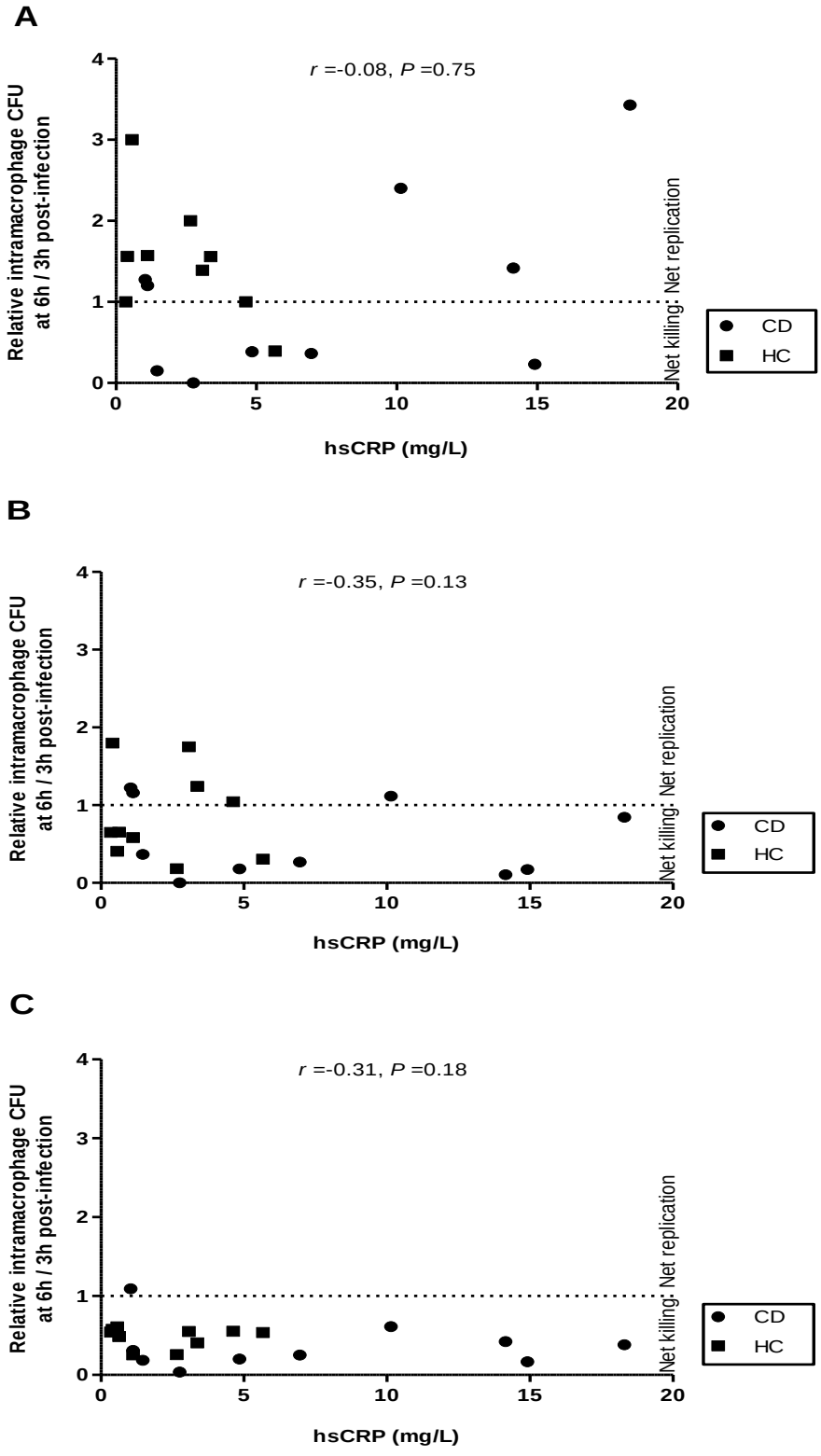

Figure 5-7: Ability of HMDMs to kill intracellular bacteria was not associated with systemic inflammation as determined by hsCRP. Each point represents individual patient mean of triplicate samples. r value obtained using Spearman's rank, P values obtained using Mann-Whitney U test. (A) HM605, (B) K12, (C) Staph. aureus Oxford strain.

5.5.6 Effect of smoking status on killing of intramacrophage bacteria

Smoking status, either current, previous or never smoker, did not affect the survival of bacteria within monocyte-derived macrophages.

For *E. coli* K12, relative change in intramacrophage bacteria over 3h in never smokers was 0.69 (n=7, 95% CI 0.24-1.14), in ex-smokers was 0.66 (n=9, 95% CI 0.37-0.90) and in current smokers was 0.82 (n=4, 95% CI 0.06-1.58) (P=0.98, ANOVA). Similarly for *E. coli* HM605, relative bacterial change in never smokers was 1.63 (n=7, 95% CI 0.64-2.62), in ex-smokers was 1.07 (n=9, 95% CI 0.61-1.52) and in current smokers was 1.09 (n=4, 95% CI 0.61-1.58) (P=0.59, ANOVA). For *S. aureus* Oxford strain, relative change in never smokers was 0.44 (n=7, 95% CI 0.30-0.58), in ex-smokers was 0.42 (n=9, 95% CI 0.22-0.62) and in current smokers was 0.38 (n=4, 95% CI 0.25-0.51) (P=0.79, ANOVA) (Figure 5-8).

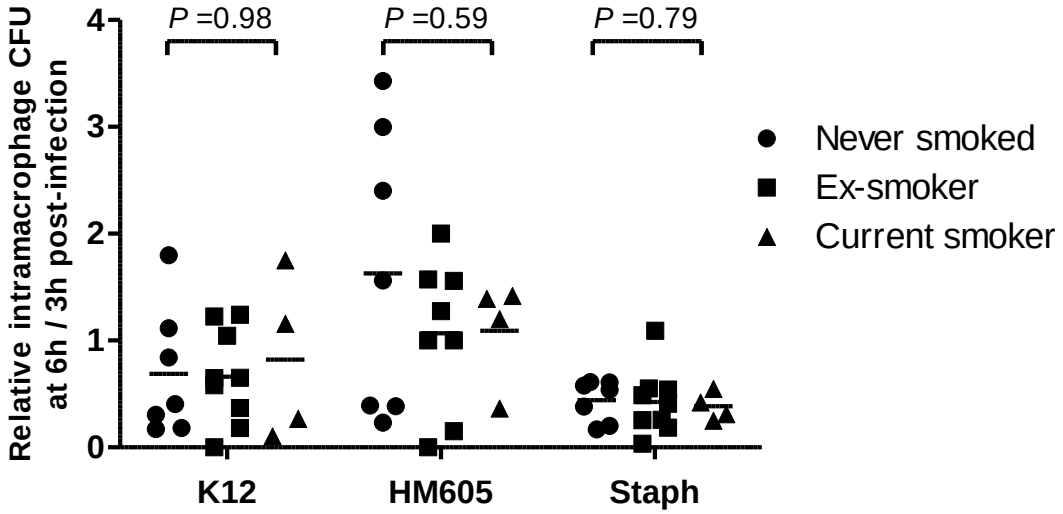

Figure 5-8: Smoking status did not affect the ability of HMDMs to kill intracellular bacteria. Each point represents individual patient mean of triplicate samples, horizontal bar indicates overall mean. P values obtained using ANOVA.

5.5.7 MDM from healthy controls and patients with Crohn's disease have equivalent ability to generate neutrophil chemoattractants in response to *E. coli* infection

E. coli infected monocyte-derived macrophages induced greater neutrophil chemotaxis than uninfected controls; for K12 infected HC derived macrophages chemotaxis relative to uninfected controls was 223% (n=7, 95% CI 186-261%), for K12 infected CD derived macrophages 220% (n=10, 178-263%), for HM605 infected HC macrophages 265% (n=7, 95% CI 246-285%) and for HM605 infected CD macrophages 255% (n=10, 95% CI 231-280%)($P<0.001$, ANOVA). No significant differences were seen in chemotaxis induced by MDMs from healthy controls and Crohn's disease patients in response to K12 infection ($P=0.89$, 2 tailed T-test) or HM605 infection ($P=0.42$, 2 tailed T-test) (Figure 5-9). No significant difference was seen in the chemotactic response generated by MDM derived from patients with active CD compared with those with inactive disease ($P = 0.60$).

Cytokine production in response to HM605 infection was equivalent in the two groups. Mean TNFα production by MDMs for healthy controls was 1823pg/mL (n=7, 95% CI 1591-2056pg/mL) and for Crohn's disease patients was 1915pg/mL (n=10, 95% CI 1711-2119pg/mL, $P=0.27$, Mann-Whitney). Mean IL-8 release by healthy controls was 6639pg/mL (n=7, 95% CI 4065-9213) and by Crohn's disease patients was 6644pg/mL (n=10, 95% CI 5066-8222pg/mL, $P=0.89$, Mann-Whitney U). For IL-6 mean levels from HC was 1210pg/mL (n=7, 95% CI 711-1709pg/mL) and from CD was 1720pg/mL (n=10, 95% CI 1178-2262 pg/mL) (Figure 5-10).

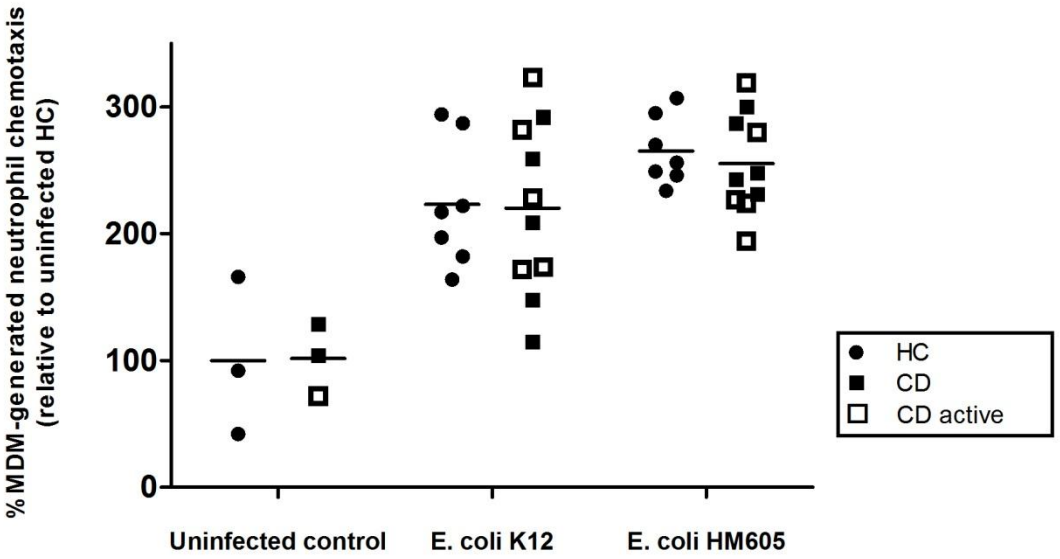

Figure 5-9: E. coli-infected MDM from patients with CD and HC have equivalent ability to generate neutrophil chemoattractants. MDM-induced neutrophil chemotaxis was equivalent between CD (N = 10) and HC (N = 7) whether uninfected or infected with E. coli (either AIEC HM605 orK12). Infected macrophages induced greater neutrophil chemotaxis than uninfected controls (P < 0.001; ANOVA).

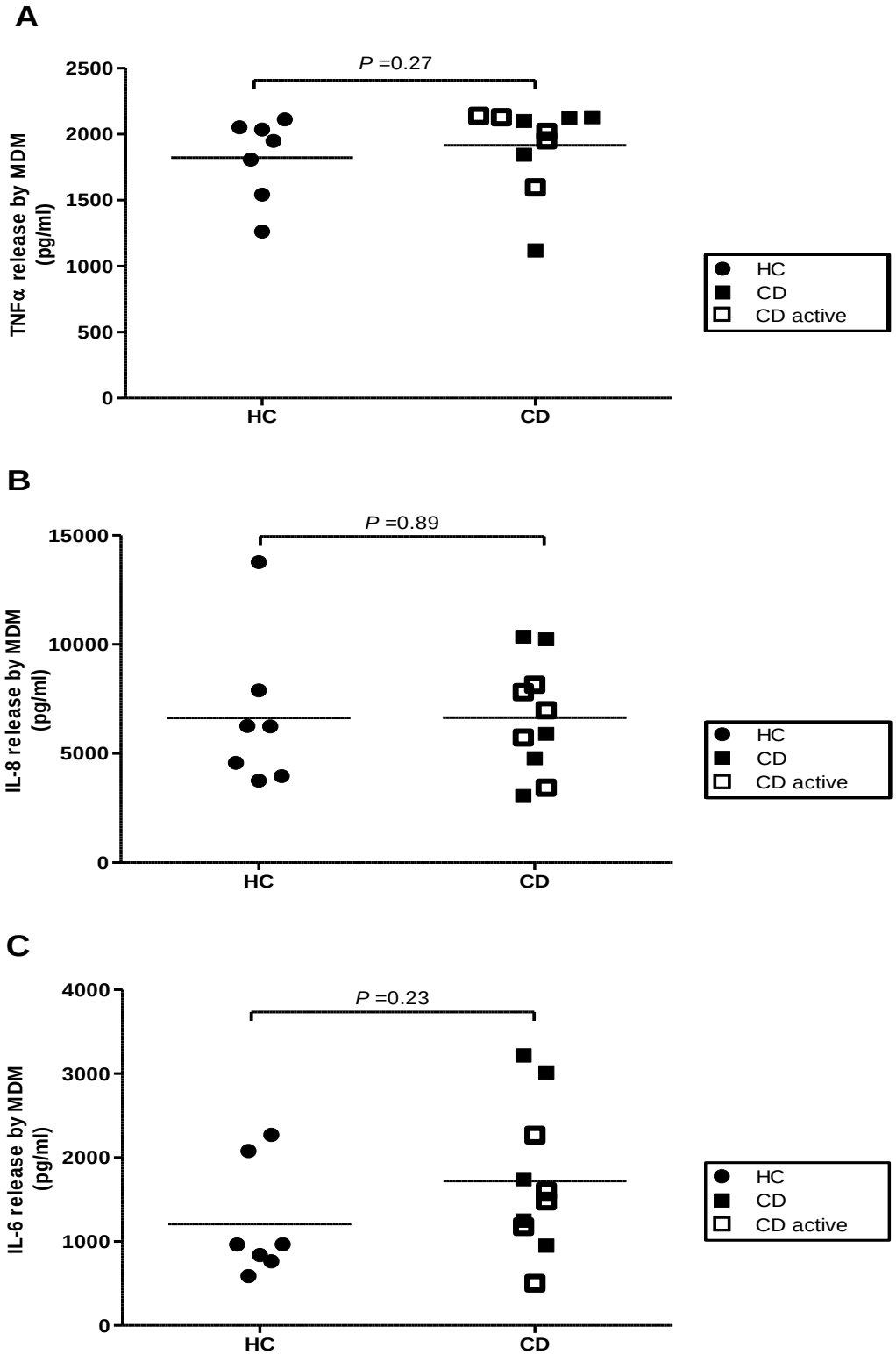

Figure 5-10: HMDM production of (A)TNF-a, (B) IL-6 and (C) IL-6 in response to HM605 infection were also equivalent between CD (N= 10) and HC (N= 7). All assays performed in triplicate. P values by Mann–Whitney U test.

5.6 Discussion

The data shown here confirm the relatively poor ability of monocyte-derived macrophages to kill intracellular *E. coli* but clearly shows no significant difference between healthy controls and Crohn's disease patients' handling of intramacrophage bacteria. Furthermore, both groups had an equivalent ability for *E. coli* infected MDMs to induce neutrophil chemotaxis and release chemoattractants, suggesting patients with Crohn's disease do not have significant innate failure of macrophage function. The ability for macrophages to kill intracellular bacteria was assessed relative to both clinical evidence of inflammation (as determined by Harvey-Bradshaw index) and serological markers (hsCRP), with no significant association demonstrated. It should however be noted that patients with active ileal disease appear to kill *E. coli* least effectively and the patient with the single poorest clearance of intramacrophage *E. coli* had both active ileal disease and was homozygous for *ATG16L1* variant (Figure 14-1, appendix 3). Due to small numbers in each group this finding could not be subjected to meaningful statistical analysis but appears to align with the more recent demonstration that active inflammation is required for patients with autophagy related polymorphisms to have a demonstrable reduction in bacterial clearance (Sadaghian Sadabad 2014). Given that *E. coli* is perhaps more important in isolated ileal disease than colonic or ileocolonic Crohn's disease (Baumgart 2007; Willing 2009) this finding warrants further specific investigation.

Thus, with the probable exception of the relatively few Crohn's disease patients who are homozygous for *ATG16L1* polymorphisms, macrophage killing of phagocytosed *E. coli* in Crohn's disease is generally normal. Segal and co-workers reported that defective neutrophil chemotaxis occurs in Crohn's disease and suggest that bacteria entering the mucosa that fail to be cleared by neutrophils are phagocytosed by macrophages with chronic intra-macrophage replication and granuloma formation as a result. Although Segal *et al* reported that this defective macrophage chemotaxis was a possible result of altered chemokine production by macrophages(Smith 2009) the studies reported in this chapter showed no such defect. Other possible mechanisms for defective neutrophil chemotaxis in Crohn's disease patients would include circulating inhibitors(Rhodes 1982b).

Give the previously documented correlation between serum CRP and mesenteric fat CRP mRNA expression, and the ability for *E. coli* to induce mesenteric fat CRP mRNA expression, it may be postulated that higher serum CRP levels may correlate with differential killing of intracellular bacteria i.e. patients may demonstrate a raised CRP in part because of disease activity and in part due to impaired killing of *E. coli* and subsequent stimulation of mesenteric fat CRP production. Whilst this study was not primarily designed to answer this question no such correlation was seen.

There is clear evidence, as previously discussed, that Crohn's disease tissue macrophages often contain intracellular *E. coli* and that *in vitro* this often results in granuloma formation. The intra-macrophage replication of *E. coli* is therefore a very

plausible therapeutic target for Crohn's disease. This will be explored further in the following chapters.

6 Investigation into the effect of hydroxychloroquine on intra-macrophage survival of mucosa-associated *E. coli*

6.1 Introduction

Occupying an intracellular niche is a recognised mechanism by which pathogens may escape killing by the immune system and demonstrate resistance to antibiotics. Clinical conditions in which intracellular bacterial replication are key pathological events include Q fever (due to *Coxiella burnetii*), Whipple's disease (due to *Tropheryma whipplei*) and tuberculosis (Rolain 2007). A shared feature of these conditions is the adaptation of the causative organism to survive and replicate within the acidic environment of macrophage vesicle, a characteristic also shown to be true for adherent invasive *E. coli* (Bringer 2006).

In both Q fever and Whipple's disease standard antibiotic regimens are often ineffective even with prolonged courses and relapse is almost universal on cessation of treatment. However enhanced killing of *C. burnetii* and *T. whipplei* can be achieved *in vitro* by raising the phagolysosomal pH using either chloroquine or hydroxychloroquine. Clinical trials in Q fever have shown a combination of hydroxychloroquine and doxycycline to be effective and lead to shorter treatment durations and fewer relapses when compared with standard therapy (Raoult 1999).

It might therefore be expected that hydroxychloroquine may also enhance killing of intra-macrophage Crohn's disease associated *E. coli* and that this might be achievable at the typical steady state serum concentrations seen with typical oral

dosing regimens (1-2µg/ml) (Carmichael 2003; Munster 2002), either alone or in combination with antibiotics. This is explored further in this chapter.

6.2 Hypothesis

Hydroxychloroquine treatment may enhance the ability of murine and human macrophages to kill intracellular *E. coli* and this effect may be synergistic with antibiotics

6.3 Aims

1. To determine the effect of hydroxychloroquine on bacterial survival in murine macrophages
2. To confirm this effect of hydroxychloroquine on bacterial survival in human monocyte derive macrophages
3. To determine the direct effect of hydroxychloroquine on bacterial viability in culture medium
4. To determine the effect of combining hydroxychloroquine with antibiotics on the survival of *E. coli* within murine macrophages

6.4 Results

6.4.1 Hydroxychloroquine enhances killing of *E. coli* within murine macrophages

Hydroxychloroquine induced a dose-dependent enhancement of intracellular killing of HM605 by J774a.1 macrophages (P< 0.001, Cuzick's test for trend). Net replication of *E. coli* (mean fold replication over 3 hr: 3.59 ± 0.35) occurred in the untreated control, but, at concentrations of HCQ ≥2 mg/mL, net killing of *E. coli* occurred. At all concentrations tested there was a reduction in viable intracellular *E. coli* relative to vehicle control (Figure 6-1).

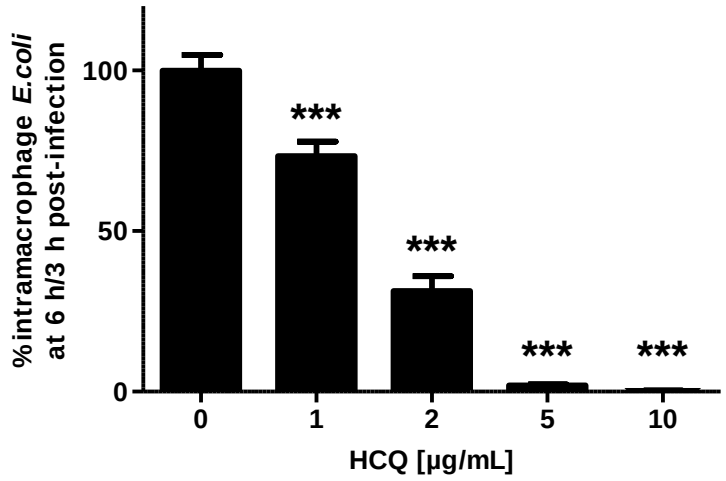

*Figure 6-1: Hydroxychloroquine (HCQ) enhances intramacrophage killing of Crohn's disease associated E. coli HM605. HCQ treatment causes a dose-dependent decrease in survival of intramacrophage bacteria J774A.1 murine macrophages (N =3, Cuzick's test for trend P < 0.001. ***P < 0.001 Dunnett's test versus control).*

6.4.2 Hydroxychloroquine enhances killing of *E. coli* within human monocyte derived macrophages

Hydroxychloroquine also induced dose-dependent enhancement of intracellular killing of HM605 in human MDM (P <0.001, Cuzick's test). Relative to vehicle control bacterial survival was reduced to 26.1 ± 8.7% at 1µg/ml, to 34.1 ± 8.1% at 2µg/ml, to 17.5 ± 11.6% at 5µg/ml and to 7.1 ± 7% at 10µg/ml(P<0.001, Dunnett's) (Figure 6-2).

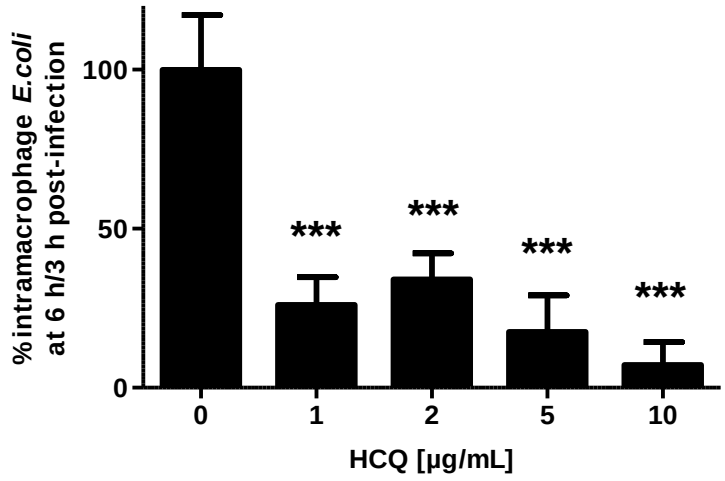

*Figure 6-2: Hydroxychloroquine (HCQ) treatment also leads to a dose-dependent enhancement of intramacrophage killing of Crohn's disease associated E. coli HM605 in human monocyte derived macrophages. (N =5, Cuzick's test for trend P < 0.001. ***P < 0.001 Dunnett's test versus control).*

6.4.3 Hydroxychloroquine does not directly affect bacterial viability

Hydroxychloroquine at concentrations ranging from 1-10µg/ml showed no significant direct effect on growth dynamics of HM605 in broth over 7 hours as measured by OD_{600} nm. In separate experiments hydroxychloroquine tested at the same concentrations had no effect on bacterial viability as determined by absolute colony-forming unit (CFU) count over a 6h period, nor on fold increase in CFU count over the same duration (Figure 6-3). These experiments confirm that the enhanced killing of intramacrophage *E. coli* seen with hydroxychloroquine treatment (section 6.4.1 and 6.4.2) is not a consequence of direct bactericidal action and is best explained by a modulation of the intracellular environment.

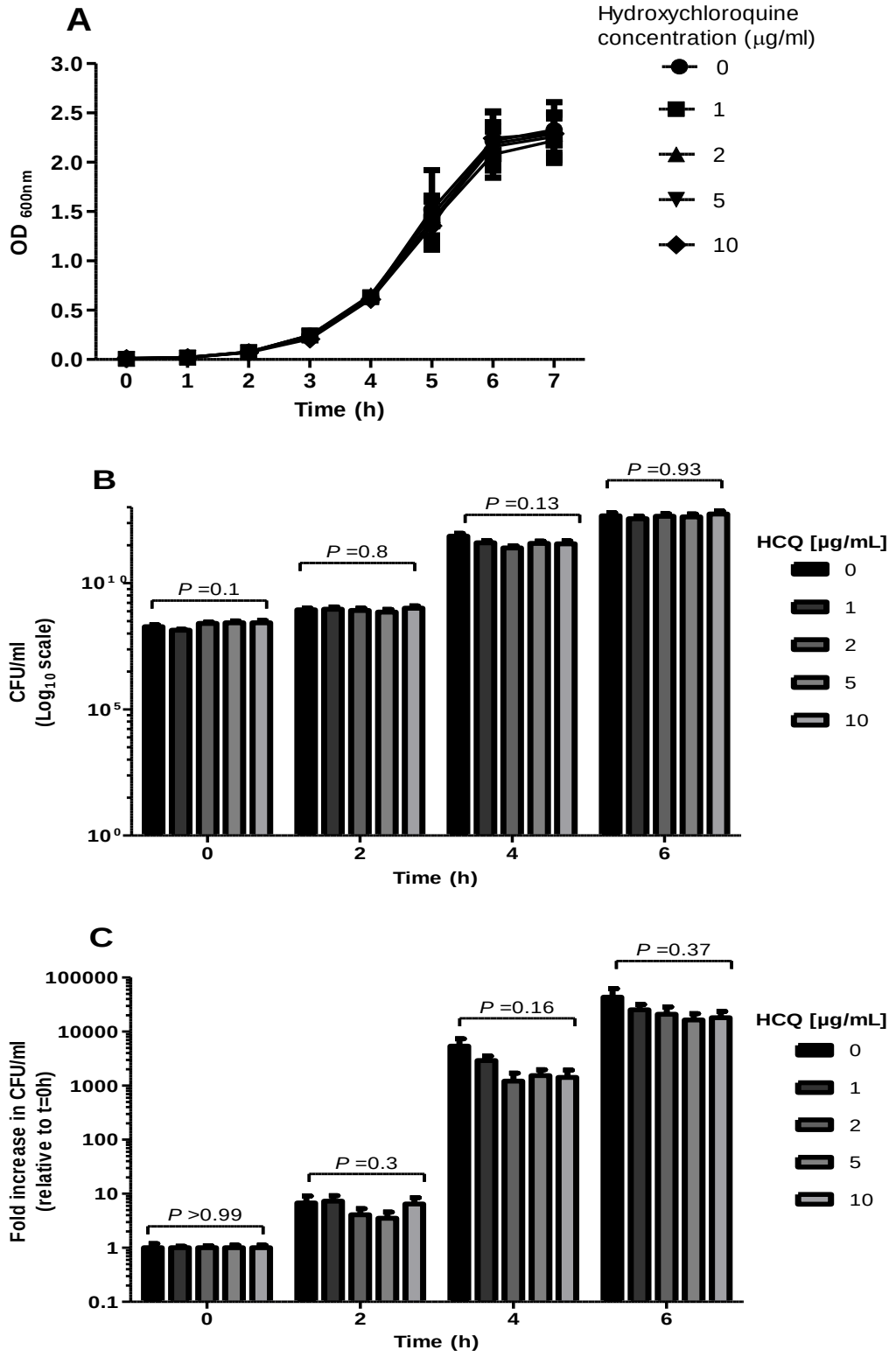

Figure 6-3: Growth of Crohn's disease colonic mucosa-associated E. coli HM605 in broth is unaffected by hydroxychloroquine. Hydroxychloroquine (HCQ), at concentrations up to 10 μg/mL, had no effect on AIEC HM605 growth over 6h as determined by OD_{600} nm (A), by absolute colony forming unit (CFU) count (B) or fold increase in CFU count from baseline(C) (n=3, ANOVA).

6.4.4 Minimum Inhibitory Concentrations for doxycycline and ciprofloxacin

Minimum inhibitory concentrations for doxycycline and ciprofloxacin against Crohn's disease associated *E. coli* HM605 and LF82 were determined in a series of three experiments against multiple drug concentrations (range 0.002-32µg/mL in 29 steps for ciprofloxacin and 0.016-256µg/mL in 29 steps for doxycycline) using Etest® gradient strips with identical results on each occasion (Figure 6-4). MIC results were compared to published typical maximum serum concentration achieved with standard oral dosing (Cmax). These confirmed the efficacy of ciprofloxacin but a relative lack of action with doxycycline as shown by an MIC in excess of the typical Cmax.

	Cmax (data from literature with reference)	**Minimum Inhibitory Concentration HM605**	**Minimum Inhibitory Concentration LF82**
Doxycycline (µg/ml)	2 (Agwuh 2006)	3.0	6.0
Ciprofloxacin (µg/ml)	4 (Gonzalez 1984; Saravolatz 2005)	0.023	0.023

Figure 6-4: Results of minimum inhibitory concentration testing for doxycycline and ciprofloxacin against Crohn's disease associated E. coli with reference maximum serum antibiotic concentrations (Cmax) after standard oral dosing as published

6.4.5 Hydroxychloroquine enhances antibiotic killing of intramacrophage *E. coli* HM605

Doxycycline at 10% Cmax was ineffective at killing intramacrophage HM605 (89.01% ± 8.57%, mean survival relative to control ± SEM, P= 0.08, ANOVA) and only achieved modest decreases in viable bacteria at Cmax (75.5% ± 6.72%, P <0.01). However, at both 10% Cmax (48.9% ±6 5.36%, P <0.001) and Cmax (34.4% ± 4.71%, P< 0.001), co-treatment with hydroxychloroquine plus doxycycline led to significant enhancement of bacterial killing relative to control (P <0.001 at both 10% Cmax and Cmax; N = 6), relative to antibiotic monotherapy (P<0.001 at both 10% Cmax and Cmax) and was also significantly more effective than hydroxychloroquine monotherapy (P<0.05 at 10% Cmax, P<0.001 at Cmax) (Figure 6-5).

Ciprofloxacin monotherapy was highly efficacious against intramacrophage HM605 at 10% Cmax (4.95% ± 0.92%, mean survival relative to control ± SEM, N=3, P<0.001) and led to near complete killing at Cmax (0.2% ± 0.0%, P< 0.001). Hydroxychloroquine combination therapy enhanced killing of intramacrophage *E. coli* at 10% Cmax (2.8% ± 0.82%, P < 0.05 relative to antibiotic monotherapy), but no such effect could be demonstrated at Cmax due to the near complete killing of intramacrophage bacteria with antibiotic monotherapy (Figure 6-6).

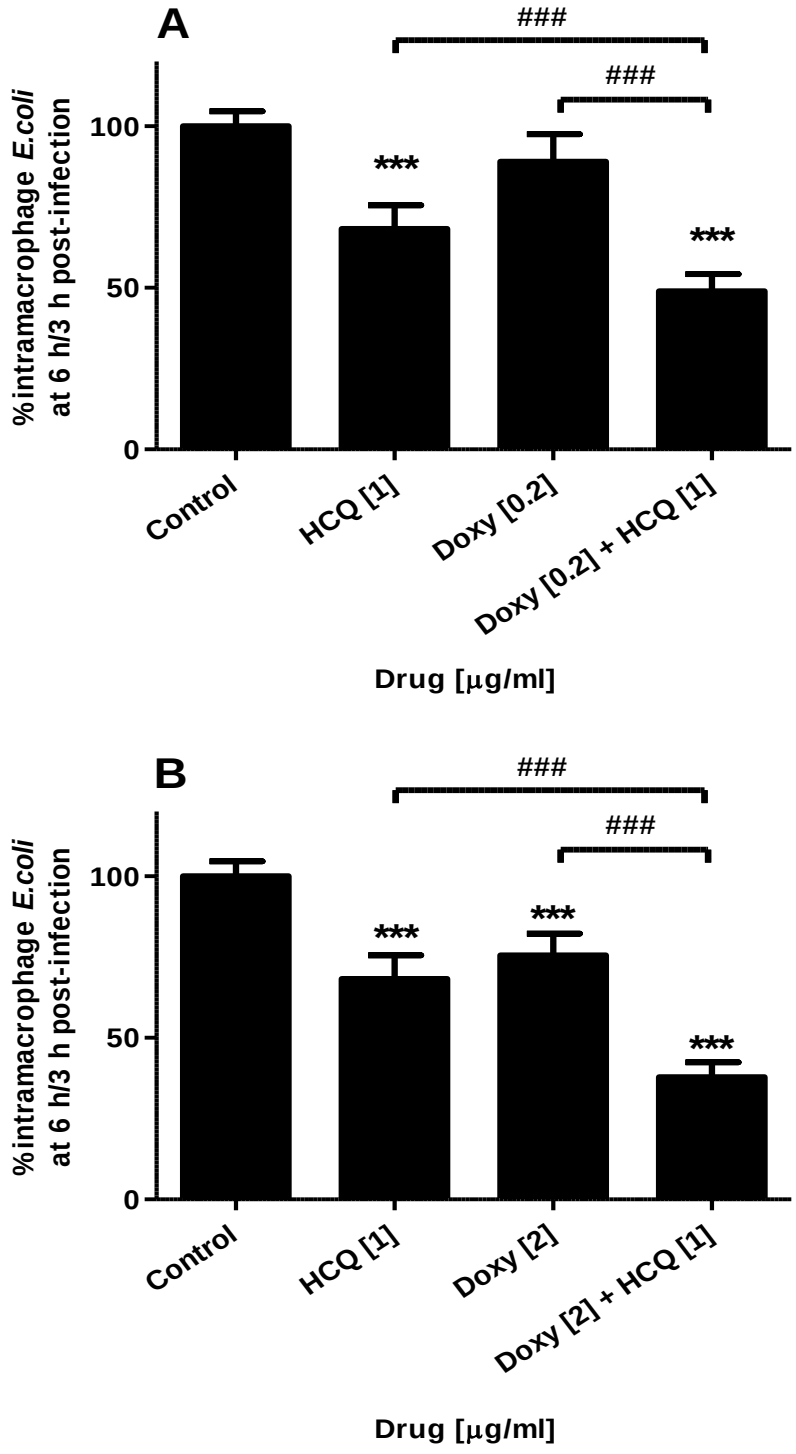

*Figure 6-5: Hydroxychloroquine (HCQ) enhances antibiotic efficacy against intramacrophage E. coli. Combination therapy (antibiotic plus HCQ 1 mg/mL) was more effective at killing HM605 within J774.A1 macrophages than both antibiotic monotherapy and hydroxychloroquine monotherapy at (A) 10% Cmax doxycycline(Doxy) and (B) Cmax doxycyline [2mg/mL] (n= 6, ***relative to control P <0.001, ### relative to monotherapy P <0.001; ANOVA).*

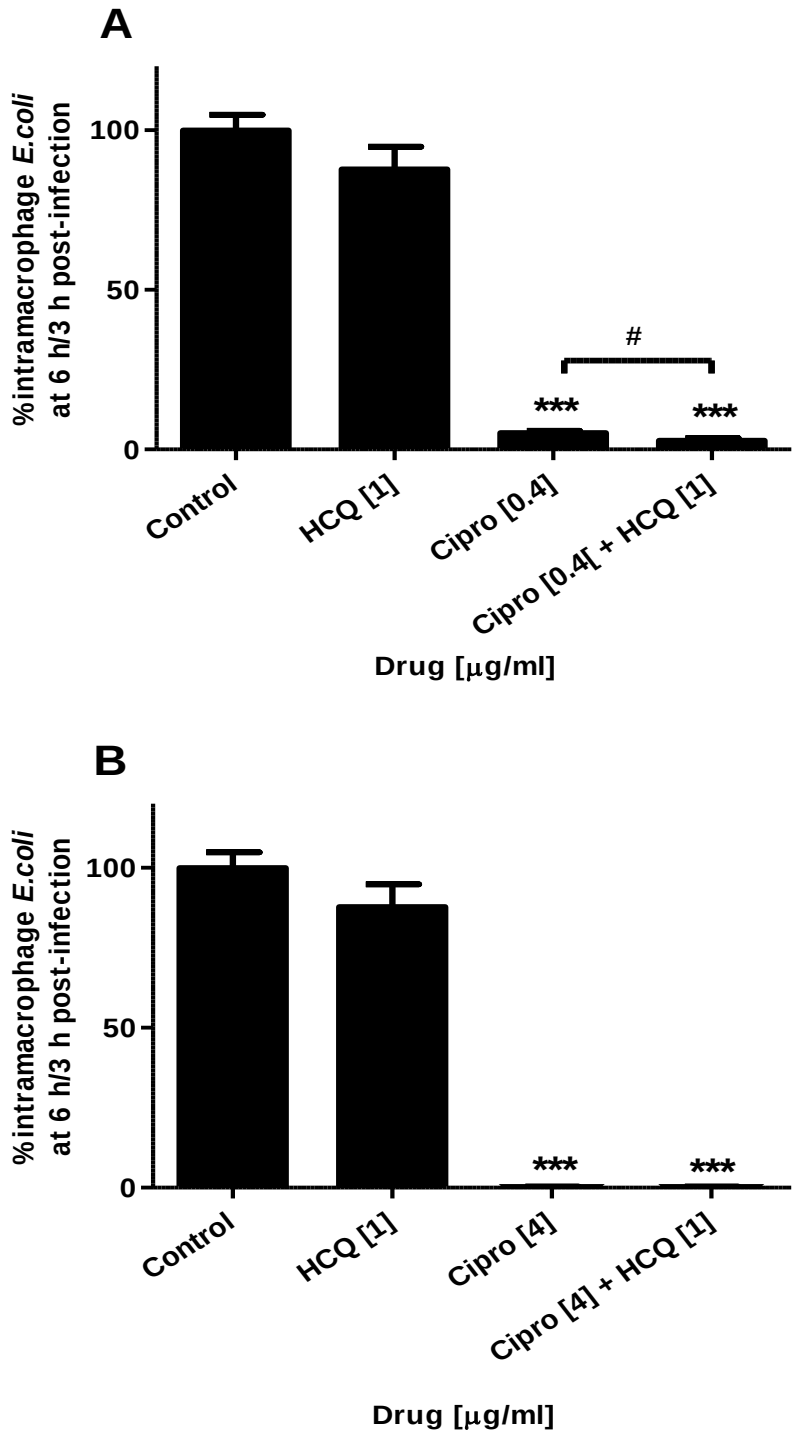

*Figure 6-6: Hydroxychloroquine (HCQ) also enhances ciprofloxacin (Cipro) efficacy against intramacrophage E. coli. Combination therapy (antibiotic plus HCQ 1 mg/mL) was more effective at killing HM605 within J774.A1 macrophages than ciprofloxacin monotherapy at 10% Cmax (A) but no additional benefit was seen Cmax (B) [4mg/mL] (n= 3, ***P <0.001 relative to control, #P<0.05, ANOVA).*

6.4.6 Hydroxychloroquine enhances antibiotic killing of intramacrophage *E. coli* LF82

In separate experiments the effect on intramacrophage survival of *E. coli* LF82 after treatment with hydroxychloroquine plus either doxycycline or ciprofloxacin treatment was similar to that seen with *E. coli* HM605.

Doxycycline monotherapy was similarly ineffective at 10% Cmax (80.9% ± 13.3%, mean survival relative to control ± SEM, N=3, P=0.17, ANOVA), whilst treatment at Cmax lead to a significant but incomplete reduction in intramacrophage bacterial survival (63.5% ± 8.4%, P<0.05, ANOVA). Similar to the effect seen with HM605, co-treatment with hydroxychloroquine at both 10% Cmax (66.9% ± 8.3%, P<0.05) and at Cmax (35.7% ± 3.3%, P<0.001) led to significant enhancement of killing of intracellular bacteria relative to control. Combination treatment was more effective than either antibiotic or hydroxychloroquine monotherapy only at Cmax (P<0.01 and P<0.001 respectively) (Figure 6-7).

Ciprofloxacin efficiently killed intramacrophage bacteria at both 10% Cmax (7.9% ± 1.3%, mean survival relative to control ± SEM, N=3, P<0.001) and at Cmax (0.17% ± 0.1%, P<0.001). Co-treatment with hydroxychloroquine was more effective than antibiotic monotherapy at 10% Cmax (P<0.05) but no effect could be demonstrated at Cmax due to the very low bacterial survival (Figure 6-8).

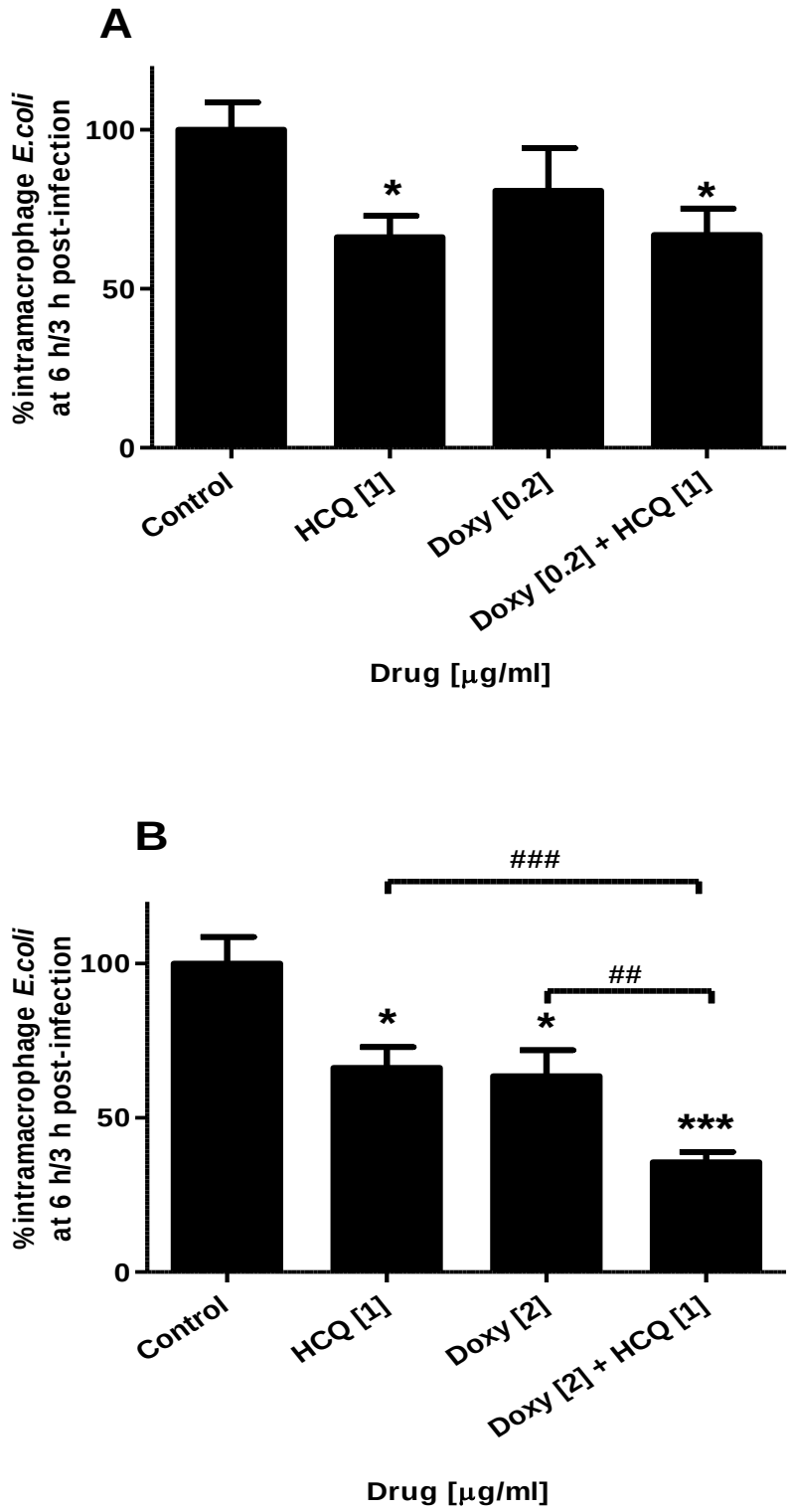

*Figure 6-7: Hydroxychloroquine (HCQ) and doxycycline (Doxy) combination therapy was significantly more effective than either hydroxychloroquine or antibiotic monotherapy in killing intramacrophage E. coli LF82 at 10% Cmax (A) and at Cmax (B). (n=3, * P<0.05 *** P<0.001 relative to control, ##P<0.01 ,###P<0.001, ANOVA)*

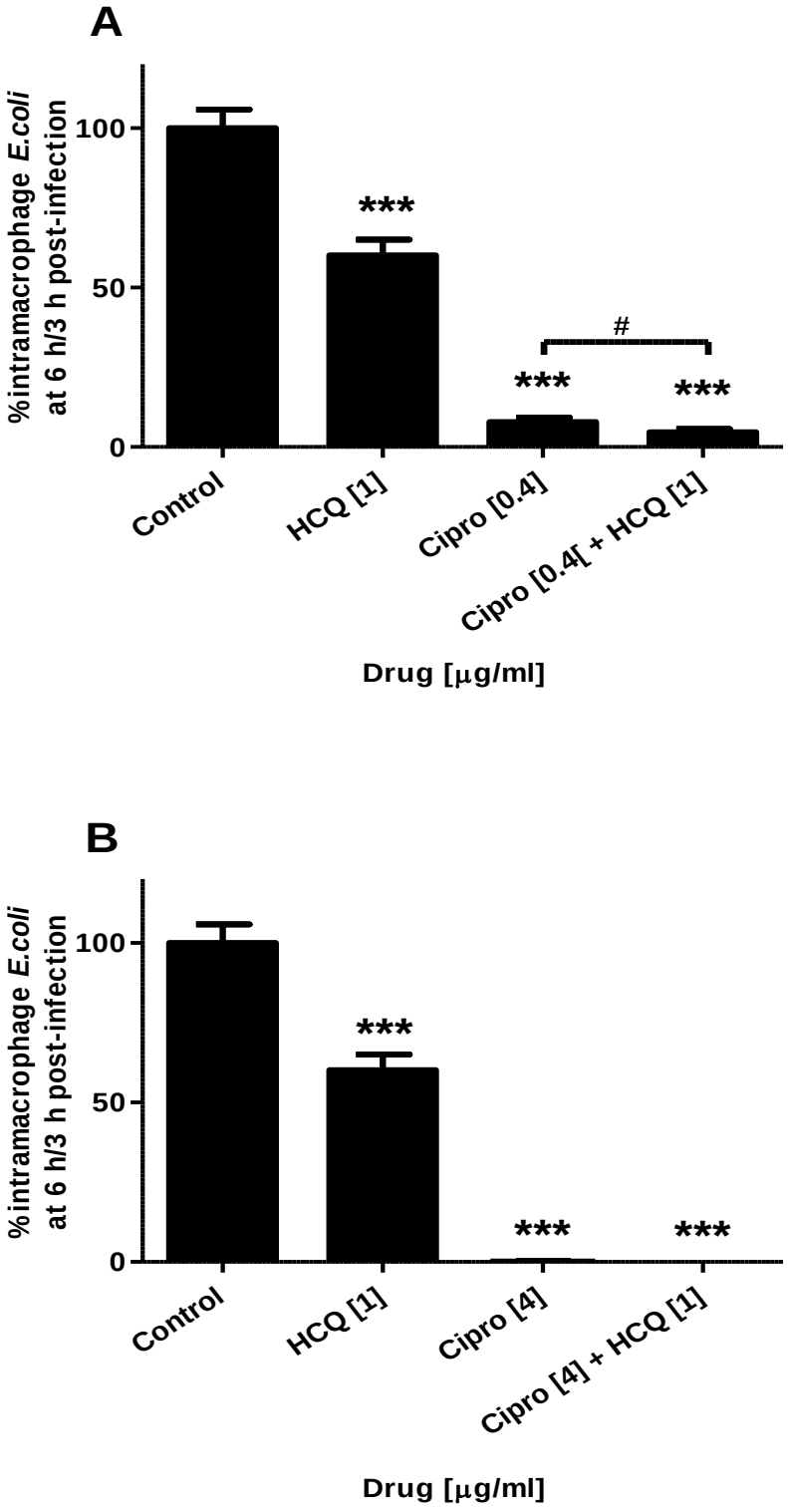

*Figure 6-8: Hydroxychloroquine (HCQ) and ciprofloxacin (Cipro) combination therapy was also significantly more effective than either hydroxychloroquine or antibiotic monotherapy in killing intramacrophage E. coli LF82 at 10% Cmax (A) but no additional benefit was seen at Cmax (B). (n=3, # P<0.05, *** P<0.001; ANOVA)*

6.5 Discussion

The data shown in this chapter demonstrate the ability of hydroxychloroquine to enhance killing of intracellular Crohn's disease-associated *E. coli* in both human and murine macrophages. Further, hydroxychloroquine enhances antimicrobial activity and these effects are independent of any direct bactericidal action.

Hydroxychloroquine undergoes steady-state pharmacokinetics but with significant variation in time to steady-state dependent on dosing regimen. Typical oral dosing (400-800mg/day) typically achieves stable serum levels of 1-2µg/ml by 5-10 weeks (Munster 2002). The data here shows enhanced killing at levels achievable with standard oral dosing.

It is notable that in Whipple's disease continued hydroxychloroquine mono-therapy at these concentrations after initial induction therapy with antibiotics, allows withdrawal of antibiotic therapy without allowing bacterial re-growth (Boulos 2004). Given the enhanced killing of intramacrophage *E. coli* seen shown here with combination antibiotics and hydroxychloroquine, a strategy of dual treatment during induction followed by period of maintenance single agent hydroxychloroquine therapy may allow clearance of Crohn's disease associated *E. coli* without the risks associated with long term antibiotic use.

One of the key concerns surrounding the therapeutic use of hydroxychloroquine is the reported risk of ocular toxicity. However the actual incidence of retinopathy is low and previous case series have reported no toxicity in patients treated for less than 6 years (Levy 1997; Mavrikakis 2003). These data suggest hydroxychloroquine

as an adjunct to antibiotics may represent a useful therapeutic option in Crohn's disease.

7 Investigation into the mechanisms by which hydroxychloroquine affects intra-macrophage *E. coli* survival

7.1 Introduction

Despite its established use in the treatment of specific infections few data exist to explain the antibacterial effect of hydroxychloroquine, with most published literature extrapolated from studies using chloroquine. Key proposed mechanisms include effects of the availability of intracellular iron (Byrd 1991; Fortier 1995) and alterations in lysosomal pH (Poole 1981) – both will be explored in this chapter.

Respiratory burst, via phosphatidylinositol-3-phosphate (PI(3)P)-dependent activation of NADPH-oxidase (nicotinamide adenine dinucleotide phosphate-oxidase), is known to have relevance in the killing of intra-macrophage *E. coli* (Berger 2010) , and may be affected by hydroxychloroquine treatment (Styrt 1986).

The optimal pH for serine protease activity is normally alkaline and in the case of Cathepsin G is 7.5 and for Macrophage elastase is 8.0 (Korkmaz 2010). We anticipate that hydroxychloroquine treatment may increase phagolysosomal pH towards the optimal environment for protease activity and thereby enhance protease killing of AIEC. The effect on cytokine production will also be explored since this has also been implicated (Jeong 1997).

7.2 Hypothesis

Hydroxychloroquine may lead to enhanced killing of intracellular bacteria by favourably altering the intracellular environment

7.3 Aims

1. To determine the effect of intracellular iron availability on the ability of hydroxychloroquine to enhance macrophage killing of *E. coli*

2. To determine the effect of hydroxychloroquine on phagolysosomal pH

3. To determine the effect of hydroxychloroquine on respiratory burst, protease function and cytokine release

7.4 Results

7.4.1 Iron treatment only partially antagonises the effect of hydroxychloroquine on bacterial survival

Data extrapolated from studies using chloroquine suggests the effect of hydroxychloroquine on bacterial survival may arise due to pH dependent alteration in the availability of intracellular iron i.e. phagolysosomal pH rises but may not be directly bactericidal, but may be sufficient to reduce bacterial survival by limiting availability of iron. To assess this *E. coli* infected macrophages were co-treated with hydroxychloroquine and ferric citrate (which allows pH-dependent release of ferric iron and thus should not reverse the effect of hydroxychloroquine on bacterial survival if the mechanism is pH-dependent alteration in iron availability) and FeNTA (allowing release of ferric iron irrespective of pH and which should, if pH-dependent alterations in iron are the key mechanism, therefore fully prevent the reduction in intra-macrophage bacterial survival observed with hydroxychloroquine treatment).

Only a limited effect was observed with only a partial reversal of the effect of hydroxychloroquine seen, and only at doses of 5 to 10 μg/mL HCQ. Supplementation with a pH-dependent iron compound had a statistically significant greater impact on hydroxychloroquine induced reduction in bacterial survival. At 5μg/ml bacterial survival was 11.8 ± 2.2% with HCQ, 31.0 ± 6.2% with FeCit ($P=0.003$ relative to monotherapy, ANOVA) and 25.5 ± 5.5% with FeNTA ($P<0.05$, ANOVA). At 10μg/ml bacterial survival was 3.0 ± 0.8% with HCQ, 8.5 ± 0.9% with FeCit ($P<0.001$ relative to monotherapy, ANOVA) and 6.6 ± 0.6% with FeNTA ($P=0.003$, ANOVA) (Figure 7-1).

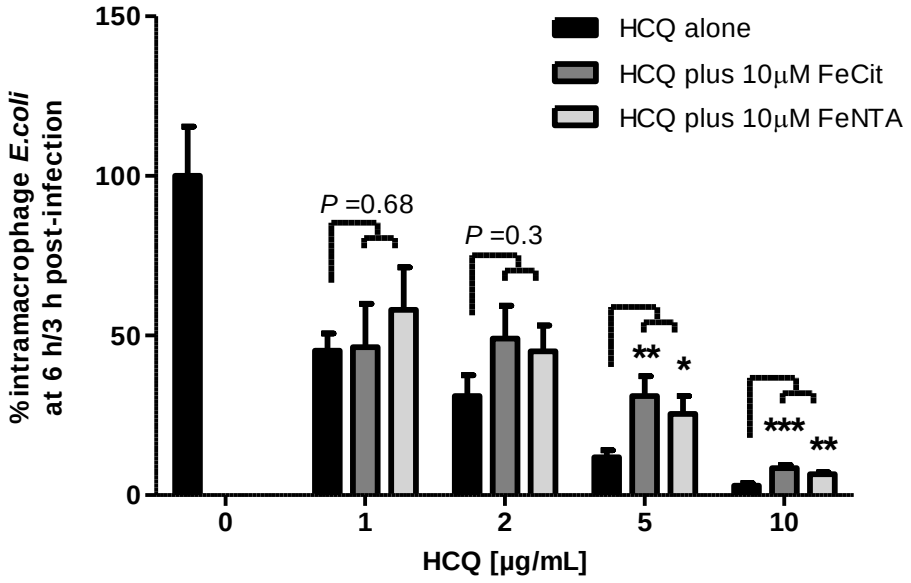

*Figure 7-1: Iron supplementation only partially antagonizes the effect of hydroxychloroquine (HCQ) on intracellular survival of E. coli HM605 in J774A.1 macrophages, with effects only seen a higher concentrations of HCQ. Significance reported relative to hydroxychloroquine monotherapy at any given HCQ concentration (N= 3; *P<0.05, **P <0.01, ***P< 0.001, ANOVA).*

7.4.2 Hydroxychloroquine increases phagolysosomal pH

Addition of HCQ to *E. coli* HM605-infected J774A.1 macrophages caused a dose-dependent increase in phagolysosomal pH (n = 6, P < 0.005, Cuzick's test for trend). From a baseline of 5.21 ± 0.31 (mean ± SEM), pH increased stepwise to 5.38 ± 0.32 at HCQ 1µg/mL, 5.55 ± 0.31 at HCQ 2µg/mL, 5.56 ± 0.36 at HCQ 5 µg/mL and 7.25 ± 0.47 at 10 µg/mL (Figure 7-2 and 7-3). Time-course experiments demonstrated the onset of pH change occurred within 30minutes, as shown in (Figure 7-2).

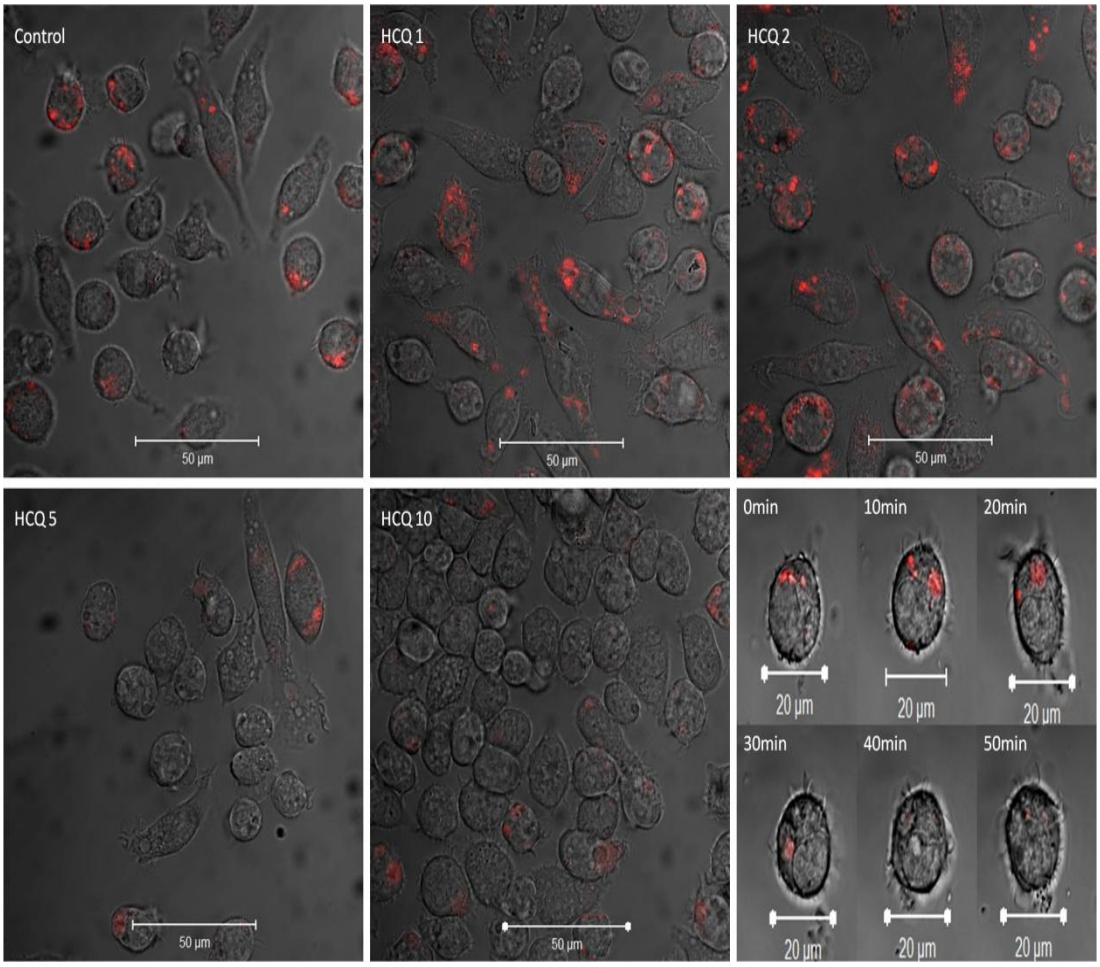

Figure 7-2: Confocal images illustrating increased phagolysosomal pH with hydroxychloroquine (HCQ) as indicated by decreased pHrodo™ fluorescence compared with untreated control. The numbers indicate the concentration of hydroxychloroquine in µg/ml (e.g. HCQ 1 = 1µg/ml). Onset of the change in pH occurs within 30 minutes (bottom right panel).

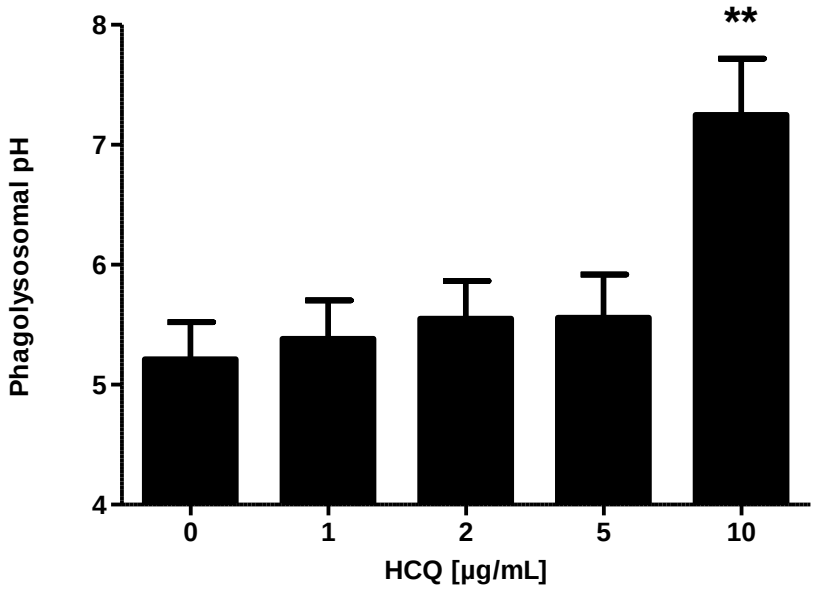

*Figure 7-3: Hydroxychloroquine increased phagolysosomal pH in E. coli HM605-infected J774A.1 macrophages as quantified by confocal microscopy measured pHrodo™ fluorescence (n= 6,Cuzick's test for trend P <0.005, **P< 0.01 Dunnett's test versus control)*

7.4.3 Hydroxychloroquine treatment of J774a.1 murine macrophages has limited effect on respiratory burst

Phorbol-12-myristate-13-acetate (PMA) induced total respiratory burst in interferon gamma (IFNγ) primed J774a.1 murine macrophages as determined by relative increase in lucigenin-mediated luminescence relative to unstimulated control was unaffected by treatment with hydroxychloroquine at any of the concentrations tested (n=5, P=0.73, Cuzick's test for trend) (Figure 7-4).

There was a significant trend towards reduced intracellular respiratory burst as determined by flow cytometric measurement of dihydrorhodamine fluorescence (n=5, P<0.05, Cuzick's) although none of the tested concentrations reached significance against control (ANOVA) (Figure 7-5).

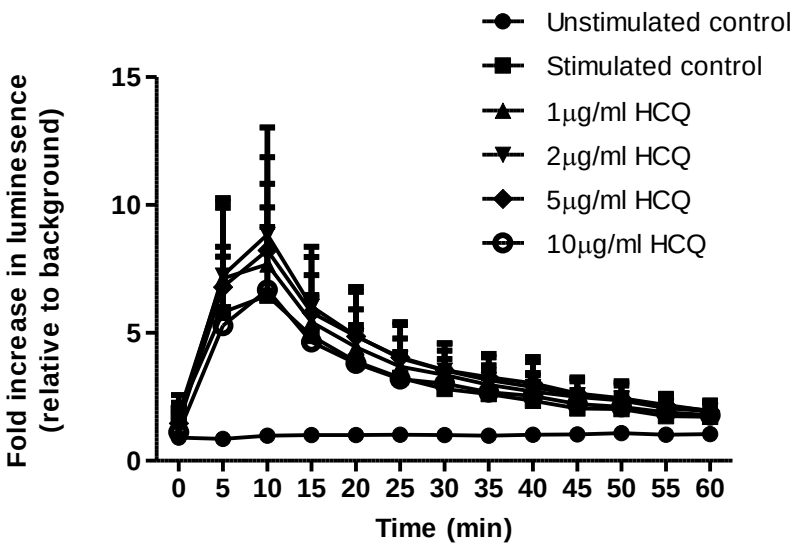

Figure 7-4: Hydroxychloroquine treatment of J774a.1 murine macrophages did not affect total respiratory burst as determined by lucigenin-mediated luminescence (n=5, Cuzick's test for trend, P=0.73)

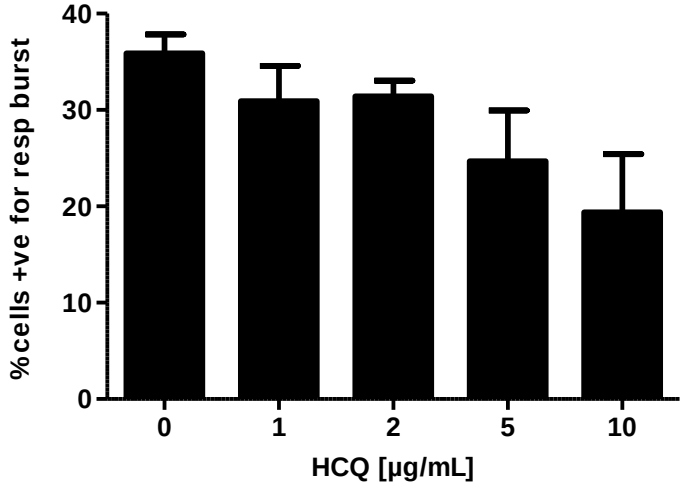

Figure 7-5: Hydroxychloroquine treatment of J774a.1 murine macrophages led to a trend towards reduced intracellular respiratory burst as determined by flow cytometry (n=5, Cuzick's test for trend P<0.05, relative to control P=0.26, ANOVA)

7.4.4 Hydroxychloroquine increases Cathepsin G and macrophage elastase activity

Cathepsin G activity in whole cell lysates of J774a.1 murine macrophages was significantly higher in *E. coli* K12 infected cells (3.11 ± 0.3 units, mean ± SEM) than in either uninfected (2.23 ± 0.2 unit, n=4, P<0.05, ANOVA) or *E. coli* HM605 infected cells (2.03 ± 0.2) (P<0.05, ANOVA) (Figure 7-6).

Baseline macrophage elastase (MMP12) activity in uninfected cells was 777.8 ± 70.6 units (mean ± SEM) and was unaffected by infection with either *E. coli* HM605 (814.0 ± 69.0 units) or *E. coli* K12 (902.0 ± 71.4 units) (n=4, P=0.44, ANOVA) (Figure 7-7).

A subtle but statistically significant trend to increased Cathepsin G activity was seen in HM605 infected murine macrophages following treatment with hydroxychloroquine, rising from a baseline of 2.03 ± 0.23 units (mean ± SEM) to 2.78 ± 0.2 units with HCQ 10µg/mL (n=4, P=0.03, Cuzick's) (Figure 7-8).

Similarly, a modest increase in macrophage elastase activity was observed in HM605 infected macrophages treated with hydroxychloroquine, rising from a baseline of 814.0 ± 69.0 units (mean ± SEM) to 1089.0 ± 75.8 units with HCQ 10µg/mL (n=4, P<0.05, Cuzick's) (Figure 7-9).

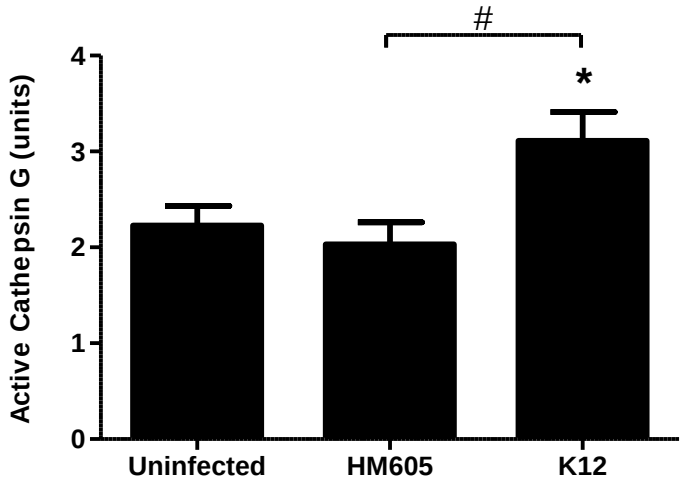

*Figure 7-6: Cathepsin G activity in whole cell lysates of murine macrophages is increased by infection with E. coli K12, but not HM605, relative to uninfected controls (n=4, *P<0.05, #P<0.05, Bonferroni) .*

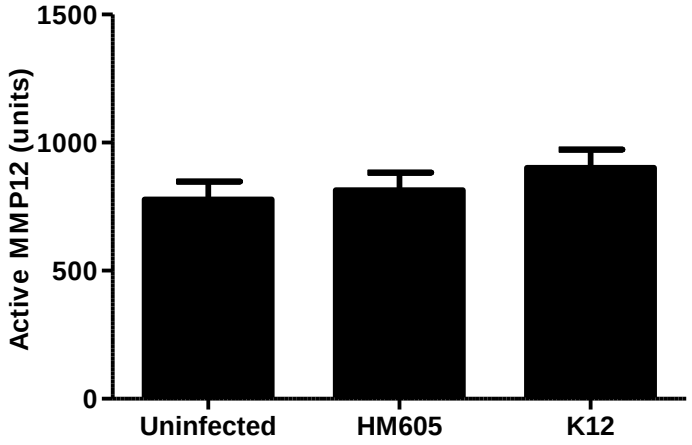

Figure 7-7: MMP12 activity in whole cell lysates of murine macrophages is unaffected by infection with E. coli relative to uninfected controls (n=4, P=0.44, ANOVA).

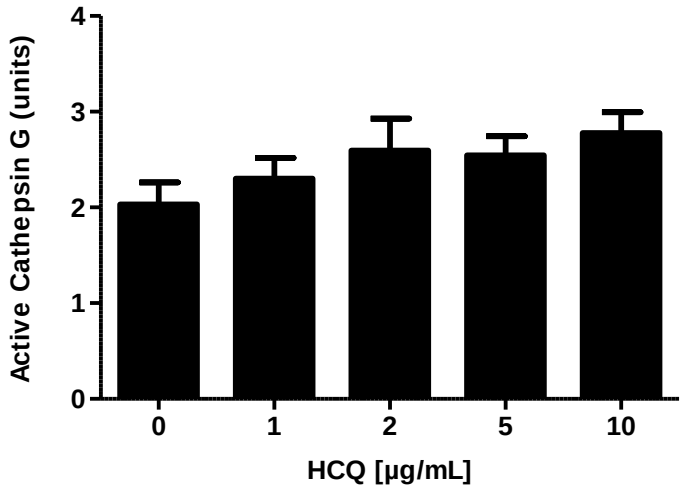

Figure 7-8: Hydroxychloroquine increases Cathepsin G activity in whole cell lysates of HM605 infected J774A.1 murine macrophages (n=4, P=0.03; Cuzick's test for trend)

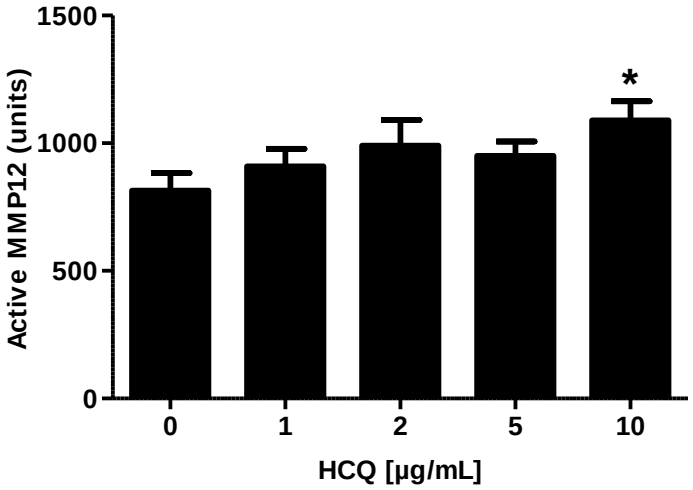

*Figure 7-9: Hydroxychloroquine increases MMP12 activity in whole cell lysates of HM605 infected J774A.1 murine macrophages (n=4, P=0.04 Cuzick's; *P<0.05 Dunnett's test).*

7.4.5 Hydroxychloroquine does not affect murine macrophage cytokine release

Mean TNFα release from *E. coli* HM605 infected J774a.1 murine macrophages was 511.7 ± 78.8pg/mL (mean ± SEM). Hydroxychloroquine treatment did not lead to any significant difference in TNFα production relative to vehicle treated control (n=3, P=0.31, Cuzick's) (Figure 7-10).

Similarly *E.coli* HM605 generated IL-6 release to a mean concentration ± SEM of 246.9 ± 41.7 pg/mL. Again this was unaffected by co-treatment with hydroxychloroquine relative to vehicle control (n=3, P=0.79, Cuzick's) (Figure 7-11).

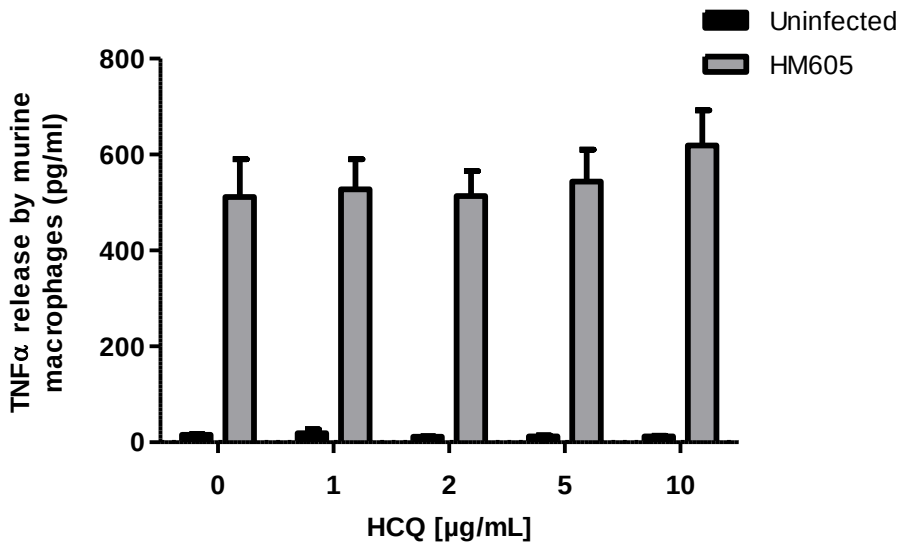

Figure 7-10: Hydroxychloroquine (HCQ) did not induce significant TNFα release in the absence of infection, nor alter production in Crohn's disease colonic mucosa-associated E.coli HM605 infected J774A.1 murine macrophages (n=3, P=0.31, Cuzick's test for trend).

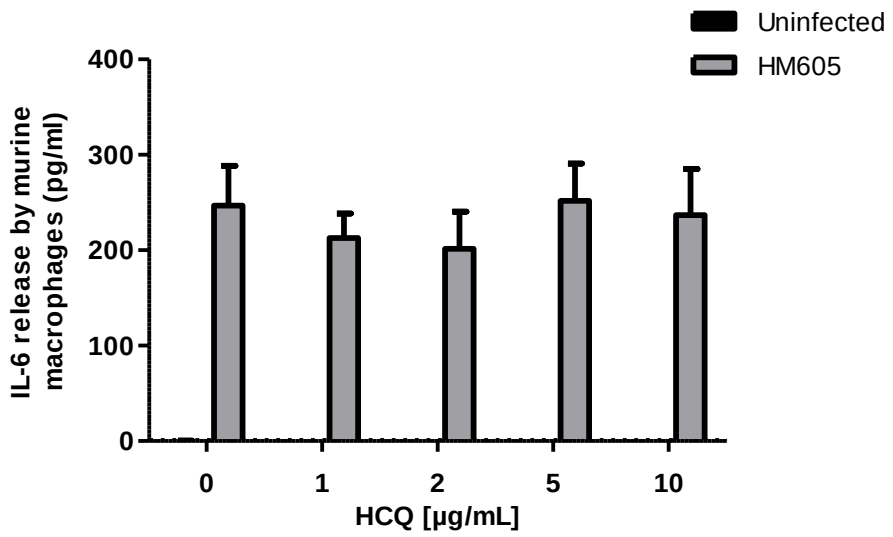

Figure 7-11: Hydroxychloroquine (HCQ) did not induce significant IL-6 release in the absence of infection, nor alter production in Crohn's disease colonic mucosa-associated E.coli HM605 infected J774A.1 murine macrophages (n=3, P=0.79, Cuzick's test for trend).

7.5 Discussion

These data confirm data extrapolated from experiments using chloroquine and for the first time demonstrate a significant effect of hydroxychloroquine treatment on phagolysosomal pH. Relative to untreated control this effect on pH only reached significance at the highest concentration tested but the fact that alterations in bacterial survival can be demonstrated at lower concentrations of hydroxychloroquine is almost certainly a reflection of the technically challenging nature of phagolysosomal pH measurement and a lack of sensitivity of the assay in demonstrating subtle pH change. It seems probable that these subtle pH changes are sufficient to alter bacterial survival but that the magnitude of pH change is insufficient for individual doses to reach significance relative to control. This is supported by the analysis of the data set as a whole when a statistically significant trend was demonstrated across all concentrations by Cuzick's test.

A modest role was seen for alterations in iron metabolism and hydroxychloroquine had little effect on respiratory burst, protease activity and cytokine release.

Hydroxychloroquine has been used to successfully treat chronic granulomatous disease (CGD), a condition where defective NADPH oxidase function is a central feature and in which a Crohn's disease like colitis is common (Arlet 2008). It might therefore have been expected that an increase in respiratory burst would have been seen with hydroxychloroquine treatment but these data show a negligible and negative effect. It should however be noted that the mechanism of action in GCD has not been elucidated and it remains possible that alterations in bacterial survival underpin efficacy in keeping with the data shown here.

Following internalisation bacteria are normally processed through the macrophage phagosomal pathway leading to the ultimate formation of the acidic mature phagolysosome - in the context of *E. coli* infection phagosomal pH has been reported to rapidly fall to around 5 (Berger 2010). Crohn's disease associated *E. coli* demonstrate resistance to this acidification, a recognised mechanism by which bacterial avoid killing (Flannagan 2009), to preferentially replicate within the acidic mature phagolysosome (Bringer 2006; Subramanian 2008). Manipulation of compartmental pH is has been previously shown to achieve clinically relevant enhancement of *C. burnetii* and *T. whipplei* killing (Fenollar 2014; Maurin 1992) and these data suggest the rise in pH seen following treatment is the predominant mechanism through which hydroxychloroquine exerts its effect on intra-macrophage bacterial survival.

8 Investigation into the effect of Vitamin D on intra-macrophage survival of mucosa-associated *E. coli*

8.1 Introduction

In the last decade huge interest has surrounded the role of Vitamin D in the immune system. Vitamin D modulates monocyte function (Nelson 2011), is required for adequate antimicrobial peptide secretion from human macrophages (Fabri 2011), stimulates *NOD2* expression and thereby defensin production (Wang 2010), and also affects T-cell function (Hewison 2012). In light of the growing evidence for a role in both innate and adaptive immunity the definition of adequate serum levels has been frequently debated and revised, with current evidence and consensus now defining deficiency as <20nM (Hewison 2012; Rosen 2011) and >80nM as sufficient for optimal immune cell function (>80nM) (Alappat 2010; Fabri 2011; Hewison 2012).

Vitamin D deficiency is common in Crohn's disease, active disease is associated with lower serum levels (Jorgensen 2013), polymorphisms in the vitamin D receptor are associated with a risk of developing Crohn's disease (Simmons 2000) and conversely higher predicted levels are associated with a reduced risk of disease development (Ananthakrishnan 2012).

In animal models of a spontaneous inflammatory bowel disease like illness, knockout mice supplemented with vitamin D are prevented from developing disease (Cantorna 2000). In humans supplementation reduces the risk of needing IBD –related surgery, non-significantly reduces the risk of clinical relapse and is

associated with a reduced requirement for healthcare (Ananthakrishnan 2013a; Jorgensen 2010; Kabbani 2016).

8.2 Hypothesis

Vitamin D supplementation may enhance the ability of macrophages to kill intracellular bacteria

8.3 Aims

1. To determine the effects of Vitamin D supplementation on intramacrophage bacterial survival

2. To determine the effect of Vitamin D supplementation on respiratory burst

3. To assess the relationship between serum Vitamin D concentration and the ability of human monocyte derived macrophages to kill intracellular bacteria

8.3.1 Vitamin D enhances killing of *E. coli* HM605 within murine macrophages

1, 25-dihydroxyvitamin D3 treatment led to a dose-dependent reduction in survival of *E. coli* HM605 within J774a.1 murine macrophages relative to vehicle treated control (n=3, P< 0.001, Cuzick's test for trend). In the absence of vitamin D supplementation HM605 replicated 6.45 ± 0.53 fold over 3h. A relative reduction in bacterial survival was seen at 20nM vitamin D, with net killing seen at 80nM, and both concentrations reaching significance relative to control (n=3, P<0.001, Dunnett's) (Figure 8-1).

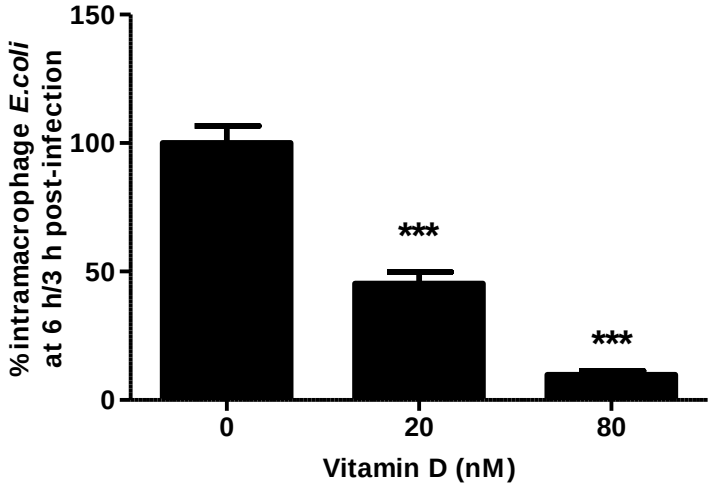

*Figure 8-1: Vitamin D enhances intramacrophage killing of E. coli HM605. 1,25 OH_2-vitamin D3 caused dose-dependent decrease in intramacrophage survival of HM605 in J774A.1 murine macrophages (N =3, P< 0.001 Cuzick's test for trend, ***P< 0.001 Dunnett's test versus control.)*

8.3.2 Vitamin D supplementation enhances *E. coli* killing by human monocyte-derived macrophages

Similarly, vitamin D supplementation significantly decreased intramacrophage survival of *E. coli* HM605 within human monocyte-derived macrophages in a dose-dependent manner (n=3, P=0.012 Cuzick's test for trend). When considering individual doses relative to vehicle-treated control, only 80nM reached significance (n=3, P<0.05, Dunnett's) (Figure 8-2).

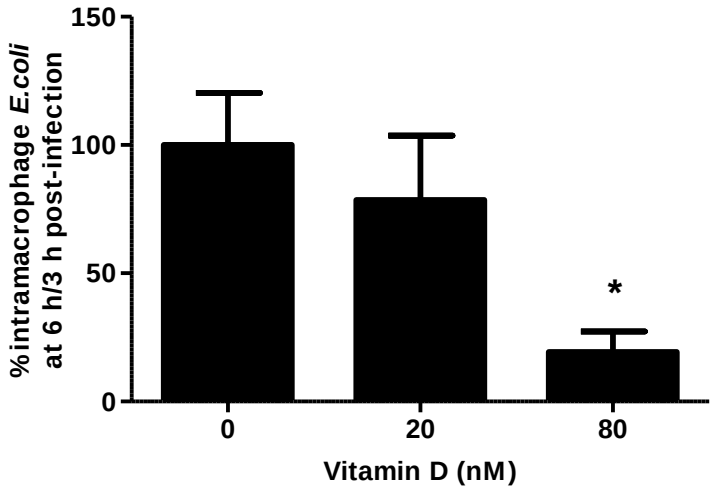

*Figure 8-2: 1,25 OH_2-vitamin D3 also led to a dose-dependent decrease in intramacrophage survival of HM605 in human MDM (N=3, P=0.012 Cuzick's test for trend, *P< 0.05 Dunnett's test versus control).*

8.3.3 Vitamin D treatment of murine macrophages increases intracellular respiratory burst but does not affect total respiratory burst

Phorbol-12-myristate-13-acetate (PMA) induced intracellular respiratory burst in interferon gamma (IFNγ) primed J774a.1 murine macrophages as determined by flow cytometric quantification of dihydrorhodamine fluorescence was increased by treatment with vitamin D (n=5, P<0.01, Cuzick's). Relative to vehicle treated control only treatment with 80nM vitamin D significantly increased intracellular respiratory burst (n=5, P<0.05, Dunnett's) (Figure 8-3).

No significant effect on total respiratory burst as determined by lucigenin mediated luminescence was demonstrated (n=5, P=0.39, Cuzick's) (Figure 8-4).

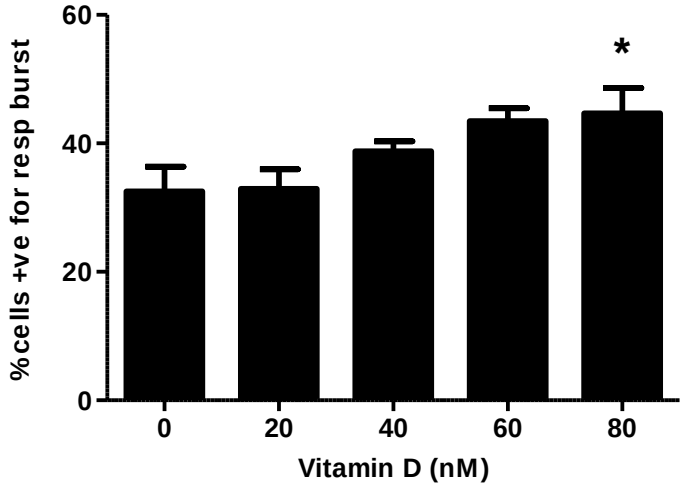

*Figure 8-3: Treatment of J774a.1 murine macrophages with 1,25OH$_2$Vitamin D3 increased intracellular respiratory burst as determined by flow cytometry (n=5, Cuzick's test for trend P<0.01, *P<0.05, Dunnett's test versus control)*

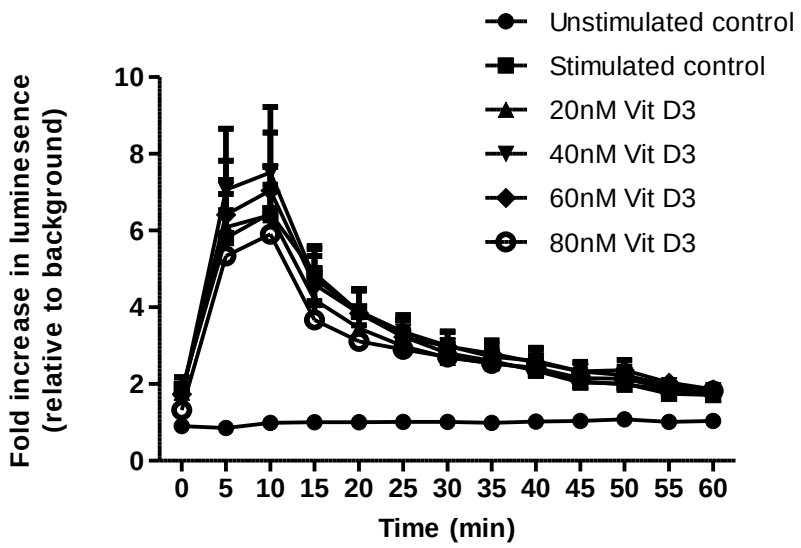

Figure 8-4: Treatment of J774a.1 murine macrophages with 1,25OH$_2$Vitamin D3 did not affect total respiratory burst as determined by lucigenin mediated luminescence (n=5, Cuzick's test for trend, P=0.39).

8.3.4 The ability of human MDM to kill intracellular bacteria was not correlated with serum vitamin D levels

Serum vitamin D levels, as quantified by the Royal Liverpool University Teaching Hospital Biochemistry Department from serum samples obtained from patients and healthy volunteers, did not correlate with the ability of MDMs to kill intracellular bacteria. Given the lack of difference between the ability of MDMs derived from healthy controls and patients with Crohn's disease to kill intracellular bacteria, all samples were pooled for analysis. Spearman's rank coefficient for *E. coli* HM605 was 0.051 (n=20, P=0.84, Mann-Whitney U), for *E. coli* K12 was 0.07 (P=0.79) and for *Staph aureus* Oxford strain was 0.10 (P=0.68) (Figure 8-5).

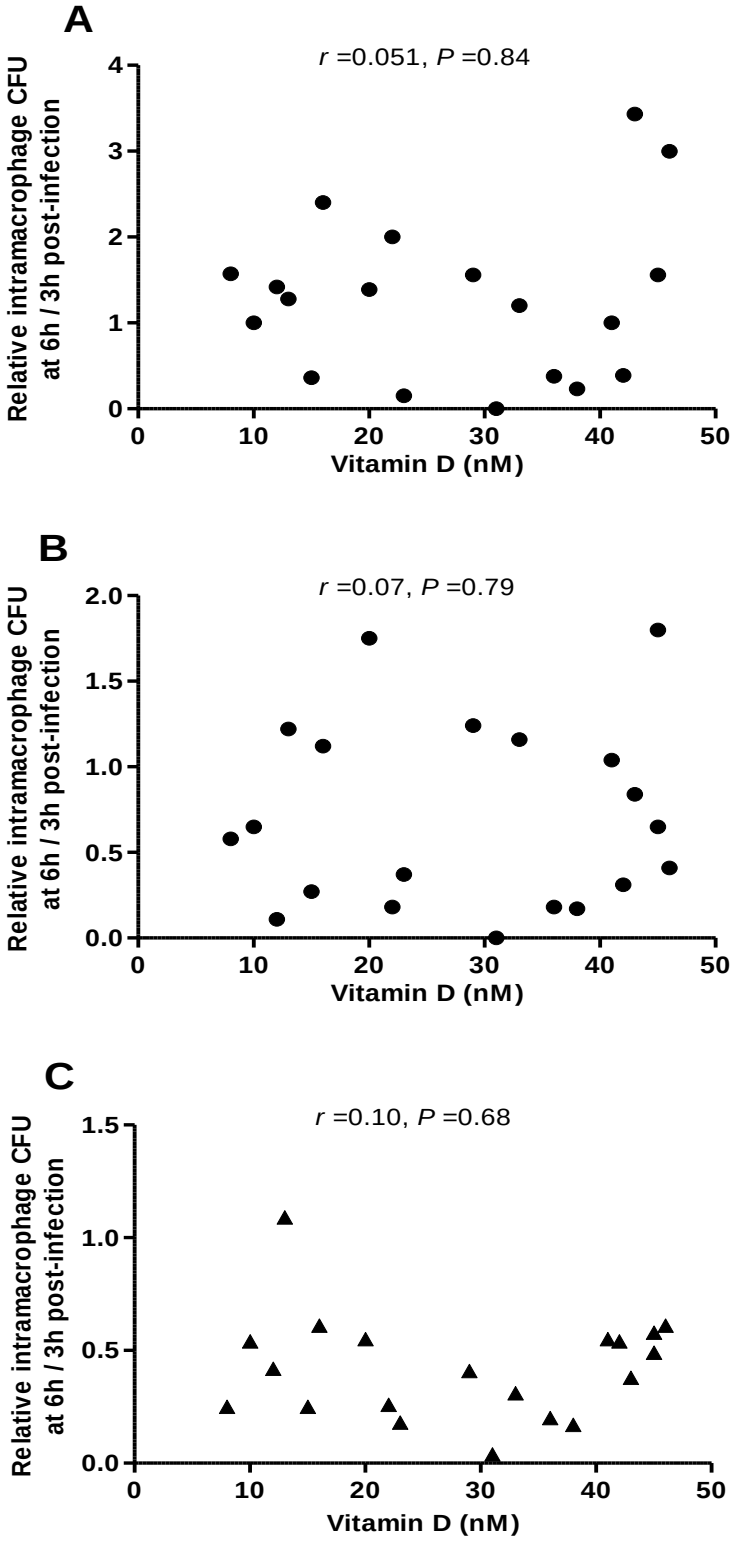

Figure 8-5: Ability of HMDMs to kill intracellular bacteria was not correlated with serum Vitamin D levels. Each point represents individual patient mean of triplicate samples. r value obtained using Spearman's rank, P values obtained using Mann-Whitney U test. (A) HM605, (B) K12, (C) Staph. aureus Oxford strain.

8.4 Discussion

A clear enhancement of the ability of macrophages, both human and murine, to kill intracellular bacteria was seen with vitamin D supplementation, largely independent of effects on respiratory burst. These data did not have sufficient power to assess whether the relative benefit of vitamin D supplementation on bacterial killing is influenced by the concentration in circulating donor serum but no correlation was seen between serum vitamin D levels and killing of intracellular bacteria in the absence of supplemental treatment.

Additional studies to determine to what extent baseline vitamin D levels affect the response to supplementation would be appropriate to explore this further, as would an assessment of the effect on tissue concentrations (rather than serum levels) and their correlation with macrophage function. It seems probable from the data here that an increase in the vitamin D concentration in the macrophage micro-environment enhances their anti-microbial activity.

In patients with deficiency (<20nM (<8ng/mL)) or insufficiency (20-75nM (8-30ng/mL)) a normal serum concentration of 75-80nM or greater (30-32ng/mL) can be be achieved with oral replacement therapy including, in some cases, over the counter preparations. Beneficial effects on serum vitamin D levels can be achieved with Cholecalciferol 400-1000 IU/day but this will commonly fail to achieve optimal levels, particularly in patients with small bowel disease where doses may need to be doubled. Dependent on baseline concentrations, doses of 5000 IU/day are commonly required and up to 10,000 IU/day considered safe (Hlavaty 2015). In light of the data shown here and the building evidence for positive effects both on

immune cell function and the clinical course of Crohn's disease it would now seem appropriate to screen all patients for deficiency on a routine basis, akin to the practice of checking B12, folate and iron levels. It might be argued that because the randomised trial of supplementation failed to reach significance more data is required before translation into clinical practice but in the context of a demonstrable vitamin deficiency withholding replacement may be hard to justify and a trial set up to investigate this in the USA (*ClinicalTrials.gov* NCT02208310) closed because of futility, presumably because vitamin deficient patients were unwilling to receive placebo.

9 Summary

- Crohn's disease associated *E. coli* survive and replicate in macrophages
- Peripheral blood monocyte derived macrophages from patients with Crohn's disease and healthy controls have equivalent ability to kill intracellular bacteria with the exception of a patient homozygous for variant *ATG16L1*
- Monocyte- derived macrophages from Crohn's disease patients had a normal ability to generate neutrophil chemotaxis and chemotactic cytokines *in vitro*
- Hydroxychloroquine enhances macrophage killing of intracellular *E. coli* in a dose-dependent manner
- Hydroxychloroquine enhances antibiotic killing of intra-macrophage *E. coli*
- Hydroxychloroquine increases phagolysosomal pH and in addition has subtle effects on intracellular iron metabolism and protease activity
- Vitamin D supplementation enhances intramacrophage killing of *E. coli* but macrophage bactericidal function is not correlated with serum Vitamin D concentrations

10 Discussion and implications for future studies

The data shown here demonstrates relatively poor killing of intracellular Crohn's disease associated *E. coli* by human monocyte-derived macrophages derived from both healthy controls and patients with Crohn's disease and without any significant differences between the two groups.

The presence of circulating bacterial DNA in the blood of patients with Crohn's disease has been consistently reported, with some studies demonstrating universally positive results in Crohn's disease patients, and most studies demonstrating higher rates in patients with active disease relative to quiescence (Gutierrez 2014; Vrakas 2017). Bacterial translocation occurs across M-cells, is increased by environmental factors including emulsifiers commonly found in western foods, is associated with *NOD2* and *ATG16L1* variants and correlates with a more aggressive disease course including a greater need for biological treatment and a higher risk of post-operative complications (Gutierrez 2014; Li 2015; Roberts 2010).

Following translocation, bacteria are ineffectively cleared from tissues and defects in neutrophil chemotaxis to the site of injury or inflammation have been consistently reported in patients with Crohn's disease with altered macrophage chemokine secretion suggested as a mechanism (Smith 2009). The data shown here suggests the previously observed failure of chemokine secretion, also variably reported elsewhere in response to MDP, cantharidin skin blisters and mucosal injury (Harbord 2006; Marks 2006; Sewell 2012), may arise not as a consequence of an inherent macrophage defect as previously postulated but perhaps arises *in situ*

following local inhibition of otherwise normal macrophages. It has already been shown that microbial mannan possess the ability to inhibit macrophage function (Mpofu 2007) and it is noteworthy that older studies have previously demonstrated circulating factors can inhibit neutrophil chemotaxis (D'Amelio 1985; Rhodes 1982b).

These data support the targeting of intra-macrophage *E. coli* as a therapeutic strategy. Data reported here clearly show enhancement of macrophage killing of *E. coli* by hydroxychloroquine, and that hydroxychloroquine acts synergistically with antibiotics to improve bacterial clearance.

In diseases where intramacrophage bacterial survival is central to pathogenesis, hydroxychloroquine in combination with antibiotics has become standard treatment. In Q fever a clinical trial of treatment with hydroxychloroquine and doxycycline targeted at the causative agent, *Coxiella burnetii,* was superior to standard therapy, shortened treatment duration and reduced the risk of relapse (Raoult 1999). Similar results are achieved in Whipple's disease where *Tropheryma whipplei* is effectively cleared from infected MRC5 cells by hydroxychloroquine and doxycycline combination (Boulos 2004). Due to the rarity of Whipple's disease a clinical trial has not been possible but this approach has now been adopted as standard treatment (Fenollar 2014).

Clinical data for the use of hydroxychloroquine as a treatment for IBD is currently lacking although in ulcerative colitis it prevents experimental colitis in rabbits (Rhodes 1982a) and chloroquine treatment has been shown to have similar short term efficacy to sulfasalazine (Goenka 1996). Hints towards potential efficacy in

Crohn's disease are given by clinical studies in both sarcoidosis and chronic granulomatous disease where is has been successfully used to treat granulomatous manifestations of the diseases (Arlet 2008; Doherty 2008). To date only one published study of only four patients exists for the use of hydroxychloroquine in Crohn's disease where hydroxychloroquine treatment failed to show a benefit of in the prevention of post-operative recurrence, although the very small numbers make it impossible to draw any firm conclusions regarding efficacy (Louis 1995). A clinical trial is therefore needed and based on this study such a trial is now recruiting, with a significant contribution to protocol development made by this author. The pilot open label trial will compare the efficacy of combination hydroxychloroquine, ciprofloxacin and doxycycline with budesonide in the treatment of active Crohn's disease (EudraCT Number: 2008-001137-99). Primary end points are remission at 10 weeks, remission at 24 weeks and remission at 52 weeks as defined by Crohn's disease activity index (CDAI) ≤150. Secondary end points include remission at 4 weeks, response at 10 weeks (fall in CDAI of >70 points), quality of life, tolerability and fall in faecal calprotectin.

Given the potential role for defective autophagy in the pathogenesis of Crohn's disease it might be argued that hydroxychloroquine, which can inhibit autophagy by blocking fusion of lysosomes and phagosomes, may represent a counterintuitive treatment (Rabinowitz 2010). It is however important to recognise that Crohn's disease associated *E. coli* typically escape autophagic pathways even in healthy phagocytes (Lapaquette 2012). Further, the effect on autophagy is a dose-

dependent phenomenon, and at low doses treatment may actually enhance endosome fusion (Bhat 2000).

Whilst primary macrophage dysfunction seems unlikely in the majority of patients with Crohn's disease the finding of poor killing by macrophages from a single patient homozygous for *ATG16L1* is notable and is comparable to findings reported by other groups since this study completed (Vazeille 2015). It has been shown that experimental inhibition of autophagy favours intracellular survival of Crohn's disease associated *E. coli* (Lapaquette 2012) suggesting a functional consequence of genetic polymorphisms may be relevant. However conflicting data are given by two further studies, only one of which addressed killing of *E. coli* (Elliott 2015; Glubb 2011), and whilst responses to MDP stimulation are attenuated by genetic variation this does not translate into a change in handling of intra-macrophage gram-negative infection (Homer 2010). This disparity may be explained by the finding that in patients homozygous for *ATG16L1* alterations in the ileal microbiome and impairment of monocyte killing of Crohn's disease mucosa-associated *E. coli* are only seen in the presence of inflammation (Sadaghian Sadabad 2014). It is noteworthy that in the study presented here there was a non-significant trend towards inferior killing in patients with active Crohn's disease, albeit in a mixed genetic population, raising the possibility that variation in macrophage function may also arise as a consequence of inflammation rather than as a primary phenomenon. Using a non-selective recruitment policy in populations with minor allele frequencies of 1-4% (*NOD2*), 9% (*IRGM)* and 53% (*ATG16L1*) (Ellinghaus 2016; Jostins 2012), a sufficiently powered study would need to be far larger than has

been possible here in order to fully explore the interplay of genetic variation, inflammation and macrophage function. Further studies are therefore indicated to undertake a targeted comparison of macrophage function in patients already known to possess autophagy-related polymorphisms and to better understand the impact of inflammation.

Vitamin D is required for interferon-dependent macrophage function, stimulates *NOD2* expression, induces autophagy and increases defensin production (Fabri 2011; Wang 2010; Yuk 2009). The data here show a clear benefit from *in vitro* vitamin D supplementation in the ability of macrophages to kill internalised *E. coli*. These data further support the use of vitamin D replacement in patients with Crohn's disease. Given that several published studies have demonstrated benefits from replacement therapy including a reduced risk of needing surgery, lower requirement for healthcare, a reduced risk of disease progression, and the ability to prevent disease in knockout mouse models, a test and treat policy appears justifiable for vitamin D, particularly as a fully powered placebo-controlled clinical trial is unlikely to be realised (Ananthakrishnan 2013a; Cantorna 2000; Kabbani 2016). Further studies to look at the impact of vitamin D treatment on autophagic pathways would be of interest, as would a prospective investigation considering the correlation between serum concentrations, disease activity and circulating markers of *E. coli* infection and how this might alter following supplementation.

Comparable to previous studies the data here shows effective clearance of Gram-positive infection by macrophages from both Crohn's disease patients and healthy controls (Mee 1980). It is however important to note that macrophages possess

specific pathways for handling of intracellular Gram-negative bacteria, specifically involving signalling lymphocytic activation molecule F1 (SLAMF1 or CD150 or commonly simply SLAM), and the relevance of this is unknown in Crohn's disease. One previous very small study demonstrated abundant SLAM positive cells in ileal resection specimens taken from patients with Crohn's disease but the functional significance has not been addressed to date (Theil 2005). SLAM has several roles including stimulation of Th2 cytokine secretion, natural killer cell development and acts a measles virus receptor. It has been shown SLAM acts as a microbial sensor where, following interaction with outer-membrane protein C (ompC) expressed by *E. coli*, SLAM is internalised along with *E. coli* and results in phosphatidylinositol-3-phosphate (PI(3)P)-dependent activation of NADPH oxidase and hence bacterial killing (Berger 2010). SLAMF2 (CD48) also acts as a receptor for FimH expressed by *E. coli* and this may also be of relevance. Future studies should consider the role of SLAM and whether this is relevant to the failed clearance of *E. coli*.

The present studies were based on peripheral blood monocyte-derived macrophages. It would be very interesting but technically much more difficult to assess the function of Crohn's disease and control tissue macrophages. Any such future study may wish to combine the approach taken here with molecular pathway analysis of tissue macrophages obtained by fluorescence-activated cell sorting.

In conclusion this study found no demonstrable difference in killing of intracellular bacteria by macrophages from patients with Crohn's disease, reaffirms the ability of Crohn's disease associated *E. coli* to survive and replicate within macrophages and

demonstrates a role for hydroxychloroquine, in combination with antibiotics, and vitamin D supplementation to enhance macrophage killing of *E. coli*. These findings are being further assessed in an ongoing clinical trial.

11 References

Abraham, C. & Cho, J.H. (2009) 'Inflammatory bowel disease', *N Engl J Med*, vol. 361, no. 21, pp. 2066-2078.

Afdhal, N.H., Long, A., Lennon, J., Crowe, J. *et al.* (1991) 'Controlled trial of antimycobacterial therapy in Crohn's disease. Clofazimine versus placebo', *Dig Dis Sci*, vol. 36, no. 4, pp. 449-453.

Agwuh, K.N. & MacGowan, A. (2006) 'Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines', *J Antimicrob Chemother*, vol. 58, no. 2, pp. 256-265.

Ahmad, T., Armuzzi, A., Bunce, M., Mulcahy-Hawes, K. *et al.* (2002) 'The molecular classification of the clinical manifestations of Crohn's disease', *Gastroenterology*, vol. 122, no. 4, pp. 854-866.

Ahuja, V. & Tandon, R.K. (2010) 'Inflammatory bowel disease in the Asia-Pacific area: a comparison with developed countries and regional differences', *J Dig Dis*, vol. 11, no. 3, pp. 134-147.

Akobeng, A.K. & Gardener, E. (2005) 'Oral 5-aminosalicylic acid for maintenance of medically-induced remission in Crohn's Disease', *Cochrane Database Syst Rev*, no. 1, p. CD003715.

Akobeng, A.K. & Thomas, A.G. (2007) 'Enteral nutrition for maintenance of remission in Crohn's disease', *Cochrane Database Syst Rev*, no. 3, p. CD005984.

Alappat, L., Valerio, M. & Awad, A.B. (2010) 'Effect of vitamin D and beta-sitosterol on immune function of macrophages', *Int Immunopharmacol*, vol. 10, no. 11, pp. 1390-1396.

Ambrose, N.S., Allan, R.N., Keighley, M.R., Burdon, D.W. *et al.* (1985) 'Antibiotic therapy for treatment in relapse of intestinal Crohn's disease. A prospective randomized study', *Dis Colon Rectum*, vol. 28, no. 2, pp. 81-85.

Ananthakrishnan, A.N., Cagan, A., Gainer, V.S., Cai, T. *et al.* (2013a) 'Normalization of plasma 25-hydroxy vitamin D is associated with reduced risk of surgery in Crohn's disease', *Inflamm Bowel Dis*, vol. 19, no. 9, pp. 1921-1927.

Ananthakrishnan, A.N., Khalili, H., Higuchi, L.M., Bao, Y. *et al.* (2012) 'Higher predicted vitamin D status is associated with reduced risk of Crohn's disease', *Gastroenterology*, vol. 142, no. 3, pp. 482-489.

Ananthakrishnan, A.N., Khalili, H., Konijeti, G.G., Higuchi, L.M. *et al.* (2013b) 'A prospective study of long-term intake of dietary fiber and risk of Crohn's disease and ulcerative colitis', *Gastroenterology*, vol. 145, no. 5, pp. 970-977.

Ananthakrishnan, A.N., McGinley, E.L. & Binion, D.G. (2009) 'Inflammatory bowel disease in the elderly is associated with worse outcomes: a national study of hospitalizations', *Inflamm Bowel Dis*, vol. 15, no. 2, pp. 182-189.

Arlet, J.B., Aouba, A., Suarez, F., Blanche, S. *et al.* (2008) 'Efficiency of hydroxychloroquine in the treatment of granulomatous complications in chronic granulomatous disease', *Eur J Gastroenterol Hepatol*, vol. 20, no. 2, pp. 142-144.

Armitage, E.L., Aldhous, M.C., Anderson, N., Drummond, H.E. *et al.* (2004) 'Incidence of juvenile-onset Crohn's disease in Scotland: association with northern latitude and affluence', *Gastroenterology*, vol. 127, no. 4, pp. 1051-1057.

Armitage, P., Berry, G. & Matthews, J.N.S. (2005) *Statistical Methods in Medical Research*, 4th edn, Blackwell.

Arnold, G.L., Beaves, M.R., Pryjdun, V.O. & Mook, W.J. (2002) 'Preliminary study of ciprofloxacin in active Crohn's disease', *Inflamm Bowel Dis*, vol. 8, no. 1, pp. 10-15.

Arnott, I.D., Landers, C.J., Nimmo, E.J., Drummond, H.E. *et al.* (2004) 'Sero-reactivity to microbial components in Crohn's disease is associated with disease severity and progression, but not NOD2/CARD15 genotype', *Am J Gastroenterol*, vol. 99, no. 12, pp. 2376-2384.

Atreya, R., Zimmer, M., Bartsch, B., Waldner, M.J. *et al.* (2011) 'Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal CD14(+) macrophages', *Gastroenterology*, vol. 141, no. 6, pp. 2026-2038.

Bach, J.F. (2002) 'The effect of infections on susceptibility to autoimmune and allergic diseases', *N Engl J Med*, vol. 347, no. 12, pp. 911-920.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. *et al.* (2005) 'Host-bacterial mutualism in the human intestine', *Science*, vol. 307, no. 5717, pp. 1915-1920.

Barclay, A.R., Russell, R.K., Wilson, M.L., Gilmour, W.H. *et al.* (2009) 'Systematic review: the role of breastfeeding in the development of pediatric inflammatory bowel disease', *J Pediatr*, vol. 155, no. 3, pp. 421-426.

Barreiro-de Acosta, M., Alvarez Castro, A., Souto, R., Iglesias, M. *et al.* (2011) 'Emigration to western industrialized countries: A risk factor for developing inflammatory bowel disease', *J Crohns Colitis*, vol. 5, no. 6, pp. 566-569.

Barrett, J.C., Hansoul, S., Nicolae, D.L., Cho, J.H. *et al.* (2008) 'Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease', *Nat Genet*, vol. 40, no. 8, pp. 955-962.

Bates, G.W. & Wernicke, J. (1971) 'The kinetics and mechanism of iron(3) exchange between chelates and transferrin. IV. The reaction of transferrin with iron(3) nitrilotriacetate', *J Biol Chem*, vol. 246, no. 11, pp. 3679-3685.

Baumgart, M., Dogan, B., Rishniw, M., Weitzman, G. *et al.* (2007) 'Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum', *ISME J*, vol. 1, no. 5, pp. 403-418.

Benjamin, J.L., Hedin, C.R., Koutsoumpas, A., Ng, S.C. *et al.* (2012) 'Smokers with active Crohn's disease have a clinically relevant dysbiosis of the gastrointestinal microbiota', *Inflamm Bowel Dis*, vol. 18, no. 6, pp. 1092-1100.

Berger, S.B., Romero, X., Ma, C., Wang, G. *et al.* (2010) 'SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages', *Nat Immunol*, vol. 11, no. 10, pp. 920-927.

Bhat, M. & Hickey, A.J. (2000) 'Effect of chloroquine on phagolysosomal fusion in cultured guinea pig alveolar macrophages: implications in drug delivery', *AAPS PharmSci*, vol. 2, no. 4, p. E34.

Bhopal, R.S., Cezard, G., Bansal, N., Ward, H.J. *et al.* (2014) 'Ethnic variations in five lower gastrointestinal diseases: Scottish health and ethnicity linkage study', *BMJ Open*, vol. 4, no. 10, p. e006120.

Blichfeldt, P., Blomhoff, J.P., Myhre, E. & Gjone, E. (1978) 'Metronidazole in Crohn's disease. A double blind cross-over clinical trial', *Scand J Gastroenterol*, vol. 13, no. 1, pp. 123-127.

Boudeau, J., Glasser, A.L., Masseret, E., Joly, B. *et al.* (1999) 'Invasive ability of an Escherichia coli strain isolated from the ileal mucosa of a patient with Crohn's disease', *Infect Immun*, vol. 67, no. 9, pp. 4499-4509.

Bouguen, G., Levesque, B.G., Feagan, B.G., Kavanaugh, A. *et al.* (2015) 'Treat to target: a proposed new paradigm for the management of Crohn's disease', *Clin Gastroenterol Hepatol*, vol. 13, no. 6, pp. 1042-1050 e1042.

Boulos, A., Rolain, J.M. & Raoult, D. (2004) 'Antibiotic susceptibility of Tropheryma whipplei in MRC5 cells', *Antimicrob Agents Chemother*, vol. 48, no. 3, pp. 747-752.

Bowman, E.J., Siebers, A. & Altendorf, K. (1988) 'Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells', *Proc Natl Acad Sci U S A*, vol. 85, no. 21, pp. 7972-7976.

Bringer, M.A., Glasser, A.L., Tung, C.H., Meresse, S. *et al.* (2006) 'The Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 replicates

in mature phagolysosomes within J774 macrophages', *Cell Microbiol*, vol. 8, no. 3, pp. 471-484.

Bryant, S.M., Guthrie, L.A., Pabst, M.J. & Johnston, R.B., Jr. (1986) 'Macrophage membrane proteins: possible role in the regulation of priming for enhanced respiratory burst activity', *Cell Immunol*, vol. 103, no. 1, pp. 216-223.

Burisch, J., Jess, T., Martinato, M. & Lakatos, P.L. (2013) 'The burden of inflammatory bowel disease in Europe', *J Crohns Colitis*, vol. 7, no. 4, pp. 322-337.

Burisch, J., Pedersen, N., Cukovic-Cavka, S., Brinar, M. *et al.* (2014a) 'East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort', *Gut*, vol. 63, no. 4, pp. 588-597.

Burisch, J., Pedersen, N., Cukovic-Cavka, S., Turk, N. *et al.* (2014b) 'Environmental factors in a population-based inception cohort of inflammatory bowel disease patients in Europe--an ECCO-EpiCom study', *J Crohns Colitis*, vol. 8, no. 7, pp. 607-616.

Burman, J.H., Thompson, H., Cooke, W.T. & Williams, J.A. (1971) 'The effects of diversion of intestinal contents on the progress of Crohn's disease of the large bowel', *Gut*, vol. 12, no. 1, pp. 11-15.

Byrd, T.F. & Horwitz, M.A. (1991) 'Chloroquine inhibits the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. A potential new mechanism for the therapeutic effect of chloroquine against intracellular pathogens', *J Clin Invest*, vol. 88, no. 1, pp. 351-357.

Cadwell, K., Liu, J.Y., Brown, S.L., Miyoshi, H. *et al.* (2008) 'A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells', *Nature*, vol. 456, no. 7219, pp. 259-263.

Calkins, B.M. (1989) 'A meta-analysis of the role of smoking in inflammatory bowel disease', *Dig Dis Sci*, vol. 34, no. 12, pp. 1841-1854.

Campbell, H., Escudier, M., Patel, P., Nunes, C. *et al.* (2011) 'Distinguishing orofacial granulomatosis from crohn's disease: two separate disease entities?', *Inflamm Bowel Dis*, vol. 17, no. 10, pp. 2109-2115.

Campbell, M.J., Julious, S.A. & Altman, D.G. (1995) 'Estimating sample sizes for binary, ordered categorical, and continuous outcomes in two group comparisons', *BMJ*, vol. 311, no. 7013, pp. 1145-1148.

Cantorna, M.T., Munsick, C., Bemiss, C. & Mahon, B.D. (2000) '1,25-Dihydroxycholecalciferol prevents and ameliorates symptoms of experimental murine inflammatory bowel disease', *J Nutr*, vol. 130, no. 11, pp. 2648-2652.

Carmichael, S.J., Charles, B. & Tett, S.E. (2003) 'Population pharmacokinetics of hydroxychloroquine in patients with rheumatoid arthritis', *Ther Drug Monit*, vol. 25, no. 6, pp. 671-681.

Castiglione, F., Diaferia, M., Morace, F., Labianca, O. *et al.* (2012) 'Risk factors for inflammatory bowel diseases according to the "hygiene hypothesis": a case-control, multi-centre, prospective study in Southern Italy', *J Crohns Colitis*, vol. 6, no. 3, pp. 324-329.

Cerrillo, E., Beltran, B., Pous, S., Echarri, A. *et al.* (2015) 'Fecal Calprotectin in Ileal Crohn's Disease: Relationship with Magnetic Resonance Enterography and a Pathology Score', *Inflamm Bowel Dis*, vol. 21, no. 7, pp. 1572-1579.

Challacombe, S.J. (1997) 'Oro-facial granulomatosis and oral Crohns disease: are they specific diseases and do they predict systemic Crohns disease?', *Oral Dis*, vol. 3, no. 3, pp. 127-129.

Chande, N., Tsoulis, D.J. & MacDonald, J.K. (2013) 'Azathioprine or 6-mercaptopurine for induction of remission in Crohn's disease', *Cochrane Database Syst Rev*, vol. 4, p. CD000545.

Charpentier, C., Salleron, J., Savoye, G., Fumery, M. *et al.* (2014) 'Natural history of elderly-onset inflammatory bowel disease: a population-based cohort study', *Gut*, vol. 63, no. 3, pp. 423-432.

Chatu, S., Subramanian, V. & Pollok, R.C. (2012) 'Meta-analysis: diagnostic medical radiation exposure in inflammatory bowel disease', *Aliment Pharmacol Ther*, vol. 35, no. 5, pp. 529-539.

Chatu, S., Subramanian, V., Saxena, S. & Pollok, R.C. (2014) 'The role of thiopurines in reducing the need for surgical resection in Crohn's disease: a systematic review and meta-analysis', *Am J Gastroenterol*, vol. 109, no. 1, pp. 23-34; quiz 35.

Choung, R.S., Princen, F., Stockfisch, T.P., Torres, J. *et al.* (2016) 'Serologic microbial associated markers can predict Crohn's disease behaviour years before disease diagnosis', *Aliment Pharmacol Ther*.

Chu, K.M., Watermeyer, G., Shelly, L., Janssen, J. *et al.* (2013) 'Childhood helminth exposure is protective against inflammatory bowel disease: a case control study in South Africa', *Inflamm Bowel Dis*, vol. 19, no. 3, pp. 614-620.

Cleynen, I., Boucher, G., Jostins, L., Schumm, L.P. *et al.* (2016) 'Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study', *Lancet*, vol. 387, no. 10014, pp. 156-167.

Colombel, J.F., Lemann, M., Cassagnou, M., Bouhnik, Y. *et al.* (1999) 'A controlled trial comparing ciprofloxacin with mesalazine for the treatment of active Crohn's disease. Groupe d'Etudes Therapeutiques des Affections

Inflammatoires Digestives (GETAID)', *Am J Gastroenterol*, vol. 94, no. 3, pp. 674-678.

Colombel, J.F., Sandborn, W.J., Reinisch, W., Mantzaris, G.J. *et al.* (2010) 'Infliximab, azathioprine, or combination therapy for Crohn's disease', *N Engl J Med*, vol. 362, no. 15, pp. 1383-1395.

Combe, C. & Saunders, H. (1813) 'A singular case of stricture and thickening of ileum', *Med Transactions of Royal College of Physicians London*, vol. 4, p. 16.

Cooney, R., Baker, J., Brain, O., Danis, B. *et al.* (2010) 'NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation', *Nat Med*, vol. 16, no. 1, pp. 90-97.

Cornish, J.A., Tan, E., Simillis, C., Clark, S.K. *et al.* (2008) 'The risk of oral contraceptives in the etiology of inflammatory bowel disease: a meta-analysis', *Am J Gastroenterol*, vol. 103, no. 9, pp. 2394-2400.

Cosnes, J., Beaugerie, L., Carbonnel, F. & Gendre, J.P. (2001) 'Smoking cessation and the course of Crohn's disease: an intervention study', *Gastroenterology*, vol. 120, no. 5, pp. 1093-1099.

Cosnes, J., Bourrier, A., Laharie, D., Nahon, S. *et al.* (2013) 'Early administration of azathioprine vs conventional management of Crohn's Disease: a randomized controlled trial', *Gastroenterology*, vol. 145, no. 4, pp. 758-765 e752; quiz e714-755.

Cosnes, J., Cattan, S., Blain, A., Beaugerie, L. *et al.* (2002) 'Long-term evolution of disease behavior of Crohn's disease', *Inflamm Bowel Dis*, vol. 8, no. 4, pp. 244-250.

Cosnes, J., Gower-Rousseau, C., Seksik, P. & Cortot, A. (2011) 'Epidemiology and natural history of inflammatory bowel diseases', *Gastroenterology*, vol. 140, no. 6, pp. 1785-1794.

Crohn, B.B., Ginzburg, L. & Oppenheim, G.D. (1932) 'Regional Ileitis: A Pathologic and Clinical Entity', *JAMA*, vol. 99, pp. 1323-1329.

D'Amelio, R., Pallone, F., Le Moli, S., Pontesilli, O. *et al.* (1985) 'Humoral inhibition of neutrophil chemotaxis in Crohn's disease', *Scand J Immunol*, vol. 22, no. 6, pp. 597-602.

D'Haens, G., Ferrante, M., Vermeire, S., Baert, F. *et al.* (2012) 'Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease', *Inflamm Bowel Dis*, vol. 18, no. 12, pp. 2218-2224.

D'Haens, G.R., Vermeire, S., Van Assche, G., Noman, M. *et al.* (2008) 'Therapy of metronidazole with azathioprine to prevent postoperative recurrence of

Crohn's disease: a controlled randomized trial', *Gastroenterology*, vol. 135, no. 4, pp. 1123-1129.

Dalziel, T.K. (1913) 'Chronic Interstitial Enteritis', *British Medical Journal*, vol. 2, pp. 1068-1070.

Danese, S., Sans, M., Scaldaferri, F., Sgambato, A. *et al.* (2006) 'TNF-alpha blockade down-regulates the CD40/CD40L pathway in the mucosal microcirculation: a novel anti-inflammatory mechanism of infliximab in Crohn's disease', *J Immunol*, vol. 176, no. 4, pp. 2617-2624.

Daperno, M., D'Haens, G., Van Assche, G., Baert, F. *et al.* (2004) 'Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD', *Gastrointest Endosc*, vol. 60, no. 4, pp. 505-512.

De Cruz, P., Kamm, M.A., Hamilton, A.L., Ritchie, K.J. *et al.* (2015) 'Crohn's disease management after intestinal resection: a randomised trial', *Lancet*, vol. 385, no. 9976, pp. 1406-1417.

Dejaco, C., Harrer, M., Waldhoer, T., Miehsler, W. *et al.* (2003) 'Antibiotics and azathioprine for the treatment of perianal fistulas in Crohn's disease', *Aliment Pharmacol Ther*, vol. 18, no. 11-12, pp. 1113-1120.

Deriy, L.V., Gomez, E.A., Zhang, G., Beacham, D.W. *et al.* (2009) 'Disease-causing mutations in the cystic fibrosis transmembrane conductance regulator determine the functional responses of alveolar macrophages', *J Biol Chem*, vol. 284, no. 51, pp. 35926-35938.

Desplat-Jego, S., Johanet, C., Escande, A., Goetz, J. *et al.* (2007) 'Update on Anti-Saccharomyces cerevisiae antibodies, anti-nuclear associated anti-neutrophil antibodies and antibodies to exocrine pancreas detected by indirect immunofluorescence as biomarkers in chronic inflammatory bowel diseases: results of a multicenter study', *World J Gastroenterol*, vol. 13, no. 16, pp. 2312-2318.

Dias de Castro, F., Boal Carvalho, P., Monteiro, S., Rosa, B. *et al.* (2015) 'Lewis Score - prognostic value in patients with isolated small bowel Crohn's disease', *J Crohns Colitis*.

Dicksved, J., Halfvarson, J., Rosenquist, M., Jarnerot, G. *et al.* (2008) 'Molecular analysis of the gut microbiota of identical twins with Crohn's disease', *ISME J*, vol. 2, no. 7, pp. 716-727.

do Carmo, A.M., Santos, F.M., Ortiz-Agostinho, C.L., Nishitokukado, I. *et al.* (2014) 'Cytomegalovirus infection in inflammatory bowel disease is not associated with worsening of intestinal inflammatory activity', *PLoS One*, vol. 9, no. 11, p. e111574.

Doherty, C.B. & Rosen, T. (2008) 'Evidence-based therapy for cutaneous sarcoidosis', *Drugs*, vol. 68, no. 10, pp. 1361-1383.

Dubinsky, M.C., Yang, H., Hassard, P.V., Seidman, E.G. *et al.* (2002) '6-MP metabolite profiles provide a biochemical explanation for 6-MP resistance in patients with inflammatory bowel disease', *Gastroenterology*, vol. 122, no. 4, pp. 904-915.

Duricova, D., Burisch, J., Jess, T., Gower-Rousseau, C. *et al.* (2014) 'Age-related differences in presentation and course of inflammatory bowel disease: an update on the population-based literature', *J Crohns Colitis*, vol. 8, no. 11, pp. 1351-1361.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E. *et al.* (2005) 'Diversity of the human intestinal microbial flora', *Science*, vol. 308, no. 5728, pp. 1635-1638.

Elkadri, A.A., Stempak, J.M., Walters, T.D., Lal, S. *et al.* (2013) 'Serum antibodies associated with complex inflammatory bowel disease', *Inflamm Bowel Dis*, vol. 19, no. 7, pp. 1499-1505.

Ellinghaus, D., Jostins, L., Spain, S.L., Cortes, A. *et al.* (2016) 'Analysis of five chronic inflammatory diseases identifies 27 new associations and highlights disease-specific patterns at shared loci', *Nat Genet*, vol. 48, no. 5, pp. 510-518.

Elliott, T.R., Hudspith, B., Karaiskos, C., Rayment, N. *et al.* (2011) 'Prolonged survival of E coli but not Staphylococcus aureus in monocytes from patients with Crohn's disease', *Gut*, vol. 60, no. Suppl 1, pp. A61-A62.

Elliott, T.R., Hudspith, B.N., Rayment, N.B., Prescott, N.J. *et al.* (2015) 'Defective macrophage handling of Escherichia coli in Crohn's disease', *J Gastroenterol Hepatol*, vol. 30, no. 8, pp. 1265-1274.

Emmendorffer, A., Hecht, M., Lohmann-Matthes, M.L. & Roesler, J. (1990) 'A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123', *J Immunol Methods*, vol. 131, no. 2, pp. 269-275.

Fabri, M., Stenger, S., Shin, D.M., Yuk, J.M. *et al.* (2011) 'Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages', *Sci Transl Med*, vol. 3, no. 104, p. 104ra102.

Falvey, J.D., Hoskin, T., Meijer, B., Ashcroft, A. *et al.* (2015) 'Disease activity assessment in IBD: clinical indices and biomarkers fail to predict endoscopic remission', *Inflamm Bowel Dis*, vol. 21, no. 4, pp. 824-831.

Fantini, M.C., Pallone, F. & Monteleone, G. (2009) 'Common immunologic mechanisms in inflammatory bowel disease and spondylarthropathies', *World J Gastroenterol*, vol. 15, no. 20, pp. 2472-2478.

Feakins, R.M. (2013) 'Inflammatory bowel disease biopsies: updated British Society of Gastroenterology reporting guidelines', *J Clin Pathol*, vol. 66, no. 12, pp. 1005-1026.

Fenollar, F., Lagier, J.C. & Raoult, D. (2014) 'Tropheryma whipplei and Whipple's disease', *J Infect*, vol. 69, no. 2, pp. 103-112.

Flannagan, R.S., Cosio, G. & Grinstein, S. (2009) 'Antimicrobial mechanisms of phagocytes and bacterial evasion strategies', *Nat Rev Microbiol*, vol. 7, no. 5, pp. 355-366.

Fleiss, J.L., Levin, B. & Paik, M.C. (2003) *Statistical Methods for Rates and Proportions*, 3rd edn, Wiley.

Florin, T.H., Paterson, E.W., Fowler, E.V. & Radford-Smith, G.L. (2006) 'Clinically active Crohn's disease in the presence of a low C-reactive protein', *Scand J Gastroenterol*, vol. 41, no. 3, pp. 306-311.

Ford, A.C., Bernstein, C.N., Khan, K.J., Abreu, M.T. *et al.* (2011a) 'Glucocorticosteroid therapy in inflammatory bowel disease: systematic review and meta-analysis', *Am J Gastroenterol*, vol. 106, no. 4, pp. 590-599; quiz 600.

Ford, A.C., Kane, S.V., Khan, K.J., Achkar, J.P. *et al.* (2011b) 'Efficacy of 5-aminosalicylates in Crohn's disease: systematic review and meta-analysis', *Am J Gastroenterol*, vol. 106, no. 4, pp. 617-629.

Ford, A.C., Khan, K.J., Talley, N.J. & Moayyedi, P. (2011c) '5-aminosalicylates prevent relapse of Crohn's disease after surgically induced remission: systematic review and meta-analysis', *Am J Gastroenterol*, vol. 106, no. 3, pp. 413-420.

Forrest, K., Symmons, D. & Foster, P. (2004) 'Systematic review: is ingestion of paracetamol or non-steroidal anti-inflammatory drugs associated with exacerbations of inflammatory bowel disease?', *Aliment Pharmacol Ther*, vol. 20, no. 10, pp. 1035-1043.

Fortier, A.H., Leiby, D.A., Narayanan, R.B., Asafoadjei, E. *et al.* (1995) 'Growth of Francisella tularensis LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth', *Infect Immun*, vol. 63, no. 4, pp. 1478-1483.

Frank, D.N., St Amand, A.L., Feldman, R.A., Boedeker, E.C. *et al.* (2007) 'Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases', *Proc Natl Acad Sci U S A*, vol. 104, no. 34, pp. 13780-13785.

Franke, A., McGovern, D.P., Barrett, J.C., Wang, K. *et al.* (2010) 'Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci', *Nat Genet*, vol. 42, no. 12, pp. 1118-1125.

Fujimura, Y., Kamoi, R. & Iida, M. (1996) 'Pathogenesis of aphthoid ulcers in Crohn's disease: correlative findings by magnifying colonoscopy, electron microscopy, and immunohistochemistry', *Gut*, vol. 38, no. 5, pp. 724-732.

Gabryel, M., Skrzypczak-Zielinska, M., Kucharski, M.A., Slomski, R. *et al.* (2016) 'The impact of genetic factors on response to glucocorticoids therapy in IBD', *Scand J Gastroenterol*, vol. 51, no. 6, pp. 654-665.

Gale, G., Sigurdsson, G.V., Ostman, S., Malmborg, P. *et al.* (2016) 'Does Crohn's Disease with Concomitant Orofacial Granulomatosis Represent a Distinctive Disease Subtype?', *Inflamm Bowel Dis*, vol. 22, no. 5, pp. 1071-1077.

Gatta, G., Di Grezia, G., Di Mizio, V., Landolfi, C. *et al.* (2012) 'Crohn's disease imaging: a review', *Gastroenterol Res Pract*, vol. 2012, p. 816920.

Gaya, D.R., Russell, R.K., Nimmo, E.R. & Satsangi, J. (2006) 'New genes in inflammatory bowel disease: lessons for complex diseases?', *Lancet*, vol. 367, no. 9518, pp. 1271-1284.

Gerich, M.E. & McGovern, D.P. (2014) 'Towards personalized care in IBD', *Nat Rev Gastroenterol Hepatol*, vol. 11, no. 5, pp. 287-299.

Gisbert, J.P. & Chaparro, M. (2014) 'Systematic review with meta-analysis: inflammatory bowel disease in the elderly', *Aliment Pharmacol Ther*, vol. 39, no. 5, pp. 459-477.

Glasser, A.L., Boudeau, J., Barnich, N., Perruchot, M.H. *et al.* (2001) 'Adherent invasive Escherichia coli strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death', *Infect Immun*, vol. 69, no. 9, pp. 5529-5537.

Glubb, D.M., Gearry, R.B., Barclay, M.L., Roberts, R.L. *et al.* (2011) 'NOD2 and ATG16L1 polymorphisms affect monocyte responses in Crohn's disease', *World J Gastroenterol*, vol. 17, no. 23, pp. 2829-2837.

Goenka, M.K., Kochhar, R., Tandia, B. & Mehta, S.K. (1996) 'Chloroquine for mild to moderately active ulcerative colitis: comparison with sulfasalazine', *Am J Gastroenterol*, vol. 91, no. 5, pp. 917-921.

Gonzalez, M.A., Uribe, F., Moisen, S.D., Fuster, A.P. *et al.* (1984) 'Multiple-dose pharmacokinetics and safety of ciprofloxacin in normal volunteers', *Antimicrob Agents Chemother*, vol. 26, no. 5, pp. 741-744.

Goodgame, R.W., Kimball, K., Akram, S., Ike, E. *et al.* (2001) 'Randomized controlled trial of clarithromycin and ethambutol in the treatment of Crohn's disease', *Aliment Pharmacol Ther*, vol. 15, no. 12, pp. 1861-1866.

Graham, D.Y., AI-Assi, M.T. & Robinson, M. (1995) 'Prolonged Remission In Crohn's Disease Following Therapy For Mycobacterium Paratuberculosis Infection', *Gastroenterology*, vol. 108, no. 4, p. A826.

Greenbloom, S.L., Steinhart, A.H. & Greenberg, G.R. (1998) 'Combination ciprofloxacin and metronidazole for active Crohn's disease', *Can J Gastroenterol*, vol. 12, no. 1, pp. 53-56.

Gui, G.P., Thomas, P.R., Tizard, M.L., Lake, J. *et al.* (1997) 'Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics', *J Antimicrob Chemother*, vol. 39, no. 3, pp. 393-400.

Guidi, L., Marzo, M., Andrisani, G., Felice, C. *et al.* (2014) 'Faecal calprotectin assay after induction with anti-Tumour Necrosis Factor alpha agents in inflammatory bowel disease: Prediction of clinical response and mucosal healing at one year', *Dig Liver Dis*, vol. 46, no. 11, pp. 974-979.

Gunesh, S., Thomas, G.A., Williams, G.T., Roberts, A. *et al.* (2008) 'The incidence of Crohn's disease in Cardiff over the last 75 years: an update for 1996-2005', *Aliment Pharmacol Ther*, vol. 27, no. 3, pp. 211-219.

Gutierrez, A., Scharl, M., Sempere, L., Holler, E. *et al.* (2014) 'Genetic susceptibility to increased bacterial translocation influences the response to biological therapy in patients with Crohn's disease', *Gut*, vol. 63, no. 2, pp. 272-280.

Hafeez, R., Greenhalgh, R., Rajan, J., Bloom, S. *et al.* (2011) 'Use of small bowel imaging for the diagnosis and staging of Crohn's disease--a survey of current UK practice', *Br J Radiol*, vol. 84, no. 1002, pp. 508-517.

Halfvarson, J. (2011) 'Genetics in twins with Crohn's disease: less pronounced than previously believed?', *Inflamm Bowel Dis*, vol. 17, no. 1, pp. 6-12.

Harbord, M.W., Marks, D.J., Forbes, A., Bloom, S.L. *et al.* (2006) 'Impaired neutrophil chemotaxis in Crohn's disease relates to reduced production of chemokines and can be augmented by granulocyte-colony stimulating factor', *Aliment Pharmacol Ther*, vol. 24, no. 4, pp. 651-660.

Hase, K., Kawano, K., Nochi, T., Pontes, G.S. *et al.* (2009) 'Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response', *Nature*, vol. 462, no. 7270, pp. 226-230.

Heap, G.A., So, K., Weedon, M., Edney, N. *et al.* (2016) 'Clinical Features and HLA Association of 5-Aminosalicylate (5-ASA)-induced Nephrotoxicity in Inflammatory Bowel Disease', *J Crohns Colitis*, vol. 10, no. 2, pp. 149-158.

Heap, G.A., Weedon, M.N., Bewshea, C.M., Singh, A. *et al.* (2014) 'HLA-DQA1-HLA-DRB1 variants confer susceptibility to pancreatitis induced by thiopurine immunosuppressants', *Nat Genet*, vol. 46, no. 10, pp. 1131-1134.

Hedin, C., van der Gast, C.J., Rogers, G.B., Cuthbertson, L. *et al.* (2016) 'Siblings of patients with Crohn's disease exhibit a biologically relevant dysbiosis in mucosal microbial metacommunities', *Gut*, vol. 65, no. 6, pp. 944-953.

Hedin, C.R., McCarthy, N.E., Louis, P., Farquharson, F.M. *et al.* (2014) 'Altered intestinal microbiota and blood T cell phenotype are shared by patients with Crohn's disease and their unaffected siblings', *Gut*, vol. 63, no. 10, pp. 1578-1586.

Heikkila, K., Madsen, I.E., Nyberg, S.T., Fransson, E.I. *et al.* (2014) 'Job strain and the risk of inflammatory bowel diseases: individual-participant meta-analysis of 95,000 men and women', *PLoS One*, vol. 9, no. 2, p. e88711.

Henderson, P., Hansen, R., Cameron, F.L., Gerasimidis, K. *et al.* (2012) 'Rising incidence of pediatric inflammatory bowel disease in Scotland', *Inflamm Bowel Dis*, vol. 18, no. 6, pp. 999-1005.

Henriksen, M., Jahnsen, J., Lygren, I., Stray, N. *et al.* (2008) 'C-reactive protein: a predictive factor and marker of inflammation in inflammatory bowel disease. Results from a prospective population-based study', *Gut*, vol. 57, no. 11, pp. 1518-1523.

Hewison, M. (2012) 'Vitamin D and immune function: an overview', *Proc Nutr Soc*, vol. 71, no. 1, pp. 50-61.

Hlavaty, T., Krajcovicova, A. & Payer, J. (2015) 'Vitamin D therapy in inflammatory bowel diseases: who, in what form, and how much?', *J Crohns Colitis*, vol. 9, no. 2, pp. 198-209.

Hoidal, J.R., Schmeling, D. & Peterson, P.K. (1981) 'Phagocytosis, bacterial killing, and metabolism by purified human lung phagocytes', *J Infect Dis*, vol. 144, no. 1, pp. 61-71.

Homer, C.R., Richmond, A.L., Rebert, N.A., Achkar, J.P. *et al.* (2010) 'ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis', *Gastroenterology*, vol. 139, no. 5, pp. 1630-1641, 1641 e1631-1632.

Hoogkamp-Korstanje, J.A., Lindner, J.G., Marcelis, J.H., den Daas-Slagt, H. *et al.* (1979) 'Composition and ecology of the human intestinal flora', *Antonie Van Leeuwenhoek*, vol. 45, no. 1, pp. 35-40.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S. *et al.* (2001) 'Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease', *Nature*, vol. 411, no. 6837, pp. 599-603.

Hugot, J.P., Laurent-Puig, P., Gower-Rousseau, C., Olson, J.M. *et al.* (1996) 'Mapping of a susceptibility locus for Crohn's disease on chromosome 16', *Nature*, vol. 379, no. 6568, pp. 821-823.

Inoue, S., Nakase, H., Matsuura, M., Ueno, S. *et al.* (2007) 'Open label trial of clarithromycin therapy in Japanese patients with Crohn's disease', *J Gastroenterol Hepatol*, vol. 22, pp. 984-988.

Irving, P.M. & Gibson, P.R. (2008) 'Infections and IBD', *Nat Clin Pract Gastroenterol Hepatol*, vol. 5, no. 1, pp. 18-27.

Ishikawa, T., Okamura, S., Oshimoto, H., Kobayashi, R. *et al.* (2003) 'Metronidazole plus ciprofloxacin therapy for active Crohn's disease', *Intern Med*, vol. 42, no. 4, pp. 318-321.

Israeli, E., Grotto, I., Gilburd, B., Balicer, R.D. *et al.* (2005) 'Anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic antibodies as predictors of inflammatory bowel disease', *Gut*, vol. 54, no. 9, pp. 1232-1236.

Jeong, J.Y. & Jue, D.M. (1997) 'Chloroquine inhibits processing of tumor necrosis factor in lipopolysaccharide-stimulated RAW 264.7 macrophages', *J Immunol*, vol. 158, no. 10, pp. 4901-4907.

Johansson, M.E., Phillipson, M., Petersson, J., Velcich, A. *et al.* (2008) 'The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria', *Proc Natl Acad Sci U S A*, vol. 105, no. 39, pp. 15064-15069.

Johnson, G.J., Cosnes, J. & Mansfield, J.C. (2005) 'Review article: smoking cessation as primary therapy to modify the course of Crohn's disease', *Aliment Pharmacol Ther*, vol. 21, no. 8, pp. 921-931.

Joossens, M., Huys, G., Cnockaert, M., De Preter, V. *et al.* (2011) 'Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives', *Gut*, vol. 60, no. 5, pp. 631-637.

Joossens, S., Vermeire, S., Van Steen, K., Godefridis, G. *et al.* (2004) 'Pancreatic autoantibodies in inflammatory bowel disease', *Inflamm Bowel Dis*, vol. 10, no. 6, pp. 771-777.

Jorgensen, S.P., Agnholt, J., Glerup, H., Lyhne, S. *et al.* (2010) 'Clinical trial: vitamin D3 treatment in Crohn's disease - a randomized double-blind placebo-controlled study', *Aliment Pharmacol Ther*, vol. 32, no. 3, pp. 377-383.

Jorgensen, S.P., Hvas, C.L., Agnholt, J., Christensen, L.A. *et al.* (2013) 'Active Crohn's disease is associated with low vitamin D levels', *J Crohns Colitis*, vol. 7, no. 10, pp. e407-413.

Jostins, L., Ripke, S., Weersma, R.K., Duerr, R.H. *et al.* (2012) 'Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease', *Nature*, vol. 491, no. 7422, pp. 119-124.

Jurgens, M., Mahachie John, J.M., Cleynen, I., Schnitzler, F. *et al.* (2011) 'Levels of C-reactive protein are associated with response to infliximab therapy in

patients with Crohn's disease', *Clin Gastroenterol Hepatol*, vol. 9, no. 5, pp. 421-427 e421.

Kabbani, T.A., Koutroubakis, I.E., Schoen, R.E., Ramos-Rivers, C. *et al.* (2016) 'Association of Vitamin D Level With Clinical Status in Inflammatory Bowel Disease: A 5-Year Longitudinal Study', *Am J Gastroenterol*, vol. 111, no. 5, pp. 712-719.

Kaplan, G.G., Pedersen, B.V., Andersson, R.E., Sands, B.E. *et al.* (2007) 'The risk of developing Crohn's disease after an appendectomy: a population-based cohort study in Sweden and Denmark', *Gut*, vol. 56, no. 10, pp. 1387-1392.

Karoui, S., Ouerdiane, S., Serghini, M., Jomni, T. *et al.* (2007) 'Correlation between levels of C-reactive protein and clinical activity in Crohn's disease', *Dig Liver Dis*, vol. 39, no. 11, pp. 1006-1010.

Kenny, E.E., Pe'er, I., Karban, A., Ozelius, L. *et al.* (2012) 'A genome-wide scan of Ashkenazi Jewish Crohn's disease suggests novel susceptibility loci', *PLoS Genet*, vol. 8, no. 3, p. e1002559.

Kevans, D., Silverberg, M.S., Borowski, K., Griffiths, A. *et al.* (2016) 'IBD Genetic Risk Profile in Healthy First-Degree Relatives of Crohn's Disease Patients', *J Crohns Colitis*, vol. 10, no. 2, pp. 209-215.

Khan, K.J., Ullman, T.A., Ford, A.C., Abreu, M.T. *et al.* (2011) 'Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis', *Am J Gastroenterol*, vol. 106, no. 4, pp. 661-673.

Korkmaz, B., Horwitz, M.S., Jenne, D.E. & Gauthier, F. (2010) 'Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases', *Pharmacol Rev*, vol. 62, no. 4, pp. 726-759.

Lakatos, P.L., Altorjay, I., Szamosi, T., Palatka, K. *et al.* (2009) 'Pancreatic autoantibodies are associated with reactivity to microbial antibodies, penetrating disease behavior, perianal disease, and extraintestinal manifestations, but not with NOD2/CARD15 or TLR4 genotype in a Hungarian IBD cohort', *Inflamm Bowel Dis*, vol. 15, no. 3, pp. 365-374.

Landers, C.J., Cohavy, O., Misra, R., Yang, H. *et al.* (2002) 'Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens', *Gastroenterology*, vol. 123, no. 3, pp. 689-699.

Lapaquette, P., Bringer, M.A. & Darfeuille-Michaud, A. (2012) 'Defects in autophagy favour adherent-invasive Escherichia coli persistence within macrophages leading to increased pro-inflammatory response', *Cell Microbiol*, vol. 14, no. 6, pp. 791-807.

Lautenbach, E., Berlin, J.A. & Lichtenstein, G.R. (1998) 'Risk factors for early postoperative recurrence of Crohn's disease', *Gastroenterology*, vol. 115, no. 2, pp. 259-267.

Lazzerini, M., Bramuzzo, M. & Ventura, A. (2014) 'Association between orofacial granulomatosis and Crohn's disease in children: systematic review', *World J Gastroenterol*, vol. 20, no. 23, pp. 7497-7504.

Lee, D., Albenberg, L., Compher, C., Baldassano, R. *et al.* (2015) 'Diet in the pathogenesis and treatment of inflammatory bowel diseases', *Gastroenterology*, vol. 148, no. 6, pp. 1087-1106.

Lees, C.W., Barrett, J.C., Parkes, M. & Satsangi, J. (2011) 'New IBD genetics: common pathways with other diseases', *Gut*, vol. 60, no. 12, pp. 1739-1753.

Lees, C.W. & Satsangi, J. (2009) 'Genetics of inflammatory bowel disease: implications for disease pathogenesis and natural history', *Expert Rev Gastroenterol Hepatol*, vol. 3, no. 5, pp. 513-534.

Leiper, K., Martin, K., Ellis, A., Watson, A.J. *et al.* (2008) 'Clinical trial: randomized study of clarithromycin versus placebo in active Crohn's disease', *Aliment Pharmacol Ther*, vol. 27, no. 12, pp. 1233-1239.

Leiper, K., Morris, A.I. & Rhodes, J.M. (2000) 'Open label trial of oral clarithromycin in active Crohn's disease', *Aliment Pharmacol Ther*, vol. 14, no. 6, pp. 801-806.

Lennard-Jones, J.E. (1989) 'Classification of inflammatory bowel disease', *Scand J Gastroenterol Suppl*, vol. 170, pp. 2-6; discussion 16-19.

Lennard-Jones, J.E. & Shivananda, S. (1997) 'Clinical uniformity of inflammatory bowel disease a presentation and during the first year of disease in the north and south of Europe. EC-IBD Study Group', *Eur J Gastroenterol Hepatol*, vol. 9, no. 4, pp. 353-359.

Levin, A.D., Wildenberg, M.E. & van den Brink, G.R. (2016) 'Mechanism of Action of Anti-TNF Therapy in Inflammatory Bowel Disease', *J Crohns Colitis*.

Levy, G.D., Munz, S.J., Paschal, J., Cohen, H.B. *et al.* (1997) 'Incidence of hydroxychloroquine retinopathy in 1,207 patients in a large multicenter outpatient practice', *Arthritis Rheum*, vol. 40, no. 8, pp. 1482-1486.

Li, X., Sundquist, J. & Sundquist, K. (2009) 'Educational level and occupation as risk factors for inflammatory bowel diseases: A nationwide study based on hospitalizations in Sweden', *Inflamm Bowel Dis*, vol. 15, no. 4, pp. 608-615.

Li, Y., Zuo, L., Zhu, W., Gong, J. *et al.* (2015) 'The impact of bacterial DNA translocation on early postoperative outcomes in Crohn's patients undergoing abdominal surgery', *J Crohns Colitis*, vol. 9, no. 3, pp. 259-265.

Lichtenstein, G.R., Targan, S.R., Dubinsky, M.C., Rotter, J.I. *et al.* (2011) 'Combination of genetic and quantitative serological immune markers are associated with complicated Crohn's disease behavior', *Inflamm Bowel Dis*, vol. 17, no. 12, pp. 2488-2496.

Lim, W.C. & Hanauer, S. (2010) 'Aminosalicylates for induction of remission or response in Crohn's disease', *Cochrane Database Syst Rev*, no. 12, p. CD008870.

Lindberg, E., Tysk, C., Andersson, K. & Jarnerot, G. (1988) 'Smoking and inflammatory bowel disease. A case control study', *Gut*, vol. 29, no. 3, pp. 352-357.

Liu, J.Z., van Sommeren, S., Huang, H., Ng, S.C. *et al.* (2015) 'Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations', *Nat Genet*, vol. 47, no. 9, pp. 979-986.

Liu, W., Yan, M., Liu, Y., McLeish, K.R. *et al.* (2012) 'Olfactomedin 4 Inhibits Cathepsin C-Mediated Protease Activities, Thereby Modulating Neutrophil Killing of Staphylococcus aureus and Escherichia coli in Mice', *J Immunol*.

Lockhart-Mummery, H.E. & Morson, B.C. (1960) 'Crohn's disease (regional enteritis) of the large intestine and its distinction from ulcerative colitis', *Gut*, vol. 1, pp. 87-105.

Lopez-Amoros, R., Comas, J. & Vives-Rego, J. (1995) 'Flow cytometric assessment of Escherichia coli and Salmonella typhimurium starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol', *Appl Environ Microbiol*, vol. 61, no. 7, pp. 2521-2526.

Louis, E. & Belaiche, J. (1995) 'Hydroxychloroquine (Plaquenil) for recurrence prevention of Crohn's disease after curative surgery', *Gastroenterol Clin Biol*, vol. 19, no. 2, pp. 233-234.

Louis, E., Collard, A., Oger, A.F., Degroote, E. *et al.* (2001) 'Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease', *Gut*, vol. 49, no. 6, pp. 777-782.

Louis, E., Mary, J.Y., Vernier-Massouille, G., Grimaud, J.C. *et al.* (2012) 'Maintenance of remission among patients with Crohn's disease on antimetabolite therapy after infliximab therapy is stopped', *Gastroenterology*, vol. 142, no. 1, pp. 63-70 e65; quiz e31.

Lunney, P.C., Kariyawasam, V.C., Wang, R.R., Middleton, K.L. *et al.* (2015) 'Smoking prevalence and its influence on disease course and surgery in Crohn's disease and ulcerative colitis', *Aliment Pharmacol Ther*, vol. 42, no. 1, pp. 61-70.

Maeda, Y., Ng, S.C., Durdey, P., Burt, C. *et al.* (2010) 'Randomized clinical trial of metronidazole ointment versus placebo in perianal Crohn's disease', *Br J Surg*, vol. 97, no. 9, pp. 1340-1347.

Mahid, S.S., Mulhall, A.M., Gholson, R.D., Eichenberger, M.R. *et al.* (2008) 'Inflammatory bowel disease and African Americans: a systematic review', *Inflamm Bowel Dis*, vol. 14, no. 7, pp. 960-967.

Malhotra, R., Turner, K., Sonnenberg, A. & Genta, R.M. (2015) 'High prevalence of inflammatory bowel disease in United States residents of Indian ancestry', *Clin Gastroenterol Hepatol*, vol. 13, no. 4, pp. 683-689.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K. *et al.* (2006) 'Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach', *Gut*, vol. 55, no. 2, pp. 205-211.

Mao, R., Xiao, Y.L., Gao, X., Chen, B.L. *et al.* (2012) 'Fecal calprotectin in predicting relapse of inflammatory bowel diseases: a meta-analysis of prospective studies', *Inflamm Bowel Dis*, vol. 18, no. 10, pp. 1894-1899.

Mardini, H.E., Kip, K.E. & Wilson, J.W. (2004) 'Crohn's disease: a two-year prospective study of the association between psychological distress and disease activity', *Dig Dis Sci*, vol. 49, no. 3, pp. 492-497.

Marks, D.J., Harbord, M.W., MacAllister, R., Rahman, F.Z. *et al.* (2006) 'Defective acute inflammation in Crohn's disease: a clinical investigation', *Lancet*, vol. 367, no. 9511, pp. 668-678.

Marks, D.J., Miyagi, K., Rahman, F.Z., Novelli, M. *et al.* (2009) 'Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease', *Am J Gastroenterol*, vol. 104, no. 1, pp. 117-124.

Martin, H.M., Campbell, B.J., Hart, C.A., Mpofu, C. *et al.* (2004) 'Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer', *Gastroenterology*, vol. 127, no. 1, pp. 80-93.

Martinez-Medina, M., Aldeguer, X., Gonzalez-Huix, F., Acero, D. *et al.* (2006) 'Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis', *Inflamm Bowel Dis*, vol. 12, no. 12, pp. 1136-1145.

Mary, J.Y. & Modigliani, R. (1989) 'Development and validation of an endoscopic index of the severity for Crohn's disease: a prospective multicentre study. Groupe d'Etudes Therapeutiques des Affections Inflammatoires du Tube Digestif (GETAID)', *Gut*, vol. 30, no. 7, pp. 983-989.

Maurin, M., Benoliel, A.M., Bongrand, P. & Raoult, D. (1992) 'Phagolysosomal alkalinization and the bactericidal effect of antibiotics: the Coxiella burnetii paradigm', *J Infect Dis*, vol. 166, no. 5, pp. 1097-1102.

Mavrikakis, I., Sfikakis, P.P., Mavrikakis, E., Rougas, K. *et al.* (2003) 'The incidence of irreversible retinal toxicity in patients treated with hydroxychloroquine: a reappraisal', *Ophthalmology*, vol. 110, no. 7, pp. 1321-1326.

Mawdsley, J.E. & Rampton, D.S. (2005) 'Psychological stress in IBD: new insights into pathogenic and therapeutic implications', *Gut*, vol. 54, no. 10, pp. 1481-1491.

Mayberry, J.F., Judd, D., Smart, H., Rhodes, J. *et al.* (1986) 'Crohn's disease in Jewish people--an epidemiological study in south-east Wales', *Digestion*, vol. 35, no. 4, pp. 237-240.

McDermott, E., Ryan, E.J., Tosetto, M., Gibson, D. *et al.* (2016) 'DNA Methylation Profiling in Inflammatory Bowel Disease Provides New Insights into Disease Pathogenesis', *J Crohns Colitis*, vol. 10, no. 1, pp. 77-86.

Meconi, S., Vercellone, A., Levillain, F., Payre, B. *et al.* (2007) 'Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro', *Cell Microbiol*, vol. 9, no. 5, pp. 1252-1261.

Mee, A.S., Szawatakowski, M. & Jewell, D.P. (1980) 'Monocytes in inflammatory bowel disease: phagocytosis and intracellular killing', *J Clin Pathol*, vol. 33, no. 10, pp. 921-925.

Moller, F.T., Andersen, V., Wohlfahrt, J. & Jess, T. (2015) 'Familial risk of inflammatory bowel disease: a population-based cohort study 1977-2011', *Am J Gastroenterol*, vol. 110, no. 4, pp. 564-571.

Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A. *et al.* (2012) 'Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review', *Gastroenterology*, vol. 142, no. 1, pp. 46-54 e42; quiz e30.

Mosli, M.H., Zou, G., Garg, S.K., Feagan, S.G. *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', *Am J Gastroenterol*, vol. 110, no. 6, pp. 802-819; quiz 820.

Mottola, G., Boucherit, N., Trouplin, V., Oury Barry, A. *et al.* (2014) 'Tropheryma whipplei, the agent of Whipple's disease, affects the early to late phagosome transition and survives in a Rab5- and Rab7-positive compartment', *PLoS One*, vol. 9, no. 2, p. e89367.

Mow, W.S., Vasiliauskas, E.A., Lin, Y.C., Fleshner, P.R. *et al.* (2004) 'Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease', *Gastroenterology*, vol. 126, no. 2, pp. 414-424.

Mpofu, C.M., Campbell, B.J., Subramanian, S., Marshall-Clarke, S. *et al.* (2007) 'Microbial mannan inhibits bacterial killing by macrophages: a possible pathogenic mechanism for Crohn's disease', *Gastroenterology*, vol. 133, no. 5, pp. 1487-1498.

Munster, T., Gibbs, J.P., Shen, D., Baethge, B.A. *et al.* (2002) 'Hydroxychloroquine concentration-response relationships in patients with rheumatoid arthritis', *Arthritis Rheum*, vol. 46, no. 6, pp. 1460-1469.

Mylonaki, M., Langmead, L., Pantes, A., Johnson, F. *et al.* (2004) 'Enteric infection in relapse of inflammatory bowel disease: importance of microbiological examination of stool', *Eur J Gastroenterol Hepatol*, vol. 16, no. 8, pp. 775-778.

Nahon, S., Lahmek, P., Macaigne, G., Faurel, J.P. *et al.* (2009) 'Socioeconomic deprivation does not influence the severity of Crohn's disease: Results of a prospective multicenter study', *Inflamm Bowel Dis*, vol. 15, no. 4, pp. 594-598.

Naismith, G.D., Smith, L.A., Barry, S.J., Munro, J.I. *et al.* (2014) 'A prospective evaluation of the predictive value of faecal calprotectin in quiescent Crohn's disease', *J Crohns Colitis*, vol. 8, no. 9, pp. 1022-1029.

Nanda Kumar, N.S., Balamurugan, R., Jayakanthan, K., Pulimood, A. *et al.* (2008) 'Probiotic administration alters the gut flora and attenuates colitis in mice administered dextran sodium sulfate', *J Gastroenterol Hepatol*, vol. 23, no. 12, pp. 1834-1839.

Nelson, C.D., Nonnecke, B.J., Reinhardt, T.A., Waters, W.R. *et al.* (2011) 'Regulation of Mycobacterium-specific mononuclear cell responses by 25-hydroxyvitamin D3', *PLoS One*, vol. 6, no. 6, p. e21674.

Neurath, M.F. & Travis, S.P. (2012) 'Mucosal healing in inflammatory bowel diseases: a systematic review', *Gut*, vol. 61, no. 11, pp. 1619-1635.

Ng, S.C., Tang, W., Leong, R.W., Chen, M. *et al.* (2015) 'Environmental risk factors in inflammatory bowel disease: a population-based case-control study in Asia-Pacific', *Gut*, vol. 64, no. 7, pp. 1063-1071.

Nguyen, G.C., Chong, C.A. & Chong, R.Y. (2014) 'National estimates of the burden of inflammatory bowel disease among racial and ethnic groups in the United States', *J Crohns Colitis*, vol. 8, no. 4, pp. 288-295.

NICE (2013) 'Faecal calprotectin diagnostic tests for inflammatory diseases of the bowel (DG11)'.

Nordgren, S., Fasth, S. & Hulten, L. (1992) 'Anal fistulas in Crohn's disease: incidence and outcome of surgical treatment', *Int J Colorectal Dis*, vol. 7, no. 4, pp. 214-218.

O'Brien, C.L., Bringer, M.A., Holt, K.E., Gordon, D.M. *et al.* (2016) 'Comparative genomics of Crohn's disease-associated adherent-invasive Escherichia coli', *Gut*.

Ohkuma, S. & Poole, B. (1978) 'Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents', *Proc Natl Acad Sci U S A*, vol. 75, no. 7, pp. 3327-3331.

Orchard, T.R., Holt, H., Bradbury, L., Hammersma, J. *et al.* (2009) 'The prevalence, clinical features and association of HLA-B27 in sacroiliitis associated with established Crohn's disease', *Aliment Pharmacol Ther*, vol. 29, no. 2, pp. 193-197.

Orholm, M., Fonager, K. & Sorensen, H.T. (1999) 'Risk of ulcerative colitis and Crohn's disease among offspring of patients with chronic inflammatory bowel disease', *Am J Gastroenterol*, vol. 94, no. 11, pp. 3236-3238.

Ostro, M.J., Greenberg, G.R. & Jeejeebhoy, K.N. (1985) 'Total parenteral nutrition and complete bowel rest in the management of Crohn's disease', *JPEN J Parenter Enteral Nutr*, vol. 9, no. 3, pp. 280-287.

Ott, C. & Scholmerich, J. (2013) 'Extraintestinal manifestations and complications in IBD', *Nat Rev Gastroenterol Hepatol*, vol. 10, no. 10, pp. 585-595.

Ott, S.J., Musfeldt, M., Wenderoth, D.F., Hampe, J. *et al.* (2004) 'Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease', *Gut*, vol. 53, no. 5, pp. 685-693.

Ouyang, Q., Tandon, R., Goh, K.L., Ooi, C.J. *et al.* (2005) 'The emergence of inflammatory bowel disease in the Asian Pacific region', *Curr Opin Gastroenterol*, vol. 21, no. 4, pp. 408-413.

Palmier, M.O. & Van Doren, S.R. (2007) 'Rapid determination of enzyme kinetics from fluorescence: overcoming the inner filter effect', *Anal Biochem*, vol. 371, no. 1, pp. 43-51.

Parkes, M., Barrett, J.C., Prescott, N.J., Tremelling, M. *et al.* (2007) 'Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility', *Nat Genet*, vol. 39, no. 7, pp. 830-832.

Paul, S., Boschetti, G., Rinaudo-Gaujous, M., Moreau, A. *et al.* (2015) 'Association of Anti-glycan Antibodies and Inflammatory Bowel Disease Course', *J Crohns Colitis*, vol. 9, no. 6, pp. 445-451.

Pavlidis, P., Romanidou, O., Roggenbuck, D., Mytilinaiou, M.G. *et al.* (2012) 'Ileal inflammation may trigger the development of GP2-specific pancreatic autoantibodies in patients with Crohn's disease', *Clin Dev Immunol*, vol. 2012, p. 640835.

Perencevich, M. & Burakoff, R. (2006) 'Use of antibiotics in the treatment of inflammatory bowel disease', *Inflamm Bowel Dis*, vol. 12, no. 7, pp. 651-664.

Peyrin-Biroulet, L., Gonzalez, F., Dubuquoy, L., Rousseaux, C. *et al.* (2012) 'Mesenteric fat as a source of C reactive protein and as a target for bacterial translocation in Crohn's disease', *Gut*, vol. 61, no. 1, pp. 78-85.

Plevy, S., Silverberg, M.S., Lockton, S., Stockfisch, T. *et al.* (2013) 'Combined serological, genetic, and inflammatory markers differentiate non-IBD, Crohn's disease, and ulcerative colitis patients', *Inflamm Bowel Dis*, vol. 19, no. 6, pp. 1139-1148.

Poole, B. & Ohkuma, S. (1981) 'Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages', *J Cell Biol*, vol. 90, no. 3, pp. 665-669.

Prantera, C., Kohn, A., Mangiarotti, R., Andreoli, A. *et al.* (1994) 'Antimycobacterial therapy in Crohn's disease: results of a controlled, double-blind trial with a multiple antibiotic regimen', *Am J Gastroenterol*, vol. 89, no. 4, pp. 513-518.

Prantera, C., Lochs, H., Campieri, M., Scribano, M.L. *et al.* (2006) 'Antibiotic treatment of Crohn's disease: results of a multicentre, double blind, randomized, placebo-controlled trial with rifaximin', *Aliment Pharmacol Ther*, vol. 23, no. 8, pp. 1117-1125.

Prantera, C., Lochs, H., Grimaldi, M., Danese, S. *et al.* (2012) 'Rifaximin-extended intestinal release induces remission in patients with moderately active Crohn's disease', *Gastroenterology*, vol. 142, no. 3, pp. 473-481 e474.

Prefontaine, E., Sutherland, L.R., Macdonald, J.K. & Cepoiu, M. (2009) 'Azathioprine or 6-mercaptopurine for maintenance of remission in Crohn's disease', *Cochrane Database Syst Rev*, no. 1, p. CD000067.

Prideaux, L., De Cruz, P., Ng, S.C. & Kamm, M.A. (2012) 'Serological antibodies in inflammatory bowel disease: a systematic review', *Inflamm Bowel Dis*, vol. 18, no. 7, pp. 1340-1355.

Prorok-Hamon, M., Friswell, M.K., Alswied, A., Roberts, C.L. *et al.* (2014) 'Colonic mucosa-associated diffusely adherent afaC+ Escherichia coli expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer', *Gut*, vol. 63, no. 5, pp. 761-770.

Qin, J., Li, R., Raes, J., Arumugam, M. *et al.* (2010) 'A human gut microbial gene catalogue established by metagenomic sequencing', *Nature*, vol. 464, no. 7285, pp. 59-65.

Quinton, J.F., Sendid, B., Reumaux, D., Duthilleul, P. *et al.* (1998) 'Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role', *Gut*, vol. 42, no. 6, pp. 788-791.

Rabinowitz, J.D. & White, E. (2010) 'Autophagy and metabolism', *Science*, vol. 330, no. 6009, pp. 1344-1348.

Ramadas, A.V., Gunesh, S., Thomas, G.A., Williams, G.T. *et al.* (2010) 'Natural history of Crohn's disease in a population-based cohort from Cardiff (1986-2003): a study of changes in medical treatment and surgical resection rates', *Gut*, vol. 59, no. 9, pp. 1200-1206.

Raoult, D., Houpikian, P., Tissot Dupont, H., Riss, J.M. *et al.* (1999) 'Treatment of Q fever endocarditis: comparison of 2 regimens containing doxycycline and ofloxacin or hydroxychloroquine', *Arch Intern Med*, vol. 159, no. 2, pp. 167-173.

Reese, G.E., Constantinides, V.A., Simillis, C., Darzi, A.W. *et al.* (2006) 'Diagnostic precision of anti-Saccharomyces cerevisiae antibodies and perinuclear antineutrophil cytoplasmic antibodies in inflammatory bowel disease', *Am J Gastroenterol*, vol. 101, no. 10, pp. 2410-2422.

Reijngoud, D. & Tager, J.M. (1975) 'Effect of ionophores and temperature on intralysosomal pH', *FEBS Lett*, vol. 54, no. 1, pp. 76-79.

Reveille, J.D. (2012) 'Genetics of spondyloarthritis--beyond the MHC', *Nat Rev Rheumatol*, vol. 8, no. 5, pp. 296-304.

Rezaie, A., Kuenzig, M.E., Benchimol, E.I., Griffiths, A.M. *et al.* (2015) 'Budesonide for induction of remission in Crohn's disease', *Cochrane Database Syst Rev*, vol. 6, p. CD000296.

Rhodes, J.M., McLaughlin, J.E., Brown, D.J., Nuttall, L.A. *et al.* (1982a) 'Inhibition of leucocyte motility and prevention of immune-complex experimental colitis by hydroxychloroquine', *Gut*, vol. 23, no. 3, pp. 181-187.

Rhodes, J.M., Potter, B.J., Brown, D.J. & Jewell, D.P. (1982b) 'Serum inhibitors of leukocyte chemotaxis in Crohn's disease and ulcerative colitis', *Gastroenterology*, vol. 82, no. 6, pp. 1327-1334.

Richman, E. & Rhodes, J.M. (2013) 'Review article: evidence-based dietary advice for patients with inflammatory bowel disease', *Aliment Pharmacol Ther*, vol. 38, no. 10, pp. 1156-1171.

Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S. *et al.* (2007) 'Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis', *Nat Genet*, vol. 39, no. 5, pp. 596-604.

Rivera-Marrero, C.A., Stewart, J., Shafer, W.M. & Roman, J. (2004) 'The down-regulation of cathepsin G in THP-1 monocytes after infection with Mycobacterium tuberculosis is associated with increased intracellular survival of bacilli', *Infect Immun*, vol. 72, no. 10, pp. 5712-5721.

Roberts, C.L., Keita, A.V., Duncan, S.H., O'Kennedy, N. *et al.* (2010) 'Translocation of Crohn's disease Escherichia coli across M-cells: contrasting effects of soluble plant fibres and emulsifiers', *Gut*, vol. 59, no. 10, pp. 1331-1339.

Roberts, C.L., Rushworth, S.L., Richman, E. & Rhodes, J.M. (2013) 'Hypothesis: Increased consumption of emulsifiers as an explanation for the rising incidence of Crohn's disease', *J Crohns Colitis*, vol. 7, no. 4, pp. 338-341.

Roggenbuck, D., Hausdorf, G., Martinez-Gamboa, L., Reinhold, D. *et al.* (2009) 'Identification of GP2, the major zymogen granule membrane glycoprotein, as the autoantigen of pancreatic antibodies in Crohn's disease', *Gut*, vol. 58, no. 12, pp. 1620-1628.

Rolain, J.M., Colson, P. & Raoult, D. (2007) 'Recycling of chloroquine and its hydroxyl analogue to face bacterial, fungal and viral infections in the 21st century', *Int J Antimicrob Agents*, vol. 30, no. 4, pp. 297-308.

Rosen, C.J. (2011) 'Clinical practice. Vitamin D insufficiency', *N Engl J Med*, vol. 364, no. 3, pp. 248-254.

Roth, M.P., Petersen, G.M., McElree, C., Feldman, E. *et al.* (1989) 'Geographic origins of Jewish patients with inflammatory bowel disease', *Gastroenterology*, vol. 97, no. 4, pp. 900-904.

Rubin, G.P., Hungin, A.P., Kelly, P.J. & Ling, J. (2000) 'Inflammatory bowel disease: epidemiology and management in an English general practice population', *Aliment Pharmacol Ther*, vol. 14, no. 12, pp. 1553-1559.

Rungoe, C., Langholz, E., Andersson, M., Basit, S. *et al.* (2014) 'Changes in medical treatment and surgery rates in inflammatory bowel disease: a nationwide cohort study 1979-2011', *Gut*, vol. 63, no. 10, pp. 1607-1616.

Rutgeerts, P., Geboes, K., Vantrappen, G., Beyls, J. *et al.* (1990) 'Predictability of the postoperative course of Crohn's disease', *Gastroenterology*, vol. 99, no. 4, pp. 956-963.

Rutgeerts, P., Hiele, M., Geboes, K., Peeters, M. *et al.* (1995) 'Controlled trial of metronidazole treatment for prevention of Crohn's recurrence after ileal resection', *Gastroenterology*, vol. 108, no. 6, pp. 1617-1621.

Rutgeerts, P., Van Assche, G., Vermeire, S., D'Haens, G. *et al.* (2005) 'Ornidazole for prophylaxis of postoperative Crohn's disease recurrence: a randomized, double-blind, placebo-controlled trial', *Gastroenterology*, vol. 128, no. 4, pp. 856-861.

Ryan, J.D., Silverberg, M.S., Xu, W., Graff, L.A. *et al.* (2013) 'Predicting complicated Crohn's disease and surgery: phenotypes, genetics, serology and psychological characteristics of a population-based cohort', *Aliment Pharmacol Ther*, vol. 38, no. 3, pp. 274-283.

Ryan, P., Kelly, R.G., Lee, G., Collins, J.K. *et al.* (2004) 'Bacterial DNA within granulomas of patients with Crohn's disease--detection by laser capture microdissection and PCR', *Am J Gastroenterol*, vol. 99, no. 8, pp. 1539-1543.

Sadaghian Sadabad, M., Regeling, A., de Goffau, M.C., Blokzijl, T. *et al.* (2014) 'The ATG16L1-T300A allele impairs clearance of pathosymbionts in the inflamed ileal mucosa of Crohn's disease patients', *Gut*, vol. epub ahead of print.

Sagiv-Friedgut, K., Karban, A., Weiss, B., Shaoul, R. *et al.* (2010) 'Early-onset Crohn disease is associated with male sex and a polymorphism in the IL-6 promoter', *J Pediatr Gastroenterol Nutr*, vol. 50, no. 1, pp. 22-26.

Sandborn, W., Gasink, C., Blank, M., Lang, Y. *et al.* (2016) 'O-001 A Multicenter, Double-Blind, Placebo-Controlled Phase3 Study of Ustekinumab, a Human IL-12/23P40 mAB, in Moderate-Service Crohn's Disease Refractory to Anti-TFNalpha: UNITI-1', *Inflamm Bowel Dis*, vol. 22 Suppl 1, p. S1.

Sandborn, W.J., Feagan, B.G., Rutgeerts, P., Hanauer, S. *et al.* (2013) 'Vedolizumab as induction and maintenance therapy for Crohn's disease', *N Engl J Med*, vol. 369, no. 8, pp. 711-721.

Sandborn, W.J., Gasink, C., Gao, L.L., Blank, M.A. *et al.* (2012) 'Ustekinumab induction and maintenance therapy in refractory Crohn's disease', *N Engl J Med*, vol. 367, no. 16, pp. 1519-1528.

Sands, B.E., Feagan, B.G., Rutgeerts, P., Colombel, J.F. *et al.* (2014) 'Effects of vedolizumab induction therapy for patients with Crohn's disease in whom tumor necrosis factor antagonist treatment failed', *Gastroenterology*, vol. 147, no. 3, pp. 618-627 e613.

Sansonetti, P.J. & Phalipon, A. (1999) 'M cells as ports of entry for enteroinvasive pathogens: mechanisms of interaction, consequences for the disease process', *Semin Immunol*, vol. 11, no. 3, pp. 193-203.

Saravolatz, L., Manzor, O., Pawlak, J. & Belian, B. (2005) 'Antimicrobial activity and a comparison of published pharmacodynamics of gemifloxacin and eight fluoroquinolones against Streptococcus pneumoniae', *Int J Antimicrob Agents*, vol. 26, no. 1, pp. 81-84.

Scanlan, P.D., Shanahan, F., O'Mahony, C. & Marchesi, J.R. (2006) 'Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease', *J Clin Microbiol*, vol. 44, no. 11, pp. 3980-3988.

Schwartz, D.A., Loftus, E.V., Jr., Tremaine, W.J., Panaccione, R. *et al.* (2002) 'The natural history of fistulizing Crohn's disease in Olmsted County, Minnesota', *Gastroenterology*, vol. 122, no. 4, pp. 875-880.

Seksik, P., Nion-Larmurier, I., Sokol, H., Beaugerie, L. *et al.* (2009) 'Effects of light smoking consumption on the clinical course of Crohn's disease', *Inflamm Bowel Dis*, vol. 15, no. 5, pp. 734-741.

Seksik, P., Rigottier-Gois, L., Gramet, G., Sutren, M. *et al.* (2003) 'Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon', *Gut*, vol. 52, no. 2, pp. 237-242.

Selby, W., Pavli, P., Crotty, B., Florin, T. *et al.* (2007) 'Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease', *Gastroenterology*, vol. 132, no. 7, pp. 2313-2319.

Severs, M., van Erp, S.J., van der Valk, M.E., Mangen, M.J. *et al.* (2016) 'Smoking is Associated With Extra-intestinal Manifestations in Inflammatory Bowel Disease', *J Crohns Colitis*, vol. 10, no. 4, pp. 455-461.

Sewell, G.W., Rahman, F.Z., Levine, A.P., Jostins, L. *et al.* (2012) 'Defective tumor necrosis factor release from Crohn's disease macrophages in response to toll-like receptor activation: Relationship to phenotype and genome-wide association susceptibility loci', *Inflamm Bowel Dis*, vol. 18, no. 11, pp. 2120-2127.

Shaffer, J.L., Hughes, S., Linaker, B.D., Baker, R.D. *et al.* (1984) 'Controlled trial of rifampicin and ethambutol in Crohn's disease', *Gut*, vol. 25, no. 2, pp. 203-205.

Shafran, I., Kugler, L., El-Zaatari, F.A., Naser, S.A. *et al.* (2002) 'Open clinical trial of rifabutin and clarithromycin therapy in Crohn's disease', *Dig Liver Dis*, vol. 34, no. 1, pp. 22-28.

Shivananda, S., Lennard-Jones, J., Logan, R., Fear, N. *et al.* (1996) 'Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD)', *Gut*, vol. 39, no. 5, pp. 690-697.

Silverberg, M.S., Mirea, L., Bull, S.B., Murphy, J.E. *et al.* (2003) 'A population- and family-based study of Canadian families reveals association of HLA DRB1*0103 with colonic involvement in inflammatory bowel disease', *Inflamm Bowel Dis*, vol. 9, no. 1, pp. 1-9.

Silverberg, M.S., Satsangi, J., Ahmad, T., Arnott, I.D. *et al.* (2005) 'Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology', *Can J Gastroenterol*, vol. 19 Suppl A, pp. 5-36.

Simmons, J.D., Mullighan, C., Welsh, K.I. & Jewell, D.P. (2000) 'Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility', *Gut*, vol. 47, no. 2, pp. 211-214.

Simpson, K.W., Dogan, B., Rishniw, M., Goldstein, R.E. *et al.* (2006) 'Adherent and invasive Escherichia coli is associated with granulomatous colitis in boxer dogs', *Infect Immun*, vol. 74, no. 8, pp. 4778-4792.

Sipponen, T., Karkkainen, P., Savilahti, E., Kolho, K.L. *et al.* (2008) 'Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings', *Aliment Pharmacol Ther*, vol. 28, no. 10, pp. 1221-1229.

Smith, A.M., Rahman, F.Z., Hayee, B., Graham, S.J. *et al.* (2009) 'Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease', *J Exp Med*, vol. 206, no. 9, pp. 1883-1897.

Smith, M.A., Blaker, P., Marinaki, A.M., Anderson, S.H. *et al.* (2012) 'Optimising outcome on thiopurines in inflammatory bowel disease by co-prescription of allopurinol', *J Crohns Colitis*, vol. 6, no. 9, pp. 905-912.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O. *et al.* (2008) 'Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients', *Proc Natl Acad Sci U S A*, vol. 105, no. 43, pp. 16731-16736.

Sokol, H., Seksik, P., Rigottier-Gois, L., Lay, C. *et al.* (2006) 'Specificities of the fecal microbiota in inflammatory bowel disease', *Inflamm Bowel Dis*, vol. 12, no. 2, pp. 106-111.

Sonnenberg, A. & Walker, J.T. (2012) 'Occupational mortality associated with inflammatory bowel disease in the United States 1984-1998', *Inflamm Bowel Dis*, vol. 18, no. 7, pp. 1249-1253.

Standish, A.J. & Weiser, J.N. (2009) 'Human neutrophils kill Streptococcus pneumoniae via serine proteases', *J Immunol*, vol. 183, no. 4, pp. 2602-2609.

Stange, E.F., Travis, S.P., Vermeire, S., Beglinger, C. *et al.* (2006) 'European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis', *Gut*, vol. 55 Suppl 1, pp. i1-15.

Stawczyk-Eder, K., Eder, P., Lykowska-Szuber, L., Krela-Kazmierczak, I. *et al.* (2015) 'Is faecal calprotectin equally useful in all Crohn's disease locations? A prospective, comparative study', *Arch Med Sci*, vol. 11, no. 2, pp. 353-361.

Steed, H., Walsh, S. & Reynolds, N. (2010) 'Crohn's disease incidence in NHS Tayside', *Scott Med J*, vol. 55, no. 3, pp. 22-25.

Steinhart, A.H., Feagan, B.G., Wong, C.J., Vandervoort, M. *et al.* (2002) 'Combined budesonide and antibiotic therapy for active Crohn's disease: a randomized controlled trial', *Gastroenterology*, vol. 123, no. 1, pp. 33-40.

Styrt, B. & Klempner, M.S. (1986) 'Inhibition of neutrophil oxidative metabolism by lysosomotropic weak bases', *Blood*, vol. 67, no. 2, pp. 334-342.

Subramanian, S., Roberts, C.L., Hart, C.A., Martin, H.M. *et al.* (2008) 'Replication of Colonic Crohn's Disease Mucosal Escherichia coli Isolates within Macrophages and Their Susceptibility to Antibiotics', *Antimicrob Agents Chemother*, vol. 52, no. 2, pp. 427-434.

Suibhne, T.N., Cox, G., Healy, M., O'Morain, C. *et al.* (2012) 'Vitamin D deficiency in Crohn's disease: prevalence, risk factors and supplement use in an outpatient setting', *J Crohns Colitis*, vol. 6, no. 2, pp. 182-188.

Summers, R.W., Switz, D.M., Sessions, J.T., Jr., Becktel, J.M. *et al.* (1979) 'National Cooperative Crohn's Disease Study: results of drug treatment', *Gastroenterology*, vol. 77, no. 4 Pt 2, pp. 847-869.

Sutherland, L., Singleton, J., Sessions, J., Hanauer, S. *et al.* (1991) 'Double blind, placebo controlled trial of metronidazole in Crohn's disease', *Gut*, vol. 32, no. 9, pp. 1071-1075.

Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S. *et al.* (2002) 'Mucosal flora in inflammatory bowel disease', *Gastroenterology*, vol. 122, no. 1, pp. 44-54.

Swift, G.L., Srivastava, E.D., Stone, R., Pullan, R.D. *et al.* (1994) 'Controlled trial of anti-tuberculous chemotherapy for two years in Crohn's disease', *Gut*, vol. 35, no. 3, pp. 363-368.

Targan, S.R., Landers, C.J., Yang, H., Lodes, M.J. *et al.* (2005) 'Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease', *Gastroenterology*, vol. 128, no. 7, pp. 2020-2028.

Terdiman, J.P., Gruss, C.B., Heidelbaugh, J.J., Sultan, S. *et al.* (2013) 'American Gastroenterological Association Institute guideline on the use of thiopurines, methotrexate, and anti-TNF-alpha biologic drugs for the induction and maintenance of remission in inflammatory Crohn's disease', *Gastroenterology*, vol. 145, no. 6, pp. 1459-1463.

Theil, D., Farina, C. & Meinl, E. (2005) 'Differential expression of CD150 (SLAM) on monocytes and macrophages in chronic inflammatory contexts: abundant in Crohn's disease, but not in multiple sclerosis', *J Clin Pathol*, vol. 58, no. 1, pp. 110-111.

Thia, K.T., Mahadevan, U., Feagan, B.G., Wong, C. *et al.* (2009) 'Ciprofloxacin or metronidazole for the treatment of perianal fistulas in patients with Crohn's

disease: a randomized, double-blind, placebo-controlled pilot study', *Inflamm Bowel Dis*, vol. 15, no. 1, pp. 17-24.

Thia, K.T., Sandborn, W.J., Harmsen, W.S., Zinsmeister, A.R. *et al.* (2010) 'Risk factors associated with progression to intestinal complications of Crohn's disease in a population-based cohort', *Gastroenterology*, vol. 139, no. 4, pp. 1147-1155.

Thomas, G.A., Swift, G.L., Green, J.T., Newcombe, R.G. *et al.* (1998) 'Controlled trial of antituberculous chemotherapy in Crohn's disease: a five year follow up study', *Gut*, vol. 42, no. 4, pp. 497-500.

Tiede, I., Fritz, G., Strand, S., Poppe, D. *et al.* (2003) 'CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes', *J Clin Invest*, vol. 111, no. 8, pp. 1133-1145.

To, N., Gracie, D.J. & Ford, A.C. (2016) 'Systematic review with meta-analysis: the adverse effects of tobacco smoking on the natural history of Crohn's disease', *Aliment Pharmacol Ther*, vol. 43, no. 5, pp. 549-561.

Tobin, M.V., Logan, R.F., Langman, M.J., McConnell, R.B. *et al.* (1987) 'Cigarette smoking and inflammatory bowel disease', *Gastroenterology*, vol. 93, no. 2, pp. 316-321.

Tontini, G.E., Bisschops, R. & Neumann, H. (2014) 'Endoscopic scoring systems for inflammatory bowel disease: pros and cons', *Expert Rev Gastroenterol Hepatol*, vol. 8, no. 5, pp. 543-554.

Trinder, D. & Morgan, E. (1998) 'Mechanisms of ferric citrate uptake by human hepatoma cells', *Am J Physiol*, vol. 275, no. 2 Pt 1, pp. G279-286.

Uhlig, H.H., Schwerd, T., Koletzko, S., Shah, N. *et al.* (2014) 'The diagnostic approach to monogenic very early onset inflammatory bowel disease', *Gastroenterology*, vol. 147, no. 5, pp. 990-1007 e1003.

Van Assche, G., Dignass, A., Panes, J., Beaugerie, L. *et al.* (2010) 'The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Definitions and diagnosis', *J Crohns Colitis*, vol. 4, no. 1, pp. 7-27.

Van Kruiningen, H.J., Ganley, L.M. & Freda, B.J. (1997) 'The role of Peyer's patches in the age-related incidence of Crohn's disease', *J Clin Gastroenterol*, vol. 25, no. 2, pp. 470-475.

Van Limbergen, J., Russell, R.K., Nimmo, E.R., Drummond, H.E. *et al.* (2008) 'Autophagy gene ATG16L1 influences susceptibility and disease location but not childhood-onset in Crohn's disease in Northern Europe', *Inflamm Bowel Dis*, vol. 14, no. 3, pp. 338-346.

van Rheenen, P.F., Van de Vijver, E. & Fidler, V. (2010) 'Faecal calprotectin for screening of patients with suspected inflammatory bowel disease: diagnostic meta-analysis', *BMJ*, vol. 341, p. c3369.

van Schaik, F.D., Oldenburg, B., Hart, A.R., Siersema, P.D. *et al.* (2013) 'Serological markers predict inflammatory bowel disease years before the diagnosis', *Gut*, vol. 62, no. 5, pp. 683-688.

Vande Casteele, N., Ferrante, M., Van Assche, G., Ballet, V. *et al.* (2015) 'Trough concentrations of infliximab guide dosing for patients with inflammatory bowel disease', *Gastroenterology*, vol. 148, no. 7, pp. 1320-1329 e1323.

Vasiliauskas, E.A., Plevy, S.E., Landers, C.J., Binder, S.W. *et al.* (1996) 'Perinuclear antineutrophil cytoplasmic antibodies in patients with Crohn's disease define a clinical subgroup', *Gastroenterology*, vol. 110, no. 6, pp. 1810-1819.

Vavricka, S.R., Rogler, G., Gantenbein, C., Spoerri, M. *et al.* (2015) 'Chronological Order of Appearance of Extraintestinal Manifestations Relative to the Time of IBD Diagnosis in the Swiss Inflammatory Bowel Disease Cohort', *Inflamm Bowel Dis*, vol. 21, no. 8, pp. 1794-1800.

Vazeille, E., Buisson, A., Bringer, M.A., Goutte, M. *et al.* (2015) 'Monocyte-derived macrophages from Crohn's disease patients are impaired in the ability to control intracellular adherent-invasive Escherichia coli and exhibit disordered cytokine secretion profile', *J Crohns Colitis*, vol. 9, no. 5, pp. 410-420.

Ventham, N.T., Kennedy, N.A., Nimmo, E.R. & Satsangi, J. (2013) 'Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics', *Gastroenterology*, vol. 145, no. 2, pp. 293-308.

Vermeire, S., Schreiber, S., Sandborn, W.J., Dubois, C. *et al.* (2010a) 'Correlation between the Crohn's disease activity and Harvey-Bradshaw indices in assessing Crohn's disease severity', *Clin Gastroenterol Hepatol*, vol. 8, no. 4, pp. 357-363.

Vermeire, S., Van Assche, G. & Rutgeerts, P. (2010b) 'Role of genetics in prediction of disease course and response to therapy', *World J Gastroenterol*, vol. 16, no. 21, pp. 2609-2615.

Vrakas, S., Mountzouris, K.C., Michalopoulos, G., Karamanolis, G. *et al.* (2017) 'Intestinal Bacteria Composition and Translocation of Bacteria in Inflammatory Bowel Disease', *PLoS One*, vol. 12, no. 1, p. e0170034.

Wang, T.T., Dabbas, B., Laperriere, D., Bitton, A.J. *et al.* (2010) 'Direct and indirect induction by 1,25-dihydroxyvitamin D3 of the NOD2/CARD15-defensin beta2 innate immune pathway defective in Crohn disease', *J Biol Chem*, vol. 285, no. 4, pp. 2227-2231.

Wehkamp, J., Salzman, N.H., Porter, E., Nuding, S. *et al.* (2005) 'Reduced Paneth cell alpha-defensins in ileal Crohn's disease', *Proc Natl Acad Sci U S A*, vol. 102, no. 50, pp. 18129-18134.

Wellens, A., Garofalo, C., Nguyen, H., Van Gerven, N. *et al.* (2008) 'Intervening with urinary tract infections using anti-adhesives based on the crystal structure of the FimH-oligomannose-3 complex', *PLoS One*, vol. 3, no. 4, p. e2040.

West, R.L., van der Woude, C.J., Hansen, B.E., Felt-Bersma, R.J. *et al.* (2004) 'Clinical and endosonographic effect of ciprofloxacin on the treatment of perianal fistulae in Crohn's disease with infliximab: a double-blind placebo-controlled study', *Aliment Pharmacol Ther*, vol. 20, no. 11-12, pp. 1329-1336.

Willing, B., Halfvarson, J., Dicksved, J., Rosenquist, M. *et al.* (2009) 'Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn's disease', *Inflamm Bowel Dis*, vol. 15, no. 5, pp. 653-660.

Wilson, K. & Walker, J. (2010) Spectroscopic techniques: I Spectrophotometric techniques, in, *Principles and Techniques of Biochemistry and Molecular Biology*, 7 edn, Cambridge University Press.

Winchell, C.G., Graham, J.G., Kurten, R.C. & Voth, D.E. (2014) 'Coxiella burnetii type IV secretion-dependent recruitment of macrophage autophagosomes', *Infect Immun*, vol. 82, no. 6, pp. 2229-2238.

Wright, E.K., Kamm, M.A., De Cruz, P., Hamilton, A.L. *et al.* (2015) 'Measurement of fecal calprotectin improves monitoring and detection of recurrence of Crohn's disease after surgery', *Gastroenterology*, vol. 148, no. 5, pp. 938-947 e931.

WTCCC (2007) 'Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls', *Nature*, vol. 447, no. 7145, pp. 661-678.

Yang, H., McElree, C., Roth, M.P., Shanahan, F. *et al.* (1993) 'Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews', *Gut*, vol. 34, no. 4, pp. 517-524.

Yang, S.K., Yun, S., Kim, J.H., Park, J.Y. *et al.* (2008) 'Epidemiology of inflammatory bowel disease in the Songpa-Kangdong district, Seoul, Korea, 1986-2005: a KASID study', *Inflamm Bowel Dis*, vol. 14, no. 4, pp. 542-549.

Yuk, J.M., Shin, D.M., Lee, H.M., Yang, C.S. *et al.* (2009) 'Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin', *Cell Host Microbe*, vol. 6, no. 3, pp. 231-243.

Zachos, M., Tondeur, M. & Griffiths, A.M. (2007) 'Enteral nutritional therapy for induction of remission in Crohn's disease', *Cochrane Database Syst Rev*, no. 1, p. CD000542.

Zelinkova, Z., Stokkers, P.C., van der Linde, K., Kuipers, E.J. *et al.* (2012) 'Maternal imprinting and female predominance in familial Crohn's disease', *J Crohns Colitis*, vol. 6, no. 7, pp. 771-776.

Zhang, F.R., Huang, W., Chen, S.M., Sun, L.D. *et al.* (2009) 'Genomewide association study of leprosy', *N Engl J Med*, vol. 361, no. 27, pp. 2609-2618.

12 Appendix 1

12.1 Monocyte derived macrophage study: Inclusion/exclusion criteria

Inclusion/Exclusion criteria	
Inclusion	Patient or control is willing to participate in the study and has provided informed consent Patients or controls aged 16 or over Patients' with Crohn's disease diagnosed by conventional clinical, radiological and histological criteria. The disease can be either active or inactive Controls: Patients with a diagnosis of Irritable bowel syndrome diagnosed by standard means, or normal volunteers with no GI disorder and no personal or family history of inflammatory bowel disease
Exclusion	Patients under 16 or unable to give informed consent. Patients receiving anti-tissue necrosis factor (anti-TNF) medications Patients receiving corticosteroids Patients on immunosuppressants Patients who are pregnant, breastfeeding or <3 months postpartum

Table 8: Inclusion and exclusion criteria for recruitment to the human macrophage project. Subjects provided blood for monocyte isolation, quantification of serum Vitamin D and hsCRP levels, and genomic analysis.

12.2 Patient baseline characteristics

	Healthy controls (N=10)	Crohn's disease (N=10)	p value
Male (%)	50%	20%	0.177
Age (y)	51.7 ± 3.8	44.8 ± 5.2	0.297
Smoker (%)	10%	30%	0.290
Harvey Bradshaw Index	n/a	5.0 ± 1.7	n/a
Serum Vit D3 (nmol/L)	30.8 ± 4.7	26.0 ± 3.7	0.433
hsCRP (mg/L)	2.2 ± 0.6	7.8 ± 2.0	0.03
NOD2 homozygote w/t	90%	80%	0.556
NOD2 heterozygote	10%	20%	0.556
ATG16L1 homozygote w/t	40%	40%	0.999
ATG16L1 heterozygote	60%	50%	0.677
ATG16L1 homozygote variant	0%	10%	0.343
IRGM homozygote w/t	80%	70%	0.628
IRGM heterozygote	20%	30%	0.628

Table 9: Comparison of baseline characteristics of patients and healthy controls recruited to monocyte derived macrophage study

12.3 Complete patient characteristics

	Sex	Age	Current smoker	Montreal	Harvey Bradshaw Index	Vitamin D3 (nM)	hsCRP (mg/L)	No. of altered alleles NOD2 (rs2066844)	No. of altered alleles NOD2 (rs2066845)	No. of altered alleles NOD2 (rs2066847)	No. of altered alleles ATG16L (rs2241880)	No. of altered alleles IRGM (rs13361189)	Fold change in intra-macrophage HM605	Fold change in intra-macrophage K12	Fold change in intra-macrophage Staph	Relative MDM induced Neutrophil chemotaxis
HC 1	M	36	No	n/a	n/a	45	0.4	0	0	0	1	0	1.56	1.80	0.58	n/d
HC 2	F	59	Yes	n/a	n/a	20	3.07	0	0	0	1	0	1.39	1.75	0.55	2.95
HC 3	F	56	No	n/a	n/a	42	5.66	0	0	0	0	1	0.39	0.31	0.54	2.34
HC 4	M	51	No	n/a	n/a	46	0.57	0	0	0	0	0	3.00	0.41	0.61	2.49
HC 5	M	74	No	n/a	n/a	22	2.65	0	0	0	0	0	2.00	0.18	0.26	2.46
HC 6	F	34	No	n/a	n/a	10	0.35	0	0	0	1	0	1.00	0.65	0.54	2.70
HC 7	M	57	No	n/a	n/a	41	4.62	0	0	0	1	0	1.00	1.04	0.55	3.07
HC 8	F	43	No	n/a	n/a	29	3.37	0	0	0	1	0	1.56	1.24	0.41	2.56
HC 9	M	59	No	n/a	n/a	8	1.12	0	0	0	0	0	1.57	0.58	0.25	n/d
HC 10	F	48	No	n/a	n/a	45	0.63	1	0	0	1	1	n/d	0.65	0.49	n/d
CD 1	F	38	No	A2, L1, B3p	0	31	2.75	1	0	0	0	0	0.00	0.00	0.04	3.00
CD 2	F	64	Yes	A3, L1, B2	5	12	14.15	0	0	1	1	0	1.42	0.11	0.42	3.19
CD 3	F	45	No	A2, L1, B2	0	23	1.46	0	0	0	1	1	0.15	0.37	0.18	2.43
CD 4	F	21	No	A1, L3, B1	2	13	1.04	0	0	0	0	1	1.28	1.22	1.09	2.31
CD 5	F	48	No	A2, L1, B2	7	43	18.3	0	0	0	2	0	3.43	0.84	0.38	2.24
CD 6	M	39	No	A2, L1, B2	10	16	10.14	0	0	0	0	0	2.40	1.12	0.61	1.94
CD 7	F	73	No	A3, L2, B1	9	38	14.91	0	0	0	0	0	0.23	0.17	0.17	2.27
CD 8	M	24	No	A1, L1, B3p	1	36	3.95	0	0	0	1	1	0.38	0.18	0.20	2.87
CD 9	F	40	Yes	A2, L1, B2	16	33	4.84	0	0	0	1	0	1.20	1.16	0.31	2.80
CD10	F	56	Yes	A2, L3, B2p	0	15	6.95	0	0	0	1	0	0.36	0.27	0.25	2.48

Table 10: Detailed characteristics of patients and healthy controls recruited to monocyte derived macrophage study

13 Appendix 2

13.1 Funding applications

Initial funding for this research project was provided by the Liverpool National Institute for Health Research Biomedical Research Centre. This funding stream finished at the end of March 2012. Consequently I spent a significant part of my early research time applying for additional funding to allow completion of the project. The applications and outcomes are summarised below. No applications other than those listed below were submitted.

Awarding body	Award applied for	Date submitted	Outcome
NIHR	Biomedical research fellowship	October 2011	Successful £12,000 consumables £2,819 training fund
Crohn's and Colitis UK	Medical Research Award	November 2011	Withdrawn following successful outcome of NIHR application
Shire	Shire Innovation Fund for SpRs	January 2012	Successful £3,200 additional consumables

13.2 Output related to MD to May 2017

13.2.1 Funding

- NIHR Biomedical Research Fellowship – April 2012 to April 2013
- Shire Development fund for SpRs, project grant - £3200

13.2.2 Prizes and awards

- British Society of Gastroenterology best poster award (IBD) 2014
- ECCO IBD Advanced course (1 of 3 selected UK participants) 2013
- Mersey Gastroenterology Group Research Prize 2012
- CORE Belmont Trust Travel Award 2011
- BSG Supraregional meeting travel bursary 2011
- NIHR Biomedical Research Centre Scientific Training Fund 2011

13.2.3 Papers

- **Flanagan PK**, Chiewchengchol D, Wright HL *et al*. Killing of Escherichia coli by Crohn's Disease Monocyte-derived Macrophages and Its Enhancement by Hydroxychloroquine and Vitamin D. *Inflamm Bowel Dis*, 2015 Jul;21(7):1499-510
- Tawfik A, **Flanagan PK**, Campbell BJ. *Escherichia coli*-host macrophage interactions in the pathogenesis of inflammatory bowel disease. World J Gastroenterol, 2014; 20(27): 8751-63
- Prorok-Hamon M, Friswell MK, Alswied A, Roberts CL, Song F, **Flanagan PK** et al. Colonic mucosa-associated Diffusely-Adherent afaC+ *Escherichia coli* expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer. Gut, 2014; 63(5):761-70
- **Flanagan PK,** Campbell BJ, Rhodes JM. Lessons from diversion studies and antibacterial interventions. *Digestive Diseases*, 2012; 30 (4): 347-50
- **Flanagan P,** Campbell BJ, Rhodes JM. Bacteria in the pathogenesis of inflammatory bowel disease. *Biochem Soc Trans* 2011; 39 (4): 1067-72
- **Flanagan P,** Campbell BJ, Rhodes JM. Bacteria, good and bad - Host-microbiota interactions in inflammatory bowel disease. *The Biochemist* 2011; 33 (4): 22-25

13.2.4 Book Chapters

- **Flanagan PK**, Subramanian S. Crohn's disease. Recent advances in Gastroenterology 12. JP Medical Publishers, 2014, Ch2; 11-23
- **Flanagan PK**, Rhodes JM. Correction of microbiota disturbances or antagonism against specific pathogens in IBD. Intestinal microbiota in health and disease. CRC Press, 2014; 230-51.

13.2.5 Abstracts – Oral presentations

- **Flanagan PK,** Campbell BJ, Rhodes JM. Hydroxychloroquine as a treatment for Crohn's disease: Enhancing antibiotic efficacy and macrophage killing of *E. coli*. DDF, 2012, OC-139
- **Flanagan PK**, Campbell BJ, Rhodes JM. Hydroxychloroquine inhibits intra-macrophage replication of Crohn's E. coli –BSG Supraregional Meeting, Sept 2011

13.2.6 Abstracts - Posters

- **Flanagan PK,** Campbell BJ, Subramanian S, Rhodes JM. Crohn's disease monocyte-derived macrophages exhibit equivalent responses to intramacrophage bacterial infection relative to healthy controls. *BSG*, 2014, PWE-091
- **Flanagan PK,** Campbell BJ, Subramanian S, Rhodes JM. Intracellular killing of bacteria is not impaired in Crohn's disease monocyte-derived macrophages. *UEGW*, 2013, P1391
- **Flanagan PK**, Campbell BJ, Rhodes JM. Vitamin D enhances macrophage function and improves killing of Crohn's associated E. coli. Ecco, 2013, P026
- **Flanagan PK,** Campbell BJ, Rhodes JM. Hydroxychloroquine Inhibits Intra-Macrophage Replication of Crohn's Disease *E. coli* and Enhances the Antimicrobial Effect of Antibiotics Doxycycline and Ciprofloxacin. *UEGW*, 2011, P0513

13.2.7 Additional Oral Presentations to Learned Societies

- ASNEMGE Young Investigators Programme 2011 – Oral presentation and selected participant

13.2.8 Peer review

- Invited reviewer for Tropical doctor – IBD clinical paper, 2012

13.3 Protocol for antibiotic trial

The trial protocol has not been included here due to space limitations.

The trial is registered with ClinicalTrials.gov, is visible online and can be found using the identifier NCT01783106.

14 Appendix 3

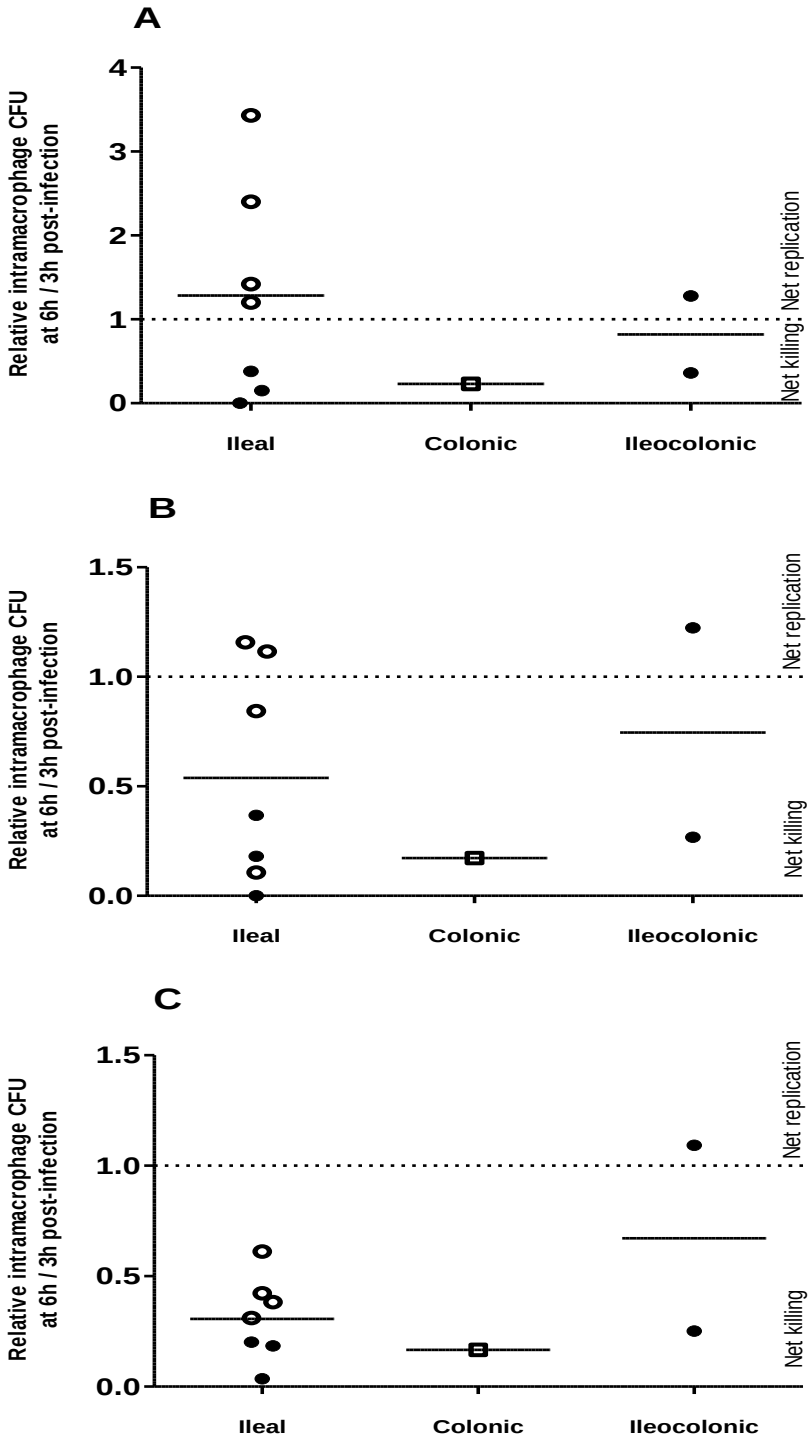

Figure 14-1. Relative intramacrophage replication of E. coli HM605 (A), E. coli K12 (B) and Staph aureus Oxford strain (C) in MDMs from patients with Crohn's disease by disease location (ileal n=7, colonic n=1, ileocolonic n=2). Active disease represented by open symbols, quiescent disease by solid symbols. Statistical analysis was prevented by insufficient patients with isolated colonic disease.